Evolutionary patterns of the human skull. A quantitative genetic analysis of craniofacial phenotypic variation

Patrons evolutius del crani humà. Anàlisi geneticoquantitativa de la variació fenotípica craniofacial

Memòria presentada per

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A los que tienen un alma flamenca

A vosotros, Ángel y Tere

A ti, David

Y, por tanto, espero que hayas comprendido que X puede resultar igual, mayor o menor que la suma de A más B. Eso depende de la interrelación de las partes. Por eso, sólo podemos afirmar con certeza que el todo es igual a la suma de las partes cuando las partes se ignoran entre sí.

Almudena Grandes, *El corazón helado*
(Barcelona, 2007)

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1.1 *The Hallstatt Project*

This thesis is the final outcome of a project called *Quantitative genetics of craniofacial traits: a functional approach to heritability,* which received support from the Wenner-Gren Foundation for Anthropological Research in 2004 (Individual Research Grant nº 7149). The main goal of this project is to integrate geometric morphometric and biodemographic tools with quantitative genetics in order to estimate the genetic variation underlying skull morphology and to assess its capability to evolve.

The analyses herein are based on the analysis of a sample of human skulls from Hallstatt, an Austrian village located in the Alps (Figure 1). The uniqueness of this sample for evolutionary anthropological studies lies in the fact that it is made up of more than 700 decorated skulls with associated genealogical data (Figure 2). This material has been accumulating in Hallstatt since 1775 and is the largest known human skull collection with pedigree information (see Appendix). Actually, it provides an unusual opportunity to apply quantitative genetic methods in a human population.

In Hallstatt has endured a singular tradition to worship the ancestors, which consisted in the following. Upon request of the family, the skeletal remains of their relatives were exhumed from their graves and were preserved at the charnel house. Long bones were just accumulated, but the skulls were given a special care. Crania were cleaned and decorated with nice paintings (Sauser 1956), and even more important, the names of the individuals were written in black ink letters on their forehead, allowing the skull identification. Originally, it was considered that this practice was carried out to give room to new burials because of the lack of space within the churchyard (Morton 1954). However, now it is regarded as a tradition connected with the Catholic Church and the celebration of All Saints' Day (Burgstaller 1961). This custom was widespread in other Austrian and German regions surrounding the Alps (Sauser 1956), but Hallstatt is the only place where this tradition persisted until

recently, since the last skull was incorporated in 1986. In other places, the practice lasted for shorter periods and almost all of those collections were reburied after the 20th century or disappeared during the World Wars. In Hallstatt, the collection is still preserved at the Catholic charnel house, where the skulls are exhibited to the public. However, the collection is not complete because some skulls were donated to different Austrian institutions. Most of these skulls can be found at the *Hallstatt Musealverein*, the *Anatomisches Institut* in Innsbruck, as well as the *Naturhistorisches Museum* and *the Österreichisches Museum für Volkskunde* in Vienna.

The skulls can be identified by its written name and the genealogical trees of the families represented at the skull sample can be reconstructed thanks to the parish records of births, deaths and marriages that are preserved at the Catholic Church of Hallstatt from as early as 1602. Therefore, the data collected to carry out this project concerned two main sources of information: craniometric and demographic data. Craniometric data was recorded by means of geometric morphometric techniques in order to provide the quantification of cranial morphology; whereas demographic data provided the necessary information to reconstruct the genealogies of the population. Finally, quantitative genetic models were used to combine these datasets and to estimate the genetic and the environmental sources of variation that determine the phenotypic variation of the human skull.

Figure 1. Panoramic view from Hallstatt.

Therefore, the gathered information was used to estimate the genetic variation of size and shape craniofacial structures, as well as for testing hypotheses about selection of cranial structures through the hominid lineage. These analyses have raised discussion regarding heritability, phenotypic selection, genetic constraints and morphological integration in the human species.

Figure 2. Decorated skulls at the charnel house.

1.1.1 HALLSTATT: BRIEF HISTORICAL AND DEMOGRAPHICAL REPORT

The village of Hallstatt (Upper Austria, Salzkammergut) is located on the Eastern Alps (47°34'N 13°39'E), approximately 70 km SE of Salzburg (Figure 3). It is surrounded by glaciers (such as the Dachstein) and lies at the end of a large lake, the Hallstätersee, formed by the Traun river. The name of the town is related to salt, since the history of Hallstatt has been linked primarily with salt extraction (UNESCO 1996). In fact, rock-salt mines have been the main resource of the area for thousands of years. The name of Hallstatt derives from the West German *hal* (salt) and the Old High German *stat* (settlement) and it was first recorded in a deed of 1305 (UNESCO 1996).

Figure 3. Map of Europe: geographic location of Hallstatt. Modified after Wikipedia.

Despite its anthropological value, Hallstatt is not especially renowned by the collection of decorated skulls, but it is famous for its prehistoric, natural and cultural legacy. Actually, the *Hallstatt-Dachstein-Salzkammergut Cultural Landscape* was included at the *UNESCO World Heritage List* in 1997 (Figure 4).

Figure 4. Stamp UNESCO Austria 2000. *Hallstatt-Dachstein-Salzkammergut*

The history of Hallstatt's human population can be traced back to the Neolithic Stone Age. As revealed by archaeological investigations, humans were already present in this area by 12000 BC (UNESCO 1996). The lithic industry recovered at the archaeological sites of the

Salzberg valley proves that the population of Hallstatt started to exploit the rich salt resources of their mountains by 5000 BC (UNESCO 1996). These discoveries established that the world's oldest known salt mine was settled in Hallstatt. However, one of the most prominent historical periods of Hallstatt occurred later, at the transition between the Bronze Age and the Iron Age. The "Hallstatt culture" developed during that period, from 1200 BC to 500 BC (Cunliffe 1997). This denomination was given after a rich Celtic prehistoric cemetery discovered in the Upper Salzberg valley by the local Johann Georg Ramsauer in 1846. Excavation works at the Dachstein massif have continued until present and more than a thousand burials have been recovered from the grounds. One hundred years before, in 1734, it had already been discovered the so-called "Man in Salt", a fully preserved body of a prehistoric miner dating back to 300 BC (Rom 1999).

The "Hallstatt culture" raised from a highly-developed culture based on salt mining and active trade (Cunliffe 1997). It was the predominant Central European culture during the transition between the Bronze Age and the Early Iron Age period (800 BC-400 BC). However, "the Hallstatt culture" was not homogeneous and two different cultural regions can be distinguished (Pydyn 1999): an eastern Hallstatt cultural zone including Croatia, Slovenia, western Hungary, Austria, Moravia region of the Czech Republic and Slovakia; and a western cultural zone which includes northern Italy, Switzerland, eastern France, southern Germany and the Bohemian region of the Czech Republic. Exchange systems and people movements also spread the Hallstatt cultural complex into the western half of the Iberian Peninsula, Great Britain and Ireland, probably within a Celtic-speaking context (Pydyn 1999).

Hallstatt is one of the richest known Celtic cemeteries, with a wide range of weapons, brooches, pins and pottery. The Hallstatt art has endured for thousands of years, especially the iron and bronze work as well as the pottery used as grave furniture and generally decorated in rigid symmetrical, repetitive, geometric patterns. This decoration style is very distinctive and similar artefacts are widespread in Europe (Cunliffe 1997).

The first people living in Hallstatt probably were the Illyrians. This was an immigrant group of cattle breeders, traders and miners coming to Europe from the East. Afterwards, the Illyrians were displaced by the arrival of the Celts to the area, who settled down the Norikum kingdom and developed "the Hallstatt culture" (Cunliffe 1997). The Celtic settlement was destroyed by a massive landslide occurred in 303 BC, which ended up with the mining activity

of this group. However, the Celts endured in Hallstatt until the start of the new era. At that time the Romans arrived to Hallstatt, defeated the Celts and incorporated the Norikum region into the provinces of the Roman Empire. Then, the village of Hallstatt was fully reconstructed and mining activities were re-established but in a different area (UNESCO 1996).

The vital significance of salt mining industry flourished again by 1000 AD and was maintained through the medieval times (UNESCO 1996). Progressively, Hallstatt became an important village and was bestowed on the rights of a market town by Queen Elisabeth in 1311, as well as on a coat of arms and brewery rights by Emperor Maximilian I in 1494. Rock-salt mining was so productive and exportation was so important for the economic subsistence of the people from the Salzkammergut region that it was the cause of a war in 1611, the Salt War, between the Habsburg Duke Maximilian of Bavaria and the Princebishop Wolf Dietrich von Raitenau of Salzburg. Anciently, salt was carried by miners from the mountain to the lakeshore and was traded through the lake; but later in 1770 a wooden pipeline was built and it transported the salt from the ancient mines down the valley to Ischl. The medieval town was a typical Gothic miner's settlement but it was almost totally destroyed by the great fire of 1750. Afterwards, the town was reconstructed in Late Baroque style (UNESCO 1996).

Christianity started to spread in the Salzkammergut around 300 AD and the first evidence of a Catholic Church is found in the 12th century: the Michaelkirche, a small Romanesque church. The present Catholic Church of St. Mary was built in the late 15th century (UNESCO 1996) jointly with St. Michael's chapel and the charnel house, where the decorated skull are stored. People from Hallstatt were mainly Catholic until the 16th century. By that time, Lutheranism was introduced in Austria (Kurz 2002) and during the Reformation it acquired many adherents among the miners and foresters of the Hallstatt region. Afterwards, Hallstatt's population was split up into two different religious communities, although Catholics have usually outnumbered Protestants (Table 1). Protestants were not permitted to exercise their faith publicly until the Edict of Toleration of 1781, enacted by Joseph II, Holy Roman Emperor and ruler of the Habsburg lands (UNESCO 1996). This Edict extended religious freedom to non-Catholic Christians living in the Austrian Empire, including Lutherans, Calvinists and the Greek Orthodox.

From 1781 to 1848, Protestant parishes were enabled to celebrate baptisms, marriages and funerals, but their congregations had to be registered both at the Protestant and the Catholic parishes (Kurz, pers.comm.). Therefore, individual registers were duplicated during this period: in Hallstatt, one copy was made at the Catholic parish of the village and another at the Protestant parish. Since Hallstatt's Protestant parish (Figure 5) was not constructed until 1837, Lutherans from Hallstatt were registered at the Protestant parish of Goisern, a nearby village. From 1848 and thereon (Kurz, pers. comm.), Catholic and Lutheran registers became independent and each individual was just registered once at his own community (see Appendix).

Figure 5. Tower of Hallstatt's Neo-Gothic Protestant parish.

Residence	Catholics	Lutherans	Total
Gosauzwang	14	10	24
Hallstatt markt	735	321	1056
Lahn	272	83	355
Obertraun	80	336	416
Salzberg	24	9	33
Winkl	-	46	46

Table 1. Number of inhabitants distributed by place of residence and religion. In 1845, Hallstatt had 1930 inhabitants, from which 58.3% were Catholics and 41.7% were Lutherans. More than 50% of the population lived in the village: either in the Hallstatt markt, which is the core of the town (see photo) or in Lahn, the southern part of the town. The rest of the people inhabited the surroundings territories, such as the Salzberg mountain or other small villages that belonged to the administrative region of Hallstatt (i.e., Obertraun).

Demographic information about Hallstatt has been provided by Michael Kurz, who has carried out a thorough research of the Salzkammergut's population: he has studied the Parish records of several villages from the Salzkammergut region and has analyzed their natality, marriage and mortality patterns (Kurz 2002). The number of inhabitants in Hallstatt from the 18th to the 20th century was about 1500. The highest population levels were achieved in 1739 and 1910: at these years the population census reported almost 2000

individuals, although these values depend on the regions considered at each census (i.e., in 1828 people living in Obersee were not included at Hallstatt's census and in 1923 people living in Obertraun were also excluded). During the 18th and 19th centuries, the number of inhabitants fluctuated between 1500 and 2000, but it started to decrease gradually from the beginnings of the 20th century (Kurz 2002). The lowest population level was achieved at the last census: in 2001, less than 1000 people were registered in Hallstatt.

In contrast to other villages from the Salzkammergut region, the population from Hallstatt has remained quite constant through the last three centuries (Kurz 2002). Conversely, the population of Ebensee, Goisern, Gosau and Ischl progressively increased from 1792 to 2001 and a peak of growth was achieved at these villages by 1950 (Kurz 2002). In Hallstatt, the pattern of growth population shows the three typical stages of the European demographic transition (Schofield et al. 1991). At the first stage, from the end of the 18th century to the last quarter of the 19th century, natality and mortality rates were rather high and similar (around 25-35‰). At the second stage, from the end of the 18th century to end of the 19th century, natality and mortality rates began to differ: first, the mortality rate started to decrease whereas the natality rate remained high, allowing a greater population growth; afterwards, the natality rate also decreased. At the third stage, from 1975 onwards, natality and mortality rates were similar again but quite low (around 10‰). A parallel demographical pattern has been described for the Österreich region (Kurz 2002).

In Hallstatt there are several excess mortality years, but the most prominent ones occurred at the beginning and at the end of the 19th century. Infant mortality was considerably high at the first stage, but steadily declined from 1875. Kurz (2002) reports that it was about 200-300‰ at the beginning of the 19th century and that it fell down to approximately 5‰ in 1997. In contrast, there are few years with significantly greater natality rates. This indicates that population growth has never been very high in Hallstatt.

Another interesting demographic characteristic of this region is that the percentage of illegitimate children was considerably high (Kurz 2002). In Hallstatt, the percentages were as follows: 2.6-5% at the 17th century, 5.1-10% at the 18th century and higher than 10% at the 19th century. The highest levels were achieved by 1850: the percentage of illegitimate born children was as much as 30% in Hallstatt and so was it at the surrounding regions (5% in Tirol,

23% in Salzburg, 19% in Oberösterreich, 24% in Steiermark, 26% in Niederösterreich, 34% in Kärnten) by the same year (Kurz 2002).

Regarding inbreeding, Sjøvold (1986, 1995) reported that in Hallstatt it was almost nonexistent since around 20% of the population was from immigrant origin. Esparza (pers. comm.) has found low levels of endogamy and consanguinity in Hallstatt between the 17th and the 19th centuries. Most probably, people immigrating to Hallstatt came from the surrounding areas attracted by the salt industry. Many of them were young individuals looking for a job in the salt mines. Such population dynamics would explain that despite immigration, the genetic background of the population was fairly homogeneous. The population definitely opened to migration exchange at the end of the 19th century, after the Industrial Revolution, with increased mobility and improved means of transport.

In fact, until the end of the 19th century Hallstatt was a rather isolated village. It could only be reached by boat across the lake or by footpaths across the mountains. The first regular transport to Hallstatt was introduced in 1862, when a small steamboat was used to travel around the lake. The first road was not built until 1875, which connected Hallstatt to Gosaumühle. The construction in 1876-1878 of a railroad along the eastern side of the lake, the "Kronprinz Rudolf Kammergut Railway", represented the opening of Hallstatt and the arrival of many people, such as immigrants and visitors (Urstöger 1984). Some years afterwards, in 1890, another road was constructed along the west shore of the lake, the Seestrasse. And finally, in 1966, the Hallstatt road tunnel was built.

As explained above, mining activities have been carried out almost during the whole history of Hallstatt, despite some peaks and declines. The last massive salt extraction was carried out at the beginning of the 19th century in order to finance the war against France. Despite the incorporation of technical innovations, such as the introduction of electric power and the construction of a rail link, the salterns were closed down in 1965. Today, salt is still extracted from the Hallstatt's mines, though the brine is now piped down the valley to a modern treatment plant at Ebersee. Salt mining is not any more the principal resource of Hallstatt. Since the mid 20th century, Hallstatt has become a touristic hotspot (UNESCO 1996) and tourism is at present the main economic activity.

1.1.2 THE HALLSTATT'S SKULL COLLECTION

Figure 6. Graveyards from the cemetery of the Catholic church.

The origins of the Hallstatt's collection of decorated skulls can be traced back to the end of the 18th century. The collection was made up after the skeletal remains recovered from the grave burials (Figure 6). When the family claimed it, the graves were opened after approximately ten years of the decease and the remains were exhumed. The gravedigger was the person in charge of digging up the bones, cleaning and decorating them with different kinds of paintings (such as flowers, leaves, wreaths and crosses) and writings, as the year of decease and the individual's name (Sauser 1956). This is in fact the distinctive characteristic of the Hallstatt skull collection in comparison to any other skull collection: the name of the individual allows his identification. Given the fact that Hallstatt is a small population, relatives can also be identified and genealogies can be reconstructed thanks to the parish demographical records (see Appendix).

The decorated skulls are exhibited at the charnel house located nearby the cemetery and below St. Michael's Chapel. This is a small rectangular room with wooden shelves placed on the lateral and back walls (Figure 7): under the shelves are accumulated the exhumed long bones (which cannot be identified) and on the shelves are placed several rows of skulls. The skulls of influential people of Hallstatt (such as priests or mayors) have a predominant central location at the charnel house. This is also the case of those skulls with special paintings, such us two skulls with a snake painted on them. These were the father and the sister of one of the gravediggers who painted the skulls. Another brother of the gravedigger died by the same time and his skull also shows a snake. This one is stored, however, in Vienna at the *Naturhistorisches Museum.*

Figure 7. Charnel house (*Beinhaus* or Bone House).

Given the exclusive nature of the Hallstatt skull collection, it is no surprise to find that it has been previously studied by several researchers. However, most of the available information about the Hallstatt skull collection is due to Dr. Torstein Sjøvold from the Stockholms Universitet. His first surveys were carried out during the seventies and since then he has reviewed and expanded his studies, which have been published in several papers (Sjøvold 1984, 1986, 1987, 1990, 1995).

According to Sjøvold, the Hallstatt skull collection can not be considered a random sample for several reasons: first, because it was restricted to Catholics; second, because it was a family matter and some families were more prone to this tradition than others (especially the families of the gravediggers, which are by far the most represented ones); and third, because there is a sex bias, appearing many more males than females. However, it seems that there is no social stratification in the sample, at least during the 19th century, when the tradition was at its very peak. During the first decades of the tradition, skull decoration was more limited to members of the upper social groups, but afterwards no social group seems to be excluded, since the skulls represent people with all kinds of occupations: the collection includes the skulls of village mayors, officials, priests, doctors, housewives, professors, miners, woodcutters, workers and inmates of the poorhouse. Moreover, the decorated skulls tend to represent individuals born in Hallstatt as well as immigrants to the village.

The skull collection is under continuous supervision but unfortunately some skulls have been lost. This is evident from photographical records of the collection and by Sjøvold's reports, who has detected that some skulls have disappeared.

1.1.3 PREVIOUS STUDIES

The first evaluation and description of the Hallstatt skull collection was carried out by the end of the 19th century by a Viennese anatomist (Zuckerkandl 1883, 1898). Professor Zuckerkandl painted in pencil a number at the parietal of some of the crania. Hallstatt skulls were reanalysed from 1948 to 1952 by a research team from the Innsbruck Anatomisches Institut led by Gustav Sauser. During this period, Professor Sauser and his disciples made an inventory of the skulls, numbered all of them with a pencil at the occipital, took some craniometric measurements and described their kind of decoration (Sauser 1956). This data was also used for some anthropological analyses. There is one

study that assessed cranial sexual dimorphism at the Hallstatt collection and showed no significant correlations between males and females (Olbrich 1962).

After these first reports, the Hallstatt skull collection was not scientifically assessed until Dr. Sjøvold focused his interest on it 25 years later. In a chapter book, Sjøvold (1987) described the vicissitudes he experienced during his first stays in Hallstatt, when he identified the decorated skulls, reconstructed the family trees and measured the skulls with traditional callipers. Sjøvold was the first researcher that used a historic human skull collection to assess the heritability of cranial traits.

At his first contribution, Sjøvold (1984) reported that 346 skulls from the Hallstatt collection turned out to fall into 91 pedigrees, in which the skulls of between 2 and 10 family members were identified, with a mean number in each of 4.29. With this data, he estimated the heritability of cranial metric and non-metric variables by means of regression techniques between first degree relatives (that is, between parents and offspring). His results showed that cranial features have significant hereditary factors. In his next paper, Sjøvold (1986) assessed the cranial morphological differences (in particular, the distribution of non-metric traits) between the people actually born in Hallstatt and the immigrant people and analyzed the influence of this immigration on the population structure of Hallstatt. Despite some significant changes that were observed for a few traits and during specific periods, results pointed out an overall homogeneity.

In his last contributions concerning Hallstatt, Sjøvold (1990, 1995) analyzed in great detail the effects of secular trend at the skull morphology of the Hallstatt sample. His results pointed out a general debrachycephalisation of the skull, at least from the end of the 18th century. The detected trend consisted in a general decrease in cranial width and a constancy of cranial length and cranial capacity. Moreover, Sjøvold (1995) reassessed the heritabilities of cranial measurements using four different parent-offspring combinations and found that few measurements showed a consistent hereditary pattern throughout all combinations. According to his results, Sjøvold (1995) suggested that some kind of sex effect could underlie the heritability of cranial morphology, since he noticed that male offspring resembled more their parents than female offspring did.

Besides Sjøvold's studies and the present one, the other studies based on the Hallstatt's collection of decorated skulls were performed recently by

Elisabeth Ann Carson, a PhD from the University of New Mexico. In these studies, the heritabilities of metric and non-metric cranial traits were reassessed using more sophisticated statistical techniques (Carson 2006a, 2006b). Carson (2006a) also reported that craniometric measurements show low to moderate heritabilities but pointed out some differences between her results and those of Sjøvold's. Regarding non-metric traits, Carson (2006b) showed that heritabilities were non significantly different from 0. The results obtained by these studies are discussed with further detail in the next introductory chapters (Quantitative Genetics, 1.5.5).

In this introductory chapter, it was important to note that this unique collection of decorated skull has been previously analyzed. The present work has further analyzed this material using different approaches and with diverse scopes. As explained above, the main goal of this thesis is to apply geometric morphometric techniques and multivariate quantitative genetics to explore the genetic patterns underlying phenotypic craniofacial variation and to use them in an evolutionary context to test hypotheses about the evolution of the human skull.

1.2 *The human skull*

The human skull is an osseous morphological complex whose architectural rules are far from being understood. In this chapter a description of the human skull is provided in order to give an insight into its anatomy, embryological origin, developmental process and functional requirements. Moreover, some emphasis is given into the current knowledge about the genetic and epigenetic factors that regulate skull development. This is a relevant background for this thesis because the analyses herein are grounded on these principles. The skull is a composite structure and its adult morphology is the result of a long and complicated ontogenetic sequence. A detailed knowledge of the events and mechanisms that lead to skull formation and growth is essential for understanding how phenotypic morphological variation is generated.

1.2.1 ANATOMY & FUNCTION

Anatomically, the skull is first differentiated into the cranium and the mandible (Aiello and Dean 1990). The cranium comprises the mid and the upper facial skeleton, the calvarium or cranial vault, which superiorly and laterally surrounds the brain, and the cranial base, which covers the inferior part of the brain (Figure 8). This thesis is focused on the cranium because the mandible and the dentition were lacking at the sampled skulls.

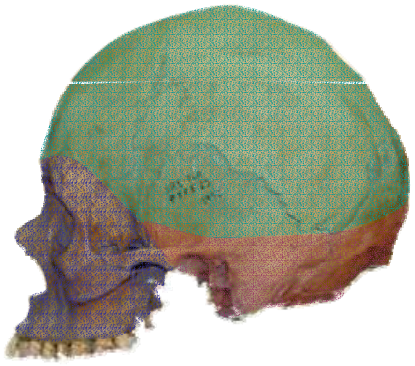

Figure 8. The three main developmental regions of the human skull. Face (blue), the cranial vault (green) and the cranial base (red).

The skull is an integrated unit that accomplishes several functions: on the one hand, it gives room and protects the brain and the sense organs of sight, smell and hearing; on the other hand, it

supports the masticatory structures. Evolution within primates is related to the differential development of these parts (Ackerman and Cheverud 2004a).

The cranial vault is formed by the fusion of several flat bones (Figure 9): the frontal (which derives from two fused halves), the paired parietal and temporal bones and the occipital bone. The non-squamous parts of the temporal and the occipital bones also participate on the formation of the cranial base, along with the sphenoid and the ethmoid. The facial complex is made by the zygomatic, maxillary, nasal, lacrimal, palatine and vomer bones.

Bones are articulated by sutures, which in general are named after the bones they articulate. However, four main sutures receive a special name: the sagittal suture, which joins the parietal bones; the coronal suture, along which the frontal, the parietal and the sphenoid bones meet; the lamboid suture, which articulates the occipital with the parietal bones; and finally the squamosal sutures, which connect the temporal and the parietal bones.

More detailed descriptions of the individual cranial bones and endocranial structures of the skull are beyond the scope of this introductory chapter because the morphological analysis presented in this thesis is limited to the external cranium. For a thorough description of the human skull anatomy see specific textbooks (Steele and Bramblett 1988, Aiello and Dean 1990, White and Folkens 1991).

1.2.2 SKULL EVOLUTIONARY TRENDS IN VERTEBRATES

In vertebrates, the skull derives from the primary cartilaginous template of fishes. From this basic structure appeared three different skull "designs" (Mooney et al. 2002). The first model concerns a complete cartilaginous skull: in Elasmobranch fishes (sharks, rays and skates), the brain is covered by a roof made of cartilage, which is not homologous to mammalian calvaria. The roof fuses with the chondrocranium but these structures never ossify. This represents the first emergence in evolution of a complete braincase. The second model is also cartilaginous, but this is not complete: it just presents some ossified regions and dermatocranial elements that form the partial roof that covers the brain. This is the case of some Osteichthyes (bony fishes). Finally, the third model represents a complete ossified braincase and is the type of skull of many teleost fishes, reptiles and mammals. The base is derived from ossification of the ventrolateral chondrocranium and the cranial vault is made exclusively from dermal (intramembranous) bone. In comparison to reptiles

and ancestral tetrapods, mammalians present a reduced number of cranial vault bones (Mooney et al. 2002).

Phylogenetically, the cranial base is the most ancient structure and derives from the cranial floor, while the cranial vault and the face are of more recent origin. The chondrocranium represents a vestigial structure of the vertebrate skull, whose basic pattern has been highly preserved through phylogeny (Carlson 1999). The cranial vault developed in order to cover the expanded brain and is derived from the evolution of incipient dermal plates of early jawless fishes (Morriss-Kay and Wilkie 2005). Finally, the facial skeleton originated from modification of branchial-arch structures (Sperber 2001).

In general, the older the structure is, the more conservative it is to evolutionary changes. The chondrocranium is strongly genetically determined, whereas the desmocranium and the splanchnocranium are thought to be more sensitive to environmental factors (Sperber 2001). One feasible explanation is that cartilage may be under more conserved genetic constraints than bone (Schilling and Thorogood 2000). The facial skeleton is perhaps the skull region that displays a wider variety of forms and is more affected by nongenetic factors, because it plays a key role in foraging and adaptation to environment and because its growth is more extended into the postnatal period (Siebert and Swindler 2002). In the present thesis we have the unique opportunity to directly measure the influence of genetic and environmental variation in the human skull phenotype.

1.2.3 MORPHOGENESIS & DEVELOPMENT

Evolution has led to a skull that is made up of three different regions that have different developmental origins. It is important to explain in some detail how the process of bone development works in the human skull. Recent investigations have revealed that skeletogenesis comprises four main processes (Hall and Miyake 2000). First, it starts with the migration of undifferentiated cells to the growth site; second, an epithelial-mesenchymal interaction is produced to activate osteogenesis at the growth site; third, this signal produces a cell condensation; and finally, cells differentiate into chondroblasts or osteoblasts that will produce bone (Richtsmeier 2002).

In the human embryo, skull primordials arise from the rostral portions of the neural tube (the notochord) as well as the pharynx, which is surrounded by a series of paired aortic arches. Between these structures and the overlying ectoderm there are large masses of neural crest and mesodermally derived

mesenchyme (Carlson 1999). Neural crest cells derive from the caudal end of the future brain (hindbrain), where the neural crest is organized in seven segmented rhombomeres (Ahlberg 1997). Both neural crest cells and mesodermal cells will give rise to the developing skull bones.

Two different ossification processes operate to give rise to the human skull: facial and cranial vault flat bones originate from intramembraneous ossification, while cranial base ones derive from endochondral ossification (Figure 9). However, both ossification types can jointly operate to produce a single bone. As stated above, both processes start with a condensation of cells but differ in the way bone is produced.

In intramembraneous ossification, mesenchymal and neural crest cells directly differentiate into osteoblasts and form ossification centers at periosteal membranes. Osteoblasts are the cell-types responsible for new bone formation. They produce osteoid, a protein-derived matrix mainly made up of Type I collagen that becomes bone after mineralization. Osteoblasts also facilitate mineral deposition (i.e. calcium, phospates) within bone matrix and produce hormones to control bone formation. Osteoblasts arise by differentiation of cells located at near bone surfaces. Differentiation is controlled by the expression of two genes, core-binding factor alpha 1 (Cba-1) and Indian hedgehog (IHH). Bone formation begins at genetically determined ossification centers and is induced by growth factors (FGF, PDGF, TGF-β) and bone morphogenetic proteins (BMPs). Bone growth occurs by deposition of osseous tissue along the periosteal membrane. Bone deposition by osteoblasts is always compensated by bone resorption by osteoclast cells. The whole bone remodelation process is under hormonal control (hypercalcemic parathyroid hormone, hypocalcemic calcitonin, as well as sex steroid hormones). The final size of a bone is a direct response of the starting time of osteogenesis: the earlier the onset, the bigger the bone is (Sperber 2001).

In endochondral ossification, the formation of bone is preceded by the formation of a cartilaginous matrix of glycoproteins that after mineralization will be replaced by endochondral bone (Sperber 2001). The process starts with the differentiation of chondroblasts, which are matured into chondrocytes that first produce a cartilaginous matrix. Afterwards, chondrocytes calcify the cartilaginous matrix and then undergo cell apoptosis. They are replaced by osteoblasts brought by blood vessels that penetrate the calcified matrix, which serves as a template to build the endochondral bone. Given that intramembraneous ossification does not require this previous step, it is faster than endochondral ossification.

Overall, the development of the skull is the result of combined morphogenesis and growth of two main regions (Carlson 1999): the braincase (neurocranium) and the facial skeleton (viscerocranium). According to their different developmental origins, the neurocranium is divided into two regions (Sperber 2001). The first region, the cranial vault or calvaria, is formed from membranous bone of paraxial mesodermal and neural crest origin, the desmocranium; while the second, the basicranium, is formed from endochondral bone that arises from a cartilaginous precursor, the chondrocranium, which originates from mesoderm (Mooney et al. 2002). The cranial vault gives room and protects the cerebral hemispheres and cerebellum, while the cranial base supports the inferior parts of the brain as well as the pons, the medulla oblongata and the brain stem (Richtsmeier 2002). The facial skeleton (the splanchnocranium or viscerocranium) ossifies intramembranously like the cranial vault but just from neural crest precursors (Sperber 2002). The splanchnocranium surrounds the pharynx and the oral and respiratory cavities, supporting the functions of feeding and breathing (Figure 9).

In the following sections, the development of each of these main regions is addressed with deeper detail.

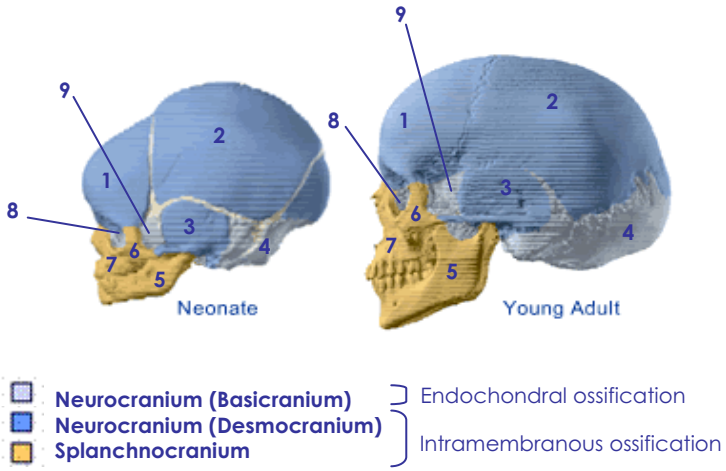

Figure 9. Ossification processes. Numbers indicate the main bones of the skull: 1, frontal; 2, parietal; 3, temporal; 4, occipital; 5, mandible; 6, zygomatic; 7, maxilla; 8, ethmoid; 9, sphenoid. Modified after the Center for Craniofacial Development and Disorders website.

Neurocranium: Desmocranium & Basicranium. Although there is still controversy about the origin of several neurocranial bones, it is likely that most cranial vault bones derive from both paraxial mesoderm and neural crest, while the cranial base is just of neural crest origin (Richtsmeier 2002). The emergence and

incorporation of neural crest cells in skull development was one of the main evolutionary innovations that led to the vertebrate skull. Neural crest cells not only participate in the formation of bones, but they also produce muscular tissue and determine highly constrained skeletomuscular connectivity (Köntges and Lumsden 1996). The experimental work carried out by these authors revealed that rhombomeres generate skeletomuscular 'packets' containing the necessary elements to produce connective muscular tissue and the two skeletal units to which the muscle attaches. Furthermore, each of these units is innervated by the same nerve. This highly integrated pattern between hard (skeletal) and soft tissue (muscular) is essential to establish and maintain the integrity and functionality of the skull. The developmental programming of the rhombomeres is of crucial importance for the coordinated growth of the skull, since it determines the formation of the viscerocranium and how this is linked to the neurocranium through muscular attachments (Ahlberg 1997).

Neurocranial development is completely dependent on the presence of the brain, whose morphogenesis is in turn controlled by different Hox genes (Sperber 2002). In absence of brain (anencephaly), cranial vault bones do not form. Neurocranial bones arise after an interaction of mesenchymal cells with epithelial structures (such as the brain), which is mediated by growth factors that interact with the extracellular matrix of bone. Skull morphogenesis is also controlled by these interactions (Carlson 1999).

Neurocranial development relies on the formation of a capsular membrane surrounding the brain (Sperber 2001). This membrane derives from mesoderm and neural crest ectomesenchyme and differentiates into two layers: the endomeninx and the ectomeninx. The endomeninx gives rise to two inner membranes, the pia mater and the arachnoid meninges, while the third meninge, the dura mater, differentiates from the ectomeninx. The extomeninx also gives rise to an outermost layer, the skeletogenic membrane, which will provide the osteogenic basis for braincase development. The two ectomeninx layers remain closely related by fibrous bands. These dural folds connect the dura mater to the sutural system of the cranial vault, constraining and guiding the direction of brain growth.

Both the cranial vault and the base derive from the osteogenesis of the ectomeninx, but as explained above, they follow different ossification processes. Primary and secondary ossification centers develop in the outer layer of the ectomeninx to form the individual bones. Cranial vault bones (paired frontal, paired parietals and squamous portions of temporal and occipital bones) develop from intramembranous direct ossification of the

ectomeninx related to the expanding brain. The formation of the desmocranium starts with the emergence of ossification centers during the 7th and 8th weeks of fetal life. The ossification process is gradual and extends postnatally.

Basicranial bones (sphenoid, petrous temporal and basioccipital) develop from endochondral ossification of the chondrified ectomeninx surrounding the floor of the brain (Sperber 2001). The inductive influence of epithelial structures on the mesenchymal condensations formed at the base of the ectomeningeal capsule activates the formation of chondrification centers, from which basal structures arise (Carlson 1999). Initially, the chondrification centers are separated but later fuse into a basal plate, which is perforated (Sperber 2001). These foramina are passage canals for blood vessels, cranial nerves and the spinal chord that contact the brain with the neural and circulatory systems. Communication with the brain is restricted to the skull base, because no channels form through the membrane calvarial bones (Morriss-Kay and Wilkie 2005).

This cartilaginous platform located beneath the forming brain is the "rough draft" of the basicranium and it first appears in the second month of embryonic life (Sperber 2001). Bone precursors consist of several sets of paired cartilages: there is one group related to the development of midline basal structures (the parachordals, the hypophyseal and the trabeculae cranii); another type of cartilages are the occipital scleretoms, which develop more caudally, and these interact with the parachordals in order to form the occipital bone; and finally, there is a set of cartilage capsules that develop surrounding the sense organs, such as the eyes and the olfactory and auditory organs.

The whole braincase forms from the differential fusion of vault and basal bones. Cranial vault sutures are fibrous tissues and are centers of bone growth that allow certain movement between bones until the skull reaches its adult final size and shape, when sutures become totally fused. Craniosynostosis affects the normal developmental morphology of both the neurocranium and the facial skeleton (Richtsmeier 2002). Recent experimental studies in mouse embryos have evidenced that the location of sutures in the skull is related to the boundaries between neural crest and mesoderm derived tissues (Morriss-Kay and Wilkie 2005).

Splanchnocranium. Facial bones develop intramembranously from the ossification centers located in the neural crest mesenchyme of the facial embryo prominences. The differentiation of facial bones starts after the

interaction between the ectomesenchyme of the prominences and the epithelium surrounding them. However, some facial bones associated with the upper and lower jaws and the middle ear develop from a cartilaginous precursor, the first arch cartilage or Meckel's cartilage.

The face is subdivided into three regions. The upper face contains the orbits; the midface includes the nasomaxillary complex and connects with the cranial base; and finally the lower face is formed by the mandible. These regions correspond to the frontonasal, maxillary and mandibular prominences of the embryo, which appear during the third intrauterine month (Sperber 2001).

The first region to develop is the upper face, because it is directly connected with the neurocranium and is influenced by the fast development of the frontal lobes of the brain. The middle and lower parts grow later and more slowly until adulthood. As in the neurocranium, ossification centers appear during the 7^{th} and 8^{th} weeks of fetal life.

Within the upper face develop the orbital cavities, which are the protective chambers for the eyes. The orbital cavities are formed by a complicate interaction of several bones: the roof is composed of the frontal bone; the lateral walls and the floor of the cavities are formed by the lacrimal, the ethmoid, the maxilla, the zygomatic and the palatine bones; and finally the posterior wall is formed by the sphenoid. The growth of the orbital cavities seems to be closely linked to that of the brain, rather than with the growth of the eyeballs, as previously thought (Siebert and Swindler 2002). The evolutionary changes that led to the frontal position of the orbits in humans seem to be related with the greater development of the visual capacity, rather than to masticatory stress (Ravosa et al. 2000).

Within the midface develops the nasal cavity, which is also a complex composite structure. It is enclosed by the nasal bones, the maxilla, several ethmoidal components and the palate, which separates it from the oral cavity. Inside the nasal cavity there are also the inferior turbinate bones, the vomer and a cartilaginous nasal septum. In comparison to other primates, the nasal cavity in humans is shorter, higher and larger. This morphology is considered as a consequence of increased basal flexion and encephalization (Siebert and Swindler 2002) and supplies humans with greater respiratory abilities.

The nasal cavity is coordinated with the maxilla, which carries out two important functions: first, it holds the upper dentition; second, it supports and dissipates from the midface the mechanical loadings of the masticatory complex. In humans, the maxillary arch undergoes resorptive growth, which

locates the maxilla in a more downward position in comparison to nonhuman primates and produces a flattened face (Siebert and Swindler 2002). Likewise, the zygomatic bones' size and shape are also influenced by chewing, since the zygomas are the insertion sites for the masseter muscles.

Facial growth is influenced by three main factors. First, it is affected by the development of the cranial base, because these regions are intimately attached through the sphenoid, the maxillary and the palatine bones. Second, it is also influenced by the development of the sense organs, such as the eye, the nasal cavity, the nasal septum and the external ear. And third, it is further regulated by the interaction with the non-osseous structures of the masticatory complex, as the tongue, the teeth and the masticatory musculature.

1.2.4 GROWTH

The neonatal human skull is made up of 45 individual bones that derive from at least 110 separate ossification centers. These bones continue to grow after birth until their final sizes and shapes are reached. Different bones meet at cranial sutures that gradually become fused. In the young adult, 22 skull bones are recognized.

At birth, the individual cranial vault bones are separated by sutures and fontanelles, which are membranous junctions of connective tissue between bones (Figure 10). The most prominent fontanelles are those located around the two parietal bones. Along with postnatal growth, fontanelles disappear and sutures become fused. The anterolateral fontanelles (which correspond to the pterion in the adult skull) close three months after birth. The posterolateral fontanelles (asterions), as the anterior one (bregma), closes during the second year of life. Finally, the posterior fontanelle (Sperber 2001) closes two months after birth.

The different skull regions grow during different developmental times and after different factors. The base is the first region to develop, followed by the cranial vault and the face (Sperber 2001). The growth of the neurocranial structures (both the base and the cranial vault) is driven by the growth of the expanding brain and occurs early during the ontogeny, during the prenatal and neonatal periods. The face develops later, once the brain has finished its growth. The face and the mandible grow during a more extended period of time, reaching its maturity at an early age (Figure 11). The cranial structures related to the development of sense organs are almost fully grown at birth.

Despite balanced growth of the skull as a whole unit, each bone has its particular timing and growth rate. Enlow (Enlow and Hans 1996, Enlow 2000) developed the idea of differential growth, assuming that the global growth of the skull is the result of a coordinated combination of several local and regional growth trajectories. According to this hypothesis, bones are changing shape and increasing in size due to local patterns of osteogenesis. Recent investigation (Bastir et al. 2006) supports the existence of craniofacial developmental levels and specifies the spatio-temporal sequence of ontogenetic maturation of the craniofacial complex.

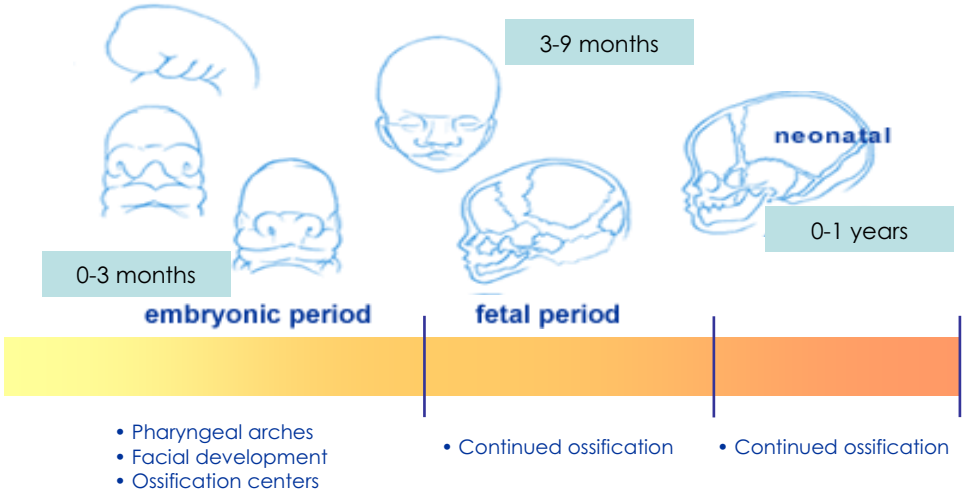

Figure 10. Cranial growth in early ontogeny. Modified after the Center for Craniofacial Development and Disorders website.

Regarding skull shape, it has been reported that the first region to reach maturation is the midline cranial base (7.7 years old), followed by the lateral cranial floor and the neurocranial outline (11.7 years). The last region to maturate is the face, which is full-grown at 15.7 years (Bastir et al. 2006). Regarding skull size, Bastir et al. (2006) also found an ordered sequence of maturation, but this is slightly different from that of shape. The neurocranial outline is the first region to attain adult size (11.4 years), the second is the midline cranial base (13.6 years), and the last ones are the lateral cranial floor together with the face (15.7 years). Another interesting finding of this research is that the basicranial region can not be considered a compact unit, at least at the ontogenetic level, since different basicranial elements (namely the midline cranial base and the lateral cranial floor) show dissociated size and shape maturation patterns (Bastir et al. 2006).

The predominance of the neurocranium over the face is greatest in the early fetus, but reduces progressively after birth (from a 8:1 proportion to 6:1

in the second year and 4:1 in the 5th year). It reaches the final proportion (2:1) by adulthood (Sperber 2001). At the age of 10, neurocranial growth is 95% complete, but the face has only achieved 65% of its total growth. Growth during adolescence is under hormonal control. In humans, as in mammals, skull growth ends around sexual maturation. However, this is not the case of many fishes, amphibians and reptiles, in which skull growth continues throughout their complete life (Morriss-Kay and Wilkie 2005).

Bone structure is also modified throughout life. For instance, calvarial bones are unilaminar and lack diploë at birth (Sperber 2001). However, this structure changes at four years old: by this time lamellar compactation of cancellous trabeculae occurs and gives rise to two cortical tables, the inner and the outer table. The inner table is more closely related to brain development and is more sensitive to intracranial pressures, whereas the outer table is more responsive to extracranial pressures (as environmental factors or musculatory mechanical loadings). Although they are not completely independent, this differentiation further isolates the brain from external stress. Several distinctive features of the human skull (which are closely related to sexual dimorphism and the development of robust skulls) result from the separation of the cortical tables and the thickening of the outer table, as the development of the glabella, the superciliary arches, the mastoid processes, the external occipital protuberance and the temporal and nuchal lines.

Expanding growing bones are the result of two processes: remodeling and transposition (Sperber 2001). On the one hand, remodeling is a combination of osteoblastic deposition and osteoclastic resorption of bone. It can be produced as a response to periosteal functional matrices and causes bone shape changes. The rate of remodeling is proportional to overall growth rate. On the other hand, transposition consists in bone displacement, which is caused by forces exerted by the surrounding soft tissues and overall bone growth. Both processes may occur at the same or at different directions.

Deposition occurs either by apposition of new bone at the surface, which increases bone thickness, or by apposition at the sutures, which allows the expansion of growing bones and facilitates displacement between them. Sutures are growth sites where bone remodeling takes place without a cartilaginous precursor and this needs to be activated by an external signal (Richtsmeier 2002). Bone growth progresses following a direction vector that is perpendicular to sutural planes (Sperber 2001). Differential apposition of bone determines the relative growth of individual sutural bones. The ossification of bone articulation causes sutural fusion and bones are thus constrained to stop

growing. Different sutures fuse at different times, but overall fusion patterns can be used as a reliable age estimator. Premature synostosis inhibits growth in the expected direction but this is compensated by stimulating an abnormal growth in other directions. Therefore, the skull keeps growing but causing specific malformations and dysmorphologies.

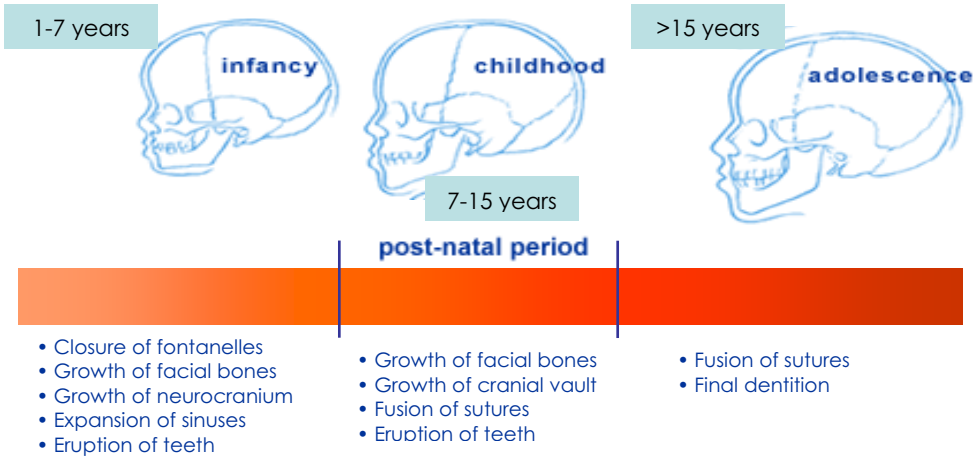

Figure 11. Cranial growth in late ontogeny. Modified after the Center for Craniofacial Development and Disorders website.

1.2.4.1 *Normal suture pattern.*

Cranial vault sutures remain open until early adulthood (around 20-25 years old). Afterwards, they began to fuse and this process can take from 15 to 20 years. The main cranial sutures become totally closed by the age of 40: the sagittal and the coronal sutures fuse a bit earlier (around 35 years old), whereas the lamboidal is the last one to close.

Cranial base sutures are called synchondroses and are growth centers allowing interstitial cartilaginous expansion between ossified portions of the cranial base (Mooney et al. 2002). Three synchondroses produce the anteroposterior growth of the skull base (the presphenoethmoidal, the midsphenoidal and the spheno-occipital). In humans, the midsphenoidal synchondrosis fuses just before birth, whereas in many mammals it does not fuse until advanced age. The presphenoethmoidal synchondrosis contributes to cranial base elongation until approximately 7 years of age and fuses later on, between puberty and adult ages. Finally, the spheno-occipital synchondrosis is the most contributing one and it is completely closed by age 20. The

basioccipit, basisphenoid, presphenoid and mesethmoid bones participate in an anteriorposterior growth during the adolescence burst.

The growth of the human cranial base can be dissected into the following phases: a rapid growth from birth to 5 years; a deceleration between 5 and 12 years of age; a parapubertal acceleration; and finally a deceleration and cessation of growth around 20 yeas of age, when the spheno-occipital synchondrosis becomes fused (Mooney et al. 2002).

Cranial base flexion is established early in ontogeny, when the ossification of the skull base starts (between the embryo 10 and 20 weeks) and does not change after two years of age. The main site of cranial base kyphosis is located at the midsphenoidal synchondrosis. There are several measures of cranial base flexion, but a widespread one is the angle Na-MST-Ba (nasion-midsella turcica-basion). In humans, it typically measures between 125º and 130º. Angulation changes are probably caused during fetal and perinatal periods (Lieberman et al. 2000a, 2000b).

1.2.4.2 *Skull growth control.*

Integration and controlled growth between craniofacial structures is necessary for normal development. Initial craniofacial morphogenesis is directly dependent on the expression of homeobox genes (Siebert and Swindler 2002), which encode transcription factors that regulate gene expression during early development. This is especially important to determine the patterning of craniofacial components.

It is well-known that afterwards genetic, environmental and mechanistic factors influence the development of the craniofacial complex (Sperber 2001). However, it remains unclear how changes in morphology and function correlate with genetic changes (Siebert and Swindler 2002). The inherited genotype settles down the genetic architectural rules to construct the skull, but because of slow and gradual bone growth and remodelation through life, its final phenotypic expression will be further modulated by the expression of genetic control mechanisms; nutritional, biochemical and physical factors; as well as functional factors depending on the development of related soft-tissues such as muscles and organs. This is the basis of the functional matrix hypothesis, postulated by Moss (Moss and Young 1960, Moss 1962, 1968, 1969, Moss and Salentijn 1969a, 1969b).

Epigenetic regulation of skull morphogenesis can occur at three different hierarchical levels: in early development, when cell differentiation within

mesenchymal condensations can be induced by epithehial-mesenchymal interactions; during growth, when interactions between neighbouring hard and soft tissue (e.g. muscle and bone, brain and cranial base) can modulate skull morphology; and finally throughout ontogeny, when further epigenetic interactions can occur between cells within a unit and the rest of the organism. These are mediated via hormones as a response to environmental influences.

1.2.4.3 *Functional Matrix Hypothesis (FMH).*

The functional matrix hypothesis is considered here with some detail because it has a long tradition in physical anthropology. Numerous studies dealing with the ontogeny, growth, development and integration patterns of the primate skull are grounded on the principles of FMH (Cheverud 1982, 1984, 1988, Enlow 1990, Richtsmeier et al. 1993b, Enlow and Hans 1996, Lieberman et al. 2000a, Marroig and Cheverud 2001, Ackerman and Cheverud 2004, Marroig et al. 2004, Bastir et al. 2004, Bastir and Rosas 2004b, 2004c, 2005, 2006). This research framework has also been adopted in order to use morphometric characters as biological markers to study the history and structure of human populations' (Pucciarelli et al. 1990, Pucciarelli et al. 2000, González-José 2003, González-José et al. 2005b, Sardi et al. 2006, Martínez-Abadías et al. 2006, Pucciarelli et al. 2006).

According to FMH, form follows function, and hence function determines, controls and regulates form. Functional components were first described by Klaauw (Klaauw 1948). The FMH framework is based on four main concepts (Moss 1962). First, it considers that the skull consists of cranial skeletal units whose origin, final size, shape, location and maintenance are the result of secondary, compensatory and obligatory responses to prior demands exerted by their neighboring nonskeletal cells, tissues, organs and operational volumes. These nonskeletal units are the so-called functional matrices and are considered the key structures of skull morphogenesis. Second, FMH claims that the factors and processes regulating morphogenesis are basically epigenetic. Third, it considers that bone growth occurs through the operation of three skeletal processes: deposition, resorption and maintenance. And finally, FMH distinguishes two types of functional matrices: periosteal and capsular matrices (Moss 1969). Periosteal matrices involve skeletal muscles that induce active growth of the bone and thus affect its final size and shape. In contrast, capsular matrices consist of soft tissue organs or cavities (e.g. the brain or the oral cavity) that cause passive growth (with no deposition or resorption). This can influence the position of the skeletal units but not their

final form. According to FHM, the sum of the functional matrix and a skeletal unit makes up a functional cranial component.

Originally, the functional matrix hypothesis argued that only epigenetic factors were responsible for bone morphogenesis (Moss and Young 1960, Moss 1962, 1968, 1969, Moss and Salentijn 1969a, 1969b). However, this assertion has been challenged with the development of molecular genetics, which have provided conclusive evidence of genetic and molecular regulatory mechanisms operating in skull ontogeny. As a response to the controversy generated by the genetic/epigenetic dichotomy, Moss revisited the functional matrix hypothesis in a series of four papers (Moss 1997a, 1997b, 1997c, 1997d). In these works he has provided further support to the primary role of function in craniofacial growth and development, but has acknowledged the genomic regulatory activity of morphogenesis.

The revision of the FMH is also relevant because it has overcome some of its main explanatory constraints. Thanks to recent advances in biomedical, bioengineering and computer sciences, it now provides a more plausible explanation for the mechanisms by which periosteal functional matrices' stimuli are transducted into regulatory signals by individual bone cells. Moreover, it explains how an intercellular communication is established to produce coordinated responses. This is a hierarchical description of a chain relating the contraction of a skeletal muscle to bone remodelation through a series of cellular and molecular signaling processes. According to Moss (1997a, 1997b), mechanotransduction in single bone cells and the presence of connected cellular networks (CNN) are the two concepts that need to be included in the new extended version of FMH.

Mechanotransduction is one type of cellular signaling that traduces an extracellular physical signal into an intracellular signal. This process allows the transmission of information from mechanoreceptors (cells that sense perturbations of their external environment) to cells that will respond to this extrinsic force (i.e. a muscular loading to bone tissue). Moss (1997a) states that when a periosteal FM loading stimulus exceeds a threshold value, the process of bone adaptation activates, inducing osteoblasts and osteoclasts to bone remodeling. The stimulus can be translated into electric (ionic) and/or mechanical signals. On the one hand, the ionic transport among cell plasma membranes and extracellular fluids creates an electric flow that is transmitted through the osseous connected cellular network, which regulates the multicellular bone responses. On the other hand, the mechanical signaling process relies on the transmembrane molecule integrin, which connects the

extracellular bone matrix with the intracellular nuclear membrane. The integrin acts as a mechanical lever that can provide a mechanical stimulus which is capable to activate the osteocytic genome and hence its phenotypic expression.

Intercellular connectivity seems to be mediated by gap junctions allowing bidirectional flow of information between osteoblasts and osteocytes (Moss 1997b). Interconnected groups of osteoblasts form a cohort, which is independent from other cohorts because gap junctions between them are closed. Therefore, the flow of information is prevented. Altogether, they make up connected cellular networks. Structurally, a CNN is non-modular and the variations in its organization permit discrete processing of differential signals. This allows the processes of bone remodeling to operate when a muscular demand is altered within a periosteal functional matrix. It is plausible that both electrical and mechanical transductive processes are operating at the same time to epigenetically regulate bone form. According to this, the plasticity of the skull relies on the ability of the bone to respond to these functional stresses.

The main conclusion of this revision work is that both genomic and epigenetic processes and mechanisms are necessary for the control of morphogenesis, but neither alone is sufficient cause. Moss (1997d) argues that only their integrated activities can generate bone growth and development. Genomic causes are thus considered as intrinsic and prior causes, whereas epigenetic factors are considered as extrinsic and proximate causes.

1.2.5 VERTEBRATE EVOLUTION OF THE BRAIN

The enlargement of the brain and the development of specialized sense organs were a consequence of cephalization, a process that involved a reorganization and concentration of nervous tissue at the cranial end of the body (Mooney et al. 2002). In vertebrates, the appearance of these structures and the need to support and to protect them yielded the formation of a rigid braincase. Cephalization also concerned the reorganization of the gill apparatus, as well as some musculatory and nervous systems.

In vertebrate evolution, the tendency to progressively produce larger brains involved the differential development of brain regions. Furthermore, the need to fit the growing brain in the protective braincase caused a special neural spatial packing. From the fishes and reptiles' neural pattern, with a horizontal arrangement of a 'poorly' developed brain, the brain became bigger, more complicated and increasingly flexed. The next step after cephalization was the cerebralization of the forebrain (Mooney et al. 2002), which initially gave rise

to the development of bilateral olfactory organs. In mammals, the brain is further characterized by a greater development of the cerebral cortex (especially the parietal and the temporal lobes), which results in the functional dominance of the cerebral hemisphere. Obviously, these brain changes were also reflected in the braincase: cephalization caused a large increase in size of the braincase, and cerebralization caused a general widening and elongation of the middle and posterior cranial fossae.

Finally, in primate evolution, the developing brain experienced a greater development of the frontal and the occipital lobes. However, the most outstanding shift was the gyrification of the entire cortical surface (Mooney et al. 2002). The convolution of the cortex allowed a high increase of the cortical surface but constrained the increase of overall brain size. These neural changes are consistent with adaptive changes to an arboreal environment. The spatial packing of the neocortex caused substantial changes in the primate skull, the most important of which is the extreme kyphosis of the basal region, although this hypothesis is not supported by other studies (Jeffery 2003). Brachycephalization also evolved as a consequence of cranial base flexion (Enlow 1990). According to Mooney et al. (2002), kyphosis is the result of a synergistic interaction of brain expansion and growth activity at the cranial base synchondroses. Cranial base sutures have a high genetic component of determination, but they can also be influenced by epigenetic factors, such as the growing neural tissue. The timing of synchondrose fusion is a crucial factor in primate skull evolution (Jeffery and Spoor 2004).

In primates, cerebralization and gyrification have also determined the evolutionary changes experienced by the capsules containing special sense organs (Mooney et al. 2002). On the one hand, the anterior expansion and gyrification of the neocortex reduced and displaced the olfactory cortex to a more ventrolateral position. The decreased development of the olfactory area is also associated to a lesser dependence of primates on the sense of smell. These neural changes are reflected in the skull morphology in several ways: the horizontally rotated and shortened cribriform plate, the overall decreased length of the nasal capsule and the more inferior position of the nasal capsule relative to the skull base. The optic capsules also experienced significant evolutionary changes along with the increasing importance of the eyes as the dominant sense organ. After cerebralization and gyrification, the optic capsules were relocated in a more antero-inferior and central position. In primates, bone orbital cavities are formed to fully protect the eyeballs. Finally, the otic capsule and the inner ear structures evolved from the chondrocranium, and

neocorticalization and extreme skull base flexion reoriented the vestibular axes of the semicircular canals (Mooney et al. 2002).

Overall, this information has been taken into account throughout the thesis in order to design the analytical framework and to put forward the hypotheses tested at each of the Results chapters. This background is relevant for understanding the human skull and its evolutionary trends, which are discussed in deeper detail in the next section.

1.3 *Evolutionary Trends in Hominid Evolution*

Although paleoanthropology is beyond the scope of this thesis, a revision of the main evolutionary trends of human evolution is relevant for several reasons. First, it is important because the Hallstatt sample represents a unique dataset that can provide information about the genetic variation underlying skull morphology, which is one of the main sources of information of fossil hominids. Second, because it is essential to detect which craniometric traits are under significant genetic control and are thus reliable phylogenetic characters. And finally, because the analyses derived from the Hallstatt sample can give insight into the evolution of modern human craniofacial form by testing the likelihood of different selective hypotheses.

Human evolutionary history encompassed many morphological, physiological and behavioral changes. However, two major trends were of crucial importance. The first one was the acquisition of bipedalism, which among other factors led to the separation between apes and hominids between 5 and 8 Ma ago. The second trend implied an increase of body size, but especially of the brain, which marked the divergence between australopithecines and early *Homo* around 1.8 Ma. Despite the magnitude of these changes, it is still controversial to define clear boundaries within the hominid phylogeny. The position of some fossil species lying in between these taxonomic groups is under continuous revision. For instance, anthropologists have not reached a consensus on which fossils could be considered as the common ancestor of hominids and great apes (possible candidates are *Sahelantropus tchadensis, Ardipithecus ramidus* and *Orrorin tunegensis*). Moreover, the assignment of *habilis* forms either within the *Homo* or within the *Australopithecus* genus is also discussed (Wood and Collard 1999, Klein 1999, Lewin 2004).

The differentiation between anatomically modern *Homo sapiens* (AMHS) and archaic *Homo* species (AH) occurred in Africa around 200 Ka and also

involved significant evolutionary changes. But again, at least at the morphological level, skull size-and-shape changes were gradual. Hence, there is neither a clear-cut between *Homo* species. The morphology of the human skull is very unique and it is the result of many evolutionary changes. Derived morphological features in AMHS (Figure 12) are a globular and expanded cranial vault, a strong cranial base flexion (kyphosis) and a smaller face that is retracted underneath the anterior cranial fossa (Aiello and Dean 1990, Lieberman et al. 2004). It is still controversial whether these differences are the result of many adaptations to diverse selection pressures or just a small number of developmental changes in early ontogeny (Lieberman et al. 2004).

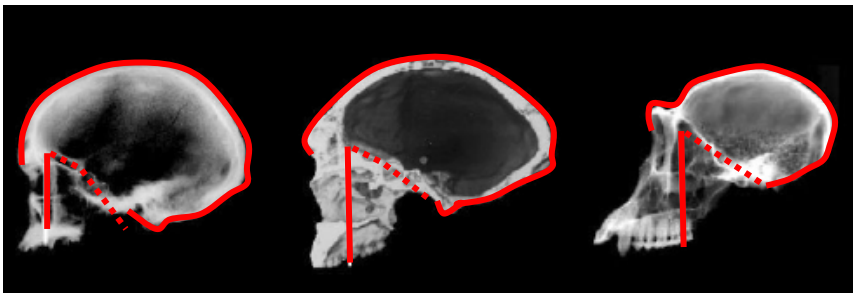

Figure 12. Skull evolution. Changes in neurocranial globular shape, facial retraction and cranial base flexion are outlined in red. Lateral view of a *Pan troglodytes* (right); an archaic *Homo* sp., Broken Hill (middle); and a modern human *H. sapiens* (left). Modified after Lieberman et al. (2004).

It has long been accepted that modern human craniofacial form has evolved as an adaptive response to changes in the brain and sensory capsules, to changes due to bipedal locomotion, as well as to dietary changes (Wolpoff 1999). It has been suggested that humankind skills, such as intelligence, language and social organization arose due to the ability of the brain to expand within an osseous hard resistant case. However, in the light of evolutionary development biology research it has been suggested that few changes related to brain shape and face size might have been sufficient to produce modern skull shape (Lieberman et al. 2002, McBratney and Lieberman 2003). According to Lieberman et al. (2004) 'AMHS autopomorphies may be by-products of more fundamental shifts rather than selected adaptations in their own right'.

The latest research shows that modern human growth and developmental patterns are relatively recent in the hominid history (Thompson et al. 2003). Actually, it is likely that these patterns evolved gradually from ancient vertebrate developmental programs, combining ancestral and derived features, as an evolutionary mosaic. The identified developmental programs required to develop a normal vertebrate skull are highly stable and its basic molecular control system has been preserved throughout evolution (Hall

1999). However, small cumulative shifts in this basic vertebrate pattern may have yielded to a wide variety of morphologies. It has been hypothesized that the present developmental program of modern humans was not fully designed until some time in the last 100,000 years (Thompson et al. 2003). However, this program is neither unique nor homogeneous: the modern ontogenetic pattern also shows significant variability that needs to be addressed. In fact, it has been reported that variability in facial ontogeny among modern human populations can be as great as variability among non-human primates (Strand Vidarsdóttir and O'Higgins 2003).

Unraveling this problem is difficult when dealing with the human skull, because it is a highly integrated structure and we have not identified yet the phenotypic units that reflect morphogenetic units. Therefore, researchers have not identified the correspondence between a given morphological change and a given shift in development (Lieberman et al. 2004).

Originally, the human craniofacial form was interpreted as an accommodation of the skull to an upright posture. According to some authors (Weidenreich 1924, Dart 1925, Schultz 1942, DuBrul 1950, Schultz 1955, DuBrul and Laskin 1961, Demes 1985), the acquisition of bipedal locomotion was the main cause of base kyphosis. According to this perspective, the foramen magnum was compelled to relocate and to move forward in a more central position in order to articulate the vertebral column vertically with the skull. Moreover, this change induced a basal flexion. Because the skull is an integrated unit, the cranial base flexion would have caused morphological changes in the adjacent regions, both in the cranial vault and in the face. The anterior cranial vault would have experienced an upwards deflection, whereas the posterior part would have deflected downwards. In turn, the face would have reached a more inferior-posterior position.

However, many authors have challenged this hypothesis and have claimed that encephalization was the main driving force of modern human craniofacial form (Bolk 1926, Weidenreich 1941, Moss 1958, Enlow 1968, Gould 1977, Stephan et al. 1981, Dean 1988, Ross and Ravosa 1993, Spoor 1997, Lieberman et al. 2000a, Lieberman et al. 2000b). According to this view, the increasingly expanding brain and the differential development of the frontal, temporal and occipital lobes generated frontal and occipital bossing, a more brachycephalic cranial vault and a more flexed cranial base (Figure 13). Nowadays, this is the more widespread and accepted opinion. However, the relationship between brain size and skull shape is not straightforward and it will considered herein with some detail.

1.3.1 ENCEPHALIZATION

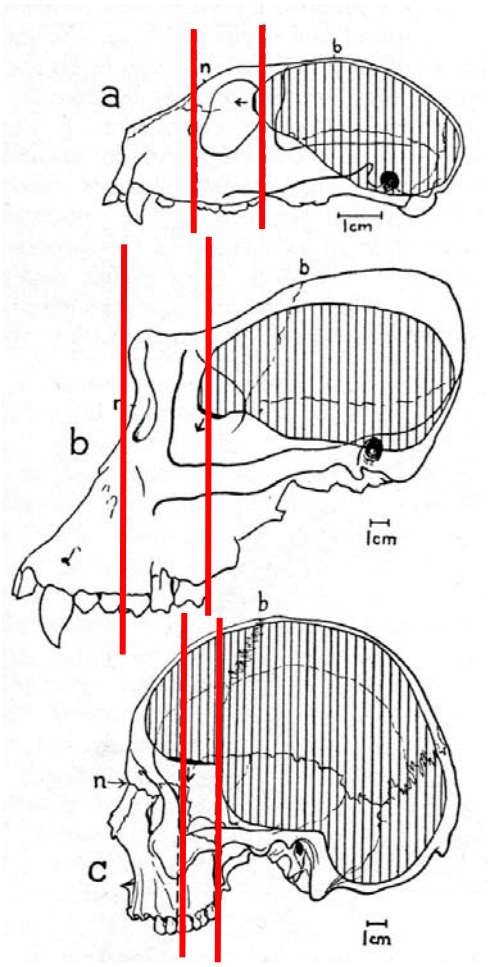

Figure 13. Encephalization. a) Lemur, b) Gorilla, c) Modern human. Modified after Weidenreich (1947).

Encephalization is an evolutionary tendency towards a larger brain (Figure 13). This is a key subject in human evolution since it has long been used as a diagnostic feature to distinguish modern humans from earlier hominids and non-human primates. The hominid brain has increased in size more than threefold during the last 2.5 million years. However, the change was not only quantitative, but also qualitative. According to (Schoenemann 2006), 'the human brain is not simply a large ape brain'.

Encephalization refers to the relationship between brain size and body size and it is generally expressed as the encephalization quotient (EQ). It is calculated as the ratio of a species' actual brain size to the size expected given its body weight (Jerison 1973). The expected brain size is usually computed using a regression of log brain to log body size. However, the brain/body relationship is nonlinear and it is not clear that the scaling association between brain and body size necessarily implies a developmental constraint between them. This scaling was first described long ago (Dubois 1913), but it is still controversial how to use and interpret EQ measures (Schoenemann 2006). EQs have been associated to estimates of intelligence and/or behavioral ability, but this assumption has long been challenged (Holloway et al. 2004). Instead, it appears that absolute brain size could have some behavioral relevance (Kappelman 1996, Schoenemann 2006).

Among mammals, primates tend to have larger brains, with EQ for anthropoids (all primates except prosimians and tarsids) averaging 2. Within anthropoids, *Pan*, *Gorilla* and *Pongo* species show absolute larger brain sizes, but

its scaling to body size is similar to other anthropoids (*Ceboidea*, *Cercopithecoidea* and *Hylobatidae*). However, human brain sizes are clear outliers within this scaling (see Schoenemann (2006) for a detailed review).

Understanding human brain evolution requires a comparative assessment of how brain has changed, both globally and regionally, among the primate lineage. That is, it is necessary to compare not only the total brain size, but also the differential development of its anatomically recognizable components (the olfactory bulb, the cerebellum, the visual cortex, the temporal lobe and the frontal lobe). In a direct way, this can only be done by comparing human brains with that of other extant living primates. Indirectly, the assessment between the brain development of humans and fossil hominids can be inferred from comparisons of endocasts, by extrapolating body and brain mass from the fossil record, or by looking at the imprints of the brain on the inner surface of the braincase.

In a comparative analysis of living hominoid brains (humans, bonobos, chimpanzees, gorillas, orangutans and gibbons), a remarkable homogeneity within the relative size of its main anatomical subdivisions was found (Semendeferi and Damasio 2000). These authors concluded that the frontal lobe is not relatively larger in humans than in apes. This finding contradicts the long claim that humans have large frontal lobes (Deacon 1988) and suggests that this is not a uniquely human feature. Instead, the temporal lobe is likely to have experienced a greater development during hominoid evolution. Further differences between humans and the rest of hominoids are found in the cerebellum, which is relatively smaller in humans. According to Semendeferi and Damasio (2000), frontal lobes might have increased in size earlier (before the split of hominoids), sometime before the Plio-Pleistocene period.

A recent review by Schoenemann (2006) gives further evidence of differential development in the human brain evolution. As stated by Semendeferi and Damasio (2000), frontal lobes in humans are as large as expected given a primate brain of our size. However, one key step in human brain evolution might have involved a biased expansion of the prefrontal cortex, which is the most anterior cortical area of the frontal lobe. In contrast, the remaining cortical areas (such as the primary motor and the premotor ones) are relatively smaller, allowing a significant change in frontal lobe structure without an apparent shift in overall size. The relevance of this restructuring might have been crucial since the prefrontal controls cortical functions important for planning, language and social interactions and coordinates other brain regions. Furthermore, recent data suggests that an

increase of white matter against grey matter in the frontal lobe composition may also have played a role in human brain evolution.

With reference to other brain regions, Schoenemann (2006) also provides a detailed review. Modern human brain is 3.1 times larger than expected from a primate brain/body-size allometric scaling. Comparatively, the olfactory bulb and the visual cortex have not increased in size in a parallel way, but have lagged behind. The lesser development of the olfactory bulb may be pointing out that the sense of smell is less important in humans. However, visual processing abilities are essential in humans. Although the human visual cortex is smaller in relative terms, it is larger in absolute terms. This quantitative difference in absolute amounts of neural tissue may be relevant for the higher visual capacities of humans in comparison to apes. On the other hand, the cerebellum experiences a similar scaling to overall brain, probably because it also participates in language processing. Finally, the brain region that in humans significantly shows larger overall and white matter volumes, and larger surface areas is the temporal lobe. In fact, all the reported evidence suggests that human behavioral skills may lie behind the greater development of the temporal lobe, since it plays a critical role in speech, auditory information processing, emotion and conceptual understanding (Schoenemann 2006).

The fossil record shows that within hominids, human evolution has led to bigger individuals with increasingly bigger brains: from small bodied (and small brained) australopithecines and habilines (Holloway et al. 2004) to modern humans with comparatively greater body and brain size. However, this trend was not a straight progression, either directional, or gradual. Body size increased with the appearance of *Homo erectus*, but comparatively these individuals showed a smaller brain size than modern humans. Neanderthals were the hominid group showing the higher estimates both in body and brain size, even greater than modern humans. Given that these were a bit smaller, it is likely that the last step towards modern humans involved a retraction in both size measures.

Recent evidence points out that within *Homo* there were at least three scaling trajectories of brain size relative to body size (Ruff et al. 1997). According to these authors, the first trajectory was defined as a long period of stasis that occurred during the Early Pleistocene (from 1.8 million years ago until 600,000 years ago) with no apparently significant changes in hominid brain size. It was followed by a period of exponential increase in encephalization during the Middle Pleistocene (from 600.000 to 150.000 years ago). The peak of this trend was reached by Neanderthals. Finally, the third

trajectory is that of modern humans: over the past 35.000 years, both brain and body sizes have decreased. However, Neanderthal's brain mass relative to body mass was slightly smaller than in early AMHS. The latter decreasing tendency continued through the Neolithic period. Recent secular trends fluctuate between positive and flat or even negative values in higher-latitude or in tropical populations.

Through a comparative analysis of endocasts, two distinct trajectories of encephalization were identified (Bruner et al. 2003): one of archaic *Homo*, in which Neanderthals would be included; and one of modern humans. These authors report that the divergent pattern would be due to a parietal expansion in modern humans. If these changes were the result of punctuational events in hominid evolution is actually under revision (Hofman 1983a, 1983b). Furthermore, it has been suggested that brain size increase during hominid evolution is not directly associated to speciation events (De Miguel and Henneberg 2001).

According to recent research (Mai et al. 1992, Kappelman 1996, Henneberg 1998), the final modern human status was achieved not by an increase in brain size, but through a reduction in body size by stopping somatic growth after the completion of brain growth. That is, the present observed pattern may have been caused by dissociation between body and brain growth through an alteration of developmental timings (Nelson et al. 2003). This would imply a heterochronic change, which is one of the main mechanisms that have been used to explain developmental changes.

The internal reorganization of the brain within hominids can also be assessed from the fossil record through the imprints on the inner surface of the braincase (for a review see Schoenemann (2006)). Despite limited and controversial, some clues can be obtained from the imprints of several neural structures, such as the position of the lunate sulcus (which defines the development of the visual cortex), the development of the Broca's area in the left prefrontal portion of the inferior frontal lobe (which is suggestive of linguistic abilities), and the presence of asymmetries in brain development (which are related to some behavior skills such as language, right-handedness and spatio-visual integration). From this evidence, it has been reported that early *Homo* (dating back to almost two million years) would have developed primary language skills (Schoenemann 2006).

Whether these changes in brain size and organization patterns are adaptive or not remains unknown. Most researchers argue that they are the result to directional selection responses. One interesting trade-off hypothesis is

the expensive tissue hypothesis (Hofman 1983b, Smith 1990, Aiello and Wheeler 1995). It suggests that the human brain increase in size was produced against strong evolutionary costs, because the brain is among the most metabolically expensive organs. These authors consider therefore that it is unlikely that this change occurred simply by drift.

Furthermore, brain evolution has been linked to evolutionary changes in life-history traits: bigger brains would have been produced at the expense of longer gestational periods, decreased number of offspring, secondary altriciality and delayed reproduction. It is likely that some adaptive factors giving more selective advantage (and improving individual's fitness) lie behind to large brained individuals. The behavioral benefits of larger brains might be, for example, greater memory, planning and linguistic abilities (Schoenemann 2006). If this is true, there should be some genetic correlation between brain anatomy and behavioral characteristics. According to Schoenemann (2006), a weak correlation would be sufficient.

The genetic variation underlying brain anatomy has been assessed through quantitative genetic studies (Winterer and Goldman 2003). The reported heritability estimates of brain size, as well as different brain areas are significantly high (more than 0.5). Similarly, cognitive abilities such as intelligence have been shown to have some genetic influence (Plomin et al. 1997). However, few studies have estimated the genetic correlations between them (Schoenemann et al. 2000). Despite results are scarce and ambiguous, and more research is needed, the existent evidence points out that the genetic correlation is not zero and that it may be significant and large enough to explain the evolution of brain size in hominids.

Moreover, hypothesis involving conceptual complexity, social abilities, language, ecological challenges, tool use and increasing longevity should be regarded as other plausible explanations. For example, given the high metabolic cost of neural tissue, the increased acquisition of meat fat and protein as energetic supply for developing bigger brains may also be considered as an important factor in human evolution. Regarding this topic, it has been hypothesized that endurance running evolved in early *Homo* for improving human performance in scavenging and hunting (Bramble and Lieberman 2004) before sophisticated toolmaking was achieved (i.e. hunting weapons as those developed in the Upper Paleolithic about 40.000 years ago). Therefore endurance running would have provided early *Homo* a richer diet and would have enhanced encephalization.

At the genetic level, recent discoveries provided by comparative genetics add further evidence to this debate (Mekel-Bobrov and Lahn 2006). Several studies have reported that genes involved in brain development and language processing have been subject to strong adaptive evolution in humans since the divergence between humans and chimpanzees. For instance, it has been reported that the transcription factor FOXP2, which is concerned with speech and language abilities, is under positive selection in humans (Enard et al. 2002).

Evans et al. (2005, 2006) found that the gene microcephalin (MCPH1), which regulates brain size during development, has evolved under strong positive selection in the human evolutionary lineage. According to their results, the frequency of a genetic variant of this gene burst out around 37.000 years ago, coinciding with the emergence of modern humans. This striking increased frequency is not consistent with neutral genetic drift (Evans et al. 2005). Moreover, there is evidence indicating that this genetic variant was already present in archaic *Homo* lineages, such as Neanderthals (Evans et al. 2006). The same research group has detected that another gene affecting brain size is undergoing strong positive selection: they have shown that one genetic variant of ASPM arose only 5800 years ago and that its frequency is still increasing (Mekel-Bobrov et al. 2005). However, Lahn and colleagues in collaboration with psychologist Rushton failed to find any correlation between the selected variants of these genes (ASPM and MCPH1) with IQ results of intelligence tests (Balter 2006).

Another gene that may be related with the evolution of AMHS skull derived characters is the MYH16 gene (Stedman et al. 2004), which contributes to myosin production and expresses at the masticatory muscles in macaque monkey. Stedman et al. (2004) found that the MYH16 gene is disabled in humans whereas is active in all apes. This is caused by a loss-of-function mutation and its morphological consequence is the development of smaller jaw muscles in humans. The authors estimated that the appearance of this mutation was about 2.5 million years ago and it has been related with the divergence of the hominid lineage from their primate ancestors. Furthermore, it has been hypothesized that the decrease in jaw-muscle size released an evolutionary constraint on brain growth and this would have enhanced the evolution of larger brains in *Homo* (Stedman et al. 2004).

1.3.2 MECHANISMS DRIVING MORPHOLOGICAL EVOLUTION

A key point in the present thesis is to investigate and to shed light into the evolutionary mechanisms that have channeled the evolution to modern

humans' craniofacial form and that have generated current human morphological variation. In this section, the main mechanisms generating novelties in morphology and the key concepts shaping skull morphology (both favoring and constraining evolution) are reviewed.

Developmental changes (such as heterochrony, heterotopy, heterotypy and heterometry), modularity and morphological integration are some of the main mechanisms driving morphological evolution. Herein, special attention is given to modularity and morphological integration, which are the issues specifically addressed in this thesis. Studies dealing with these topics in human and primate's evolution and using geometric morphometric techniques are reviewed here because this is a relevant background for this thesis.

1.3.2.1 *Mechanisms of developmental change*

Ontogeny plays a key role in the development of organisms. Although Haeckel's idea that ontogeny recapitulates phylogeny (that is, that the development of an organism mirrors the evolutionary development of the species) has been proved inconsistent, the analysis of ontogeny can clarify many unsolved aspects of evolution. Ontogeny is the result of three main processes: growth (changes of size with age), development (changes of shape with age) and ontogenetic allometry (changes of shape with size).

If evolution occurs, in part is because morphological novelties arise and differentiate taxa between them. Although this is obvious, the mechanisms by which these novelties are generated in a particular moment in ontogeny are not completely disentangled. It is important to understand the causes and the processes by which morphological novelties are produced and maintained. A lot of research has been focused on the developmental pathways underlying morphological structures. Several mechanisms have been described to produce alterations on these developmental networks and these are expected to generate new morphological patterns: they basically concern modifications of the temporal and/or spatial patterns of development. For a complete review see (Gould 1979, Montagu 1981, Shea 1989, McKinney and McNamara 1991, Verhulst 1993, Vrba 1994, Godfrey and Sutherland 1996, Zelditch and Fink 1996, Klingenberg 1998, McKinney 1998, O'Higgins and Jones 1998, Zelditch et al. 2000, Arthur 2000, Zelditch 2001, Collard and O'Higgins 2001, Cobb 2001, Minugh-Purvis and McNamara 2002, McNamara 2002, Alba 2002, Hall 2002, Shea 2002, McKinney 2002, Williams et al. 2002, Vinicius and Mirazón-Lahr 2003, Mitteroecker et al. 2004, Zollikofer and Ponce de León 2004, Mitteroecker et al. 2005, Webster and Zelditch 2005, Leigh 2006)

1.3.2.2 *Modularity*

Modularity is another important mechanism that can drive morphological evolution. The internal organization of a morphological structure determines its ability to evolve and to respond to selection or other nonadaptive microevolutionary forces such as genetic drift. One of the main concerns of the present thesis is the evolutionary potential of craniometric traits; therefore, one of the main goals is to estimate the "available" genetic variation in such traits. However, craniometric traits are not independent among them. Instead, they are correlated with other traits located within the same region, or developed within the same pathway, or involved in the same function (Winther 2001). Actually, it is considered that the mammal and the human skull are hierarchically organized into different structural regions (Lieberman et al. 2000b, Hallgrímsson et al. 2004, Lieberman et al. 2004, Bastir and Rosas 2005, Hallgrímsson et al. 2005, 2006).

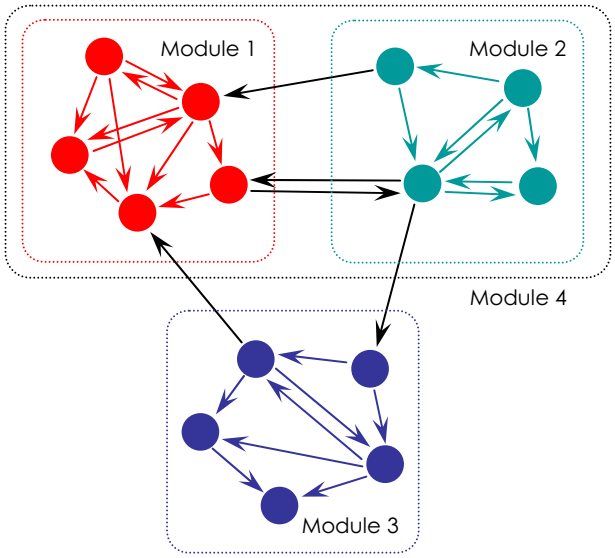

Figure 14. Hierarchical modularity. Modified after Klingenberg (2005)

The modular architecture of phenotypes is receiving an increasing attention at present research. One of the authors leading this research is Dr. Klingenberg, who has developed a theoretical and methodological framework to detect modules by combining developmental and geometric morphometric approaches (Klingenberg 2002, Klingenberg et al. 2003, Klingenberg 2003a, 2004, Klingenberg et al. 2004, Klingenberg 2005). According to Klingenberg (2004) "modules are units that are made internally coherent by manifold interactions of their parts, but are relatively autonomous from other such units with which they are connected by fewer or weaker interactions" (Figure 14). Hence, in natural conditions, the modular structure of organisms is not complete and boundaries between modules are somewhat "fuzzy", depending

on the strength of integration between parts. Modularity and integration are not mutually exclusive concepts, but complementary. A fully integrated structure is non-modular (or else it only consists of a unique module); while a modular structure consists of several modules which are more or less independent among them but highly integrated within them. To Klingenberg (2004), development is at the roots of integration, as opposed to the view of Olson and Miller (1958), Cheverud (1982, 1984) and Wagner and Altenberg (1996), which considers that functional selection is the responsible force of morphological integration.

Modularity and integration patterns are mathematically expressed as covariation between traits. Klingenberg (2004) argues that modules result from the development of spatially distinct morphogenetic fields and distinguishes between two sources of modular covariation: developmental and non-developmental. Developmental covariation is due to direct interactions of the shared developmental pathway of two given traits. These can occur by precursor partitioning (when two structures are derived by fission of a common developmental precursor) or by inductive signalling (when two traits follow two different but interrelated pathways and a signal in one pathway also affects the other one). Non-developmental covariation is caused by environmental or genetic factors that produce parallel variation, but this does not reflect modularity.

Integration seems to be pervasive in nature. Regarding the ability of an organism to adapt, integration has both a positive and a negative aspect: these are two sides of the same coin. The positive aspect is that integration may favour functional coordination between parts and enhance adaptation to a given environment. However, the negative consequence is that integration can constrain future evolution because functional systems can not evolve independently (Klingenberg 2004). Constraints can be absolute or relative and in the morphological realm these can be detected as those directions in the morphospace that would lead to "impossible" or "less-likely" phenotypes. Absolute constraints represent "forbidden" trajectories of morphological change that lack genetic variation; whereas relative constraints might be overcome depending on the amount of available genetic variation to achieve the "new" phenotypic optimum (for a detailed description on how to detect these constraints see the section Quantitative Genetics, 1.5.7).

At the genetic level, morphological constraints are due to pleiotropy, which occurs when a single gene influences multiple phenotypic traits. Pleiotropic patterns are likely to be evolutionary conservative, but changes in

direct developmental interactions can trigger a complete reorganization of pleiotropic patterns, affecting the whole modular structure of a phenotype. Through this mechanism, strong morphological changes can occur through small developmental changes in a punctuated manner (Klingenberg 2005). If such a developmental change occurs, and if this change represents a significant advantage for the organism (improving a given function and thus increasing the organism's fitness), strong directional selection might favour the new developmental system after long periods of stabilizing selection. According to Klingenberg (2005), this mechanism would provide a means to remove developmental constraints. Therefore, new phenotypic variation would be released and the evolvability of that trait would be increased.

Several attempts have been carried out in order to delimit modules in several types of organisms: from insect wings such as *Drosophila* (Klingenberg and Zaklan 2000), bumblebees (Klingenberg et al. 2001) and tsetse flies (Klingenberg and McIntyre 1998), to the mouse mandible (Klingenberg and Leamy 2001, Klingenberg et al. 2003), the mouse skull (Debat et al. 2000) and the primate skull (Hallgrímsson et al. 2004, Willmore et al. 2005). All of these studies analyzed patterns of fluctuating asymmetry to identify potential developmental modules.

Goswami (2006) used a different approach in order to compare modularity patters of craniofacial complex across mammalian taxa. This was also based in geometric morphometrics but used cluster analysis and matrix correlation methods (Goswami 2006). In this study, 106 species of mammals were compared and it was found that cranial modularity is conserved among therians, but is differentiated from that of monotremes. Therefore, these results point out that the mammal skull is modular and is an evolving character. Within therians, three modules are highly integrated: the oral-nasal, the molar and the basicranium modules. In contrast, the orbit, the zygomatic-pterygoid and the vault regions are poorly integrated. Goswami (2006) couldn't statically distinguish between developmental and functional modules, but it is apparent from the cluster analysis that modules respond to functional demands. A closer look to her results suggests that the less integrated modules are those involving several osseous structures with very different developmental/tissue origins (such as the orbit, which is formed by neural, dermal and endochondral derived bones).

As much of developmental biology research is performed upon model species (such as the house mouse), an important issue here is to compare the primate craniofacial pattern with that of mice in order to confirm if

conclusions driven from mice can be extrapolated to primates. This point was addressed by Hallgrímsson et al. (2004), who found significant similarities in the genetic and the phenotypic covariation patterns between mice and macaques, but found significant differences in modularity and in developmental stability. Hallgrímsson et al. (2004) found that the phenotypic and genetic correlations matrices derived from the macaques supported the functional-developmental modular pattern reported by Cheverud (1982, 1995): the most integrated regions were the face and the basicranium, whereas the neurocranium was lagged behind. This pattern was not confirmed in mice, in particular after the results obtained from a mutant strain. Their results show that developmental patterns are quite conservative among mammals, although they can be modified within a species by single mutations affecting craniofacial development (Hallgrímsson et al. 2004).

In a subsequent analysis, Hallgrímsson et al. (2006) identified one of such mutations: mice carrying an autosomal recessive mutation of gene Papps 2 display a reduced chondrocranial growth, which is in turn associated with a brachymorph phenotype. Mutant mice showed an altered craniofacial pattern, with an increase in phenotypic variation and morphological integration. Their results point out that the degree of cranial vaulting is correlated with the degree to which the growth of basicranium is retarded. According to them, this is a case of developmental canalization.

1.3.2.3 *Morphological Integration*

Finally, morphological integration is another key concept in evolutionary morphology that must be considered for a deeper comprehension of the skull biology. Due to its variety of functional requirements and growth patterns, the skull is a complex morphological structure (Pucciarelli et al. 1990). Thus, to analyze and further understand its biology, as well as the developmental mechanisms and microevolutionary processes by which its phenotypical variation is expressed, morphological integration between structural components related either by developmental or functional criteria must be considered (Olson and Miller 1958, Zelditch and Carmichael 1989, Zelditch et al. 1992, Roth 1996, Marroig and Cheverud 2001, Bookstein et al. 2003, Hallgrímsson et al. 2004, Lieberman et al. 2004).

The mechanisms by which the shape of a complex structure, such as the human skull (Figure 15), result from the integration of morphogenetic rules, plastic responses and evolutionary forces are not well-established (Lieberman et al. 2000a). Several factors like morphological integration (Olson and Miller

1958, Marroig and Cheverud 2001, Bookstein et al. 2003), developmental and functional constraints (Lieberman 1997, Pucciarelli et al. 2000, Lieberman et al. 2000a, 2004), as well as different levels of plasticity (Kiliaridis 1995, Wood and Lieberman 2001, Giesen et al. 2003), are thought to interact through ontogeny until the expression of adult morphology is achieved. As a result of morphological integration, it is expected that functionally and developmentally related characters will be inherited together. Environment also plays an important integrative role, since selection favors functional related traits, which evolve as a single coordinated unit (Cheverud 1995).

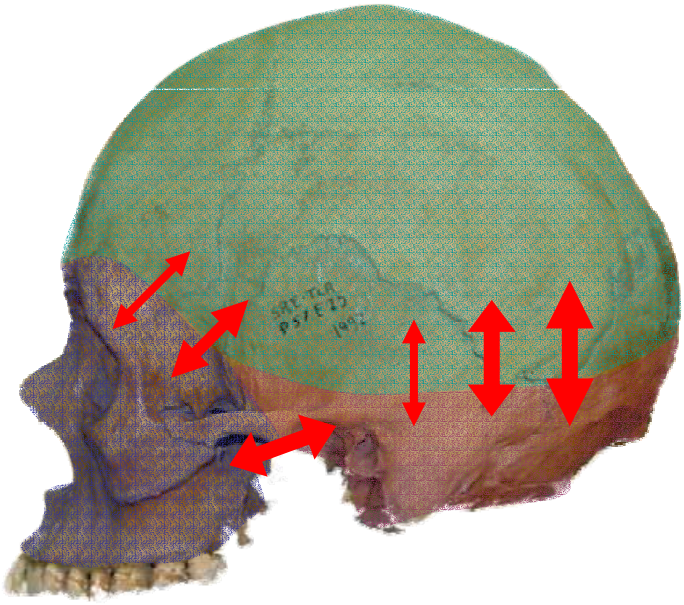

Figure 15. Morphological integration in the human skull. Arrows indicate forces and strengths of covariation between the main developmental regions of the skull.

The evolutionary trends of the human skulls rely on the amount of genetic variation. These trends are driven by microevolutionary forces such as natural selection, gene flow, genetic drift and mutation, but strongly depend on morphological integration, which can favour or constrain the evolution of complex phenotypes. Morphological integration assumes that functionally and/or developmentally related traits will be coinherited, so that trait evolution is not independent, but coordinate responses to evolution are expected (Ackerman and Cheverud 2004a).

Cheverud and colleagues (Cheverud 1982, 1988, Richtsmeier et al. 1993a, Cheverud 1995, 1996a, Marroig and Cheverud 2001, Ackermann 2002, Marroig and Cheverud 2004) have focused much of their research on

morphological integration patterns within the primate skull, comparing the patterns of extant covariation of different phylogenetic groups, namely New and Old World monkeys, apes and humans. In their approach they distinguish two main regions of the skull, the neurocranium and the face, and further subdivide them into smaller units, whose development is influenced by the growth of connected soft-tissues. Following the Functional Matrix Hypothesis (Moss and Young 1960), they consider that three regions can be distinguished within the neurocranium: the cranial vault, which is strongly influenced by brain growth; the orbit, which is affected by the eye; and the cranial base, which is dependent on brain growth and other late-somatic factors. Within the face they define the oral region, associated with the growth of teeth and oral cavity; the nasal region, related to the growth of the nasal septum; and finally the zygomatic region, influenced to mastication muscles. Their findings have provided support to the Functional Matrix Hypothesis: covariation within functional units is stronger than covariation within individual bones or osseous subdivisions, such as the splanchnocranium, the cranial base or the anterior cranial fossa.

Despite high levels of diversity within primates, their results suggest general shared patterns of integration at this Order (see Ackermann and Cheverud (2004a) for a complete revision). Both New and Old World monkeys show a basic neurosomatic integration pattern. Facial and neurocranial traits are strongly correlated within them and differences in taxa stem from the relative weighting between them. The strongest correlation tends to occur within the orofacial region. When compared to hominoids, this general pattern is consistent with just one exception. In African apes and humans, the zygomatic region is also a main source of facial integration, suggesting that mastication may play an important role in skull integration. These differences among the common primate pattern may be explained by changes in signalling factors that occur during ontogeny after the divergence of the different groups.

Ackermann and Cheverud (2004a) also reported that the cranial vault is one of the least integrated structures within the primate skull. This was interpreted as a consequence of encephalization: that is, the lack of cranial vault integration provided the skull with more capability and 'freedom' to evolve in response to the increasing brain size. This view is supported by other researchers (Goswami 2006) that have found similar patterns in mammal craniofacial patterns. However, Goswami (2006) does not confirm the finding of Lieberman et al. (2000a, b) that the cranial base is well integrated with the other skull regions. According to this, the cranial base is highly integrated

within itself and is more spatially limited than reported by Lieberman et al. (2000a, b).

Following this line of research, Bastir and Rosas (2004a, 2004b, 2004c, 2005, 2006) and Bastir et al. (2004) have produced a number of papers dealing with the ontogeny and morphological integration in humans, fossil hominids and chimpanzees. According to their results, chimpanzees and humans show differences in their integration patterns, which are higher and more widespread in chimpanzees than in humans. Bastir and Rosas (2004b) argue that the face is integrated but that it is relatively independent from the neuro- and the basicranium, a finding that has also been reported by other researchers (Lieberman et al. 2000a, 2000b, Zollikofer and Ponce de León 2004). They further suggest the existence of two developmental components: the face on the one hand, and the neurobasicranium on the other. Moreover, their results did not fit traditional hypothesis of integration (Enlow and Hans 1996) suggesting that longer faces were associated with dolicocephalic skulls (elongated and narrow braincases) and a less flexed basicranium. Conversely, Bastir and Rosas (2004b) pointed out that the dolicofacial pattern is associated with an even greater external basicranial flexion with no apparent change in the braincase length.

One possible explanation for the ontogenetic facial divergence between primate species, as well as for the lack of integration of the face within the braincase, is that facial postnatal growth is highly influenced by environmental factors during ontogeny (especially mechanical loadings) and thus it is more prone to plastic responses (Strand Vidarsdóttir et al. 2002, Bastir and Rosas 2004a). It has been suggested that from the phylogenetic point of view, facial traits would not be as informative as neuro- and basicranial, which are more conservative and would reflect more reliably the underlying genetic patterns (Collard and Wood 2000, Collard and O'Higgins 2001). These suggestions are non tested hypothesis and the results from the present heritability study will shed light on this point (Chapters 3 and 4).

Few years ago, Bookstein et al. (2003) published a paper exploring cranial integration in *Homo*, where they compared ontogenetic and phylogenetic samples using a new geometric morphometrics methodology, the singular warps. Their results pointed out that integration patterns over ontogeny and over evolution are not exactly the same and that those differences were concentrated in the cranial base. Overall similar patterns concern cranial base flexion, facial retraction and neurocranial globularity. However, Bookstein et al. (2003) advise caution when using of these traits as phylogenetic characters.

Furthermore, morphological integration in the skull is not necessarily limited to the osseous components of the skull. Given that skull and brain development are intimately linked, the integration between them should also be addressed. The first attempt to directly assess the phenotypic integration of neurocranium and brain was developed by Richtsmeier et al. (2006). This represents a significant step forward because it analyzes simultaneously the integration between two different types of tissue: the skeletal hard tissue and the neural soft tissue (Richtsmeier et al. 2006). These authors have investigated the developmental association of brain and skull through a comparative 3D geometric morphometric analysis of skull computed tomographies and brain magnetic resonance images of individuals with two types of craniosynostoses (sagittal and right unicoronal). Their results detect differences in phenotypic integration between the two samples and emphasize that the strong positive association between the skull and the brain is always maintained. As previously suggested by Hallgrímsson et al. (2005, 2006) in mice models, Richtsmeier et al. (2006) reported that for some measurements craniosynostosis in humans causes increased integration patterns. However, they found that these measures were not necessarily anatomically proximate to the prematurely closed suture, but that most of them were located in the cranial base. This finding suggests that craniosynostosis is not a strictly local phenomenon and that sutural patterns (position, patency and closure) are regulated by hierarchical processes of developmental control. Finally, they emphasized the important role that cranial suture patterns may have played in mammalian evolution. According to Richtsmeier et al. (2006), premature suture closure could be considered as the process that allowed the reduction of number of skull bones from synapsid into mammals' evolution.

To sum up, the reported evidence strengthens the consideration that phenotypic integration is an important process to the evolution and development of the primate skull.

1.4 *Geometric Morphometrics*

In this section a general introduction to geometric morphometrics (GM) is provided because this was the method used to measure and to analyse the skulls of the Hallstatt's collection. More technical and detailed explanations are presented at the Materials and Methods sections of each Results chapter. However, it is relevant to introduce here the main concepts of GM and the "philosophy" underlying it.

Geometric morphometrics has been defined as the fusion between geometry and biology. Actually, it is a useful approach for quantitative characterization, analysis and comparison of biological form (Bookstein 1991, Marcus et al. 1996, Dryden and Mardia 1998, Lele and Richtsmeier 2001). GM is a landmark-based method that was developed to analyze form, and thus morphological changes, in a bidimensional or a tridimensional space (Bookstein 1982). GM involves a growing corpus of statistical and graphical techniques for shape analysis.

It has been claimed that GM has caused an authentic "revolution" in the morphometric field (Bookstein 1991, Rohlf and Marcus 1993, Klingenberg 2002, Adams et al. 2004). This is because it provides a completely new framework and approach to the analysis and comparison of forms. The use of geometric morphometrics started in the late seventies and burst out from the mid nineties (Adams et al. 2004). A revision of the scientific literature from the last two decades points out that GM has almost replaced traditional morphometrics. The use of GM is widespread among different fields, such as biology (including systematics, evolutionary biology, physical anthropology, palaeontology, ecology, genetics, developmental biology), medicine, geology and biotechnology (animal and plant breeding).

Several reasons explain the tremendous success of GM: it is a more precise, robust and powerful tool to describe morphological trends and to

detect shape differences; it provides a better visualization of shape; everyday it becomes more accessible with the development of more sophisticated hardware and software (most of it freely available on internet); and data sampling is easier, more precise and more efficient. For instance, a digitizer tool can provide a rich dataset over large samples in a moderate lapse of time. Nevertheless, despite all of these benefits, GM also has some disadvantages, as for example the lack of sufficiently tested and user friendly software for 3D analysis and the theoretical difficulties that underlie this methodology. These are being overcome with the development of new software, the publication of GM manuals for beginners and the organization of courses, workshops and conferences all around the world.

1.4.1 AN HISTORICAL OUTLINE

The "fathers" of Biometry were outstanding researchers from the 19th and 20th centuries, namely Adolphe Quetelet (1797-1874), Francis Galton (1822-1911), Karl Pearson (1857-1936) and Ronald Fisher (1890-1962), who developed the standard statistics to analyze biological and morphological variation. During the thirties and forties, the advances introduced by these researchers were combined with the modern evolutionary synthesis and the development of population genetics. The evolutionary synthesis integrated Darwin's theory of the evolution of species by natural selection and Mendel's theory of genetics as the basis for biological inheritance.

At the beginning, biometric statistics were restricted to the analysis of single variables (or univariate analysis). Further advances reached in the seventies lead to the development of statistical methods that allowed the analysis of many variables simultaneously (or multivariate analysis). In classic or traditional morphometrics (Marcus 1990, Reyment 1991), morphological measurements were used as variables. A new approach to quantify and analyze morphological data started to develop during the eighties, the so-called GM (Rohlf and Marcus 1993). GM is based on geometrical principles and unlike traditional morphometrics, GM is based on the recording of Cartesian coordinates (*x, y* in 2D analysis; or *x, y, z* in 3D analysis). GM allows the global study of shape, because in does not require to dissect form into a number of arbitrary measurements. Thus, the relationships among traits and structures are fully preserved throughout the analysis (Richtsmeier et al. 2002). In fact, one of the main contributions of GM is that it always maintains the physical integrity of shape.

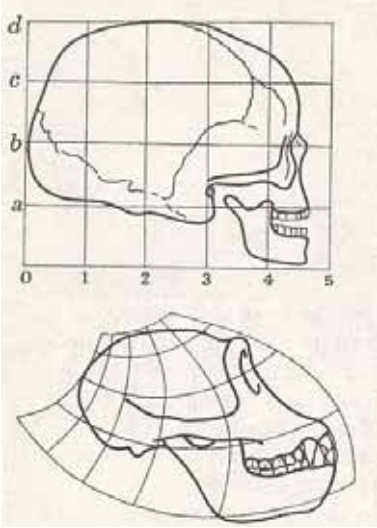

Figure 16. Transformation grids from D'Arcy Thompson's book.

The origins of GM should be sought at the beginnings of the 20th century. In 1917, D'Arcy Thompson published his hallmark book *"On growth and form",* where he first introduced the concept that morphological shape changes could be described and represented through Cartesian models, what he called "transformation grids" (Figure 16). The fatherhood of GM has been attributed to D'Arcy Thompson, but the very original idea might had arisen much before, as far as 1528, when Albrecht Dührer used it to study and display human proportions of the head. In any case, a true theoretical and quantitative basis for D'Arcy Thompson's transformation grids was not developed until the eighties (Bookstein 1978, Kendall 1984, Bookstein 1984a, 1984b, Kendall 1986). These authors provided a rigorous statistical theory for the analysis of form, what was called the "morphometric synthesis" (Bookstein 1991). It combines the advantages of multivariate statistics and those of the geometrical representation of form.

1.4.2 THE PRINCIPLES OF GM

In GM jargon, form is considered as the combination of size and shape (Lele and Richtsmeier 2001). The form of an object is recorded through Cartesian coordinates of a set of landmarks. These landmarks must be homologous between forms; that is, they must be present in all the sampled individuals and should represent some kind of biological correspondence between them (either phylogenetic, structural, functional, developmental or biochemical) (Lele and Richtsmeier 2001). The equivalence between forms can also be assessed through geometrical, allometric or biomechanical parameters. In order to describe skull morphology, anatomical landmarks are usually used (i.e. a point of convergence between sutures, a given trace of muscular insertion, the location of foramina, etc.).

The principles of GM are grounded on the capability to transform the particular geometry of specimens (recorded as a set of landmarks coordinates) into points of an abstract space called a morphospace and vice versa (Zelditch et al. 2004). The geometrical, mathematical and statistical properties of morphospaces are complex but well established. In general terms, a

morphospace can be viewed as a scatter plot where each point represents the form of an object. Moreover, every possible form corresponds to a particular point in the space. Distances between points represent the degree of similarity between forms (Zelditch et al. 2004). A particular improvement of GM methods is that they always keep the correspondence between the geometry of the morphospace and the original figures, so that the transformation is reversible and is easy to go back and forth them.

Morphospaces are usually multidimensional and non Euclidean, except when form can be just represented by the position of a single point (Bookstein 1991). The representation of morphospace gets more and more complicated as the number of landmarks increases and it is very difficult to visualize when more than three landmarks are considered. In the simplest case, the space for triangles is a sphere, a two-dimensional curved surface in a three-dimensional space (Kendall et al. 1999). The standard methods of multivariate statistics are applied onto the morphospace and the results of statistical analysis can be transformed into the original geometry of figures and visualized as morphological changes or deviations of them.

1.4.3 SIZE & SHAPE

The core concepts in GM are size and shape (Bookstein 1991), which are the main features of form that are analyzed and quantified by GM methods. Another important improvement of GM is that it has provided specific definitions for the terms of size and shape, so that every researcher understands and applies the same terminology and this prevents from confusion. Furthermore, GM can separately analyze size from shape information (Bookstein 1991).

Traditionally, the quantification of size has been controversial, because the use of different measures could yield to different results (Richtsmeier et al. 2002). The more commonly used measures of size are body mass or particular length measures, but also area and volume measures. In most studies, size is condensed in a single measure that concerns either one total length or mass measure, or the relationship between several measures (such as the arithmetic mean, the geometric mean, areas and volumes, or indices). GM provides a specific measure for size, called centroid size, which can always be obtained from a set of landmarks and is comparable between specimens. In other words, smaller individuals will have smaller centroid sizes than bigger individuals. An interesting property of centroid size is that it is independent from form under certain assumptions: in absence of allometry, centroid size is

not correlated with form variables when landmarks are evenly distributed around their means (Bookstein 1986).

While size refers to the magnitude and dimensions of an organism or of one of its parts, shape refers to the essence of its figure, to the proportions of it and the relative size and position of the parts that make it up. Shape would somehow represent the "platonic idea" of objects. Even the simplest form has multiple aspects to be described and that's why it is stated that shape is an inherently multidimensional property (Klingenberg 2005). Technically, shape is defined as all the geometric information that remains invariable after removing the effects of size and position (that is, the nuisance factors of scale, rotation and translation) (Dryden and Mardia 1998). These effects are removed through a series of transformations based on the Procrustes methods, which are described with deeper detail in the next sections.

1.4.4 TYPES OF LANDMARKS

As there is a wide variety of forms and biological forms can be rather complex, different types of landmarks have been defined (Bookstein 1991, Dryden and Mardia 1998, Lele and Richtsmeier 2001). All kinds of landmarks can be used for any kind of GM analysis, using the same techniques and procedures. The only exceptions to this rule are pseudolandmarks, which are mathematically constructed landmarks that require a special treatment. However, researchers combine the use of different landmarks to quantify as accurately as possible the organismal form they are analyzing.

There are basically three types of landmarks (Figure 17), but they have received different names by different researchers: they are called type I, II and III by Bookstein (1991); traditional, fuzzy and constructed by Lele and Richtsmeier (2001); and anatomical, mathematic and pseudolandmark by Dryden and Mardia (1998). Type I/traditional/anatomical landmarks are mathematical points that are defined after their biological meaning. In Bookstein (1991) terminology, type I landmarks are points defined by the juxtaposition of different tissues (Figure 17). Within traditional landmarks, Lele and Richtsmeier (2001) distinguish between those landmarks whose position is dependent on a coordinate system or on a particular orientation and those which are not. For example, the nasion is independent from orientation, because it is exactly located at the intersection between the nasal and the nasofrontal sutures; whereas the opisthocranion, which is defined as the most distant sagittal point from the nasion, is dependent on orientation and the skull must be oriented in Frankfurt plane to locate it correctly (Figure 17).

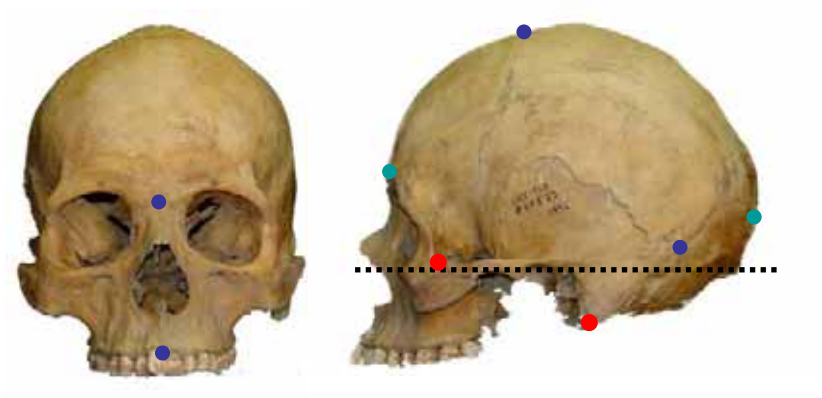

Figure 17. Different types of landmarks. These are shown on the frontal and lateral profile of a human skull. Blue points indicate type I landmarks (nasion, prosthion, bregma, asterion), red points indicate type II landmarks (jugale, mastoidale) and green points indicate type III landmarks (glabella, opisthion), whose definition depends on the orientation of the skull in the Frankfurt plane (dashed line).

Type II/fuzzy/mathematical landmarks are mathematical points that are homologous not from the biological point of view, but just geometrical. That is, they can be located in different forms because they are always positioned in the same way, although they are biologically nonsense. These are for example points of maximum curvature or the innermost points of concavities (such as bulges, saddle points, or dips). This definition would also include Bookstein's Type III landmarks (Bookstein 1991), which are considered as extreme points such as the anteriormost or posteriormost points of a structure (Figure 17). The difference with Type II landmarks is that Type III refers to features whose definition is based on the main axes of the structure, at a larger geometric scale. Lele and Richtsmeier (2001) called fuzzy landmarks to those landmarks that are not strictly located in a given point but within a certain area (i.e. the parietal and frontal protuberances on the human skull vault). Because these landmarks might be difficult to locate in 3D surface areas, they are prone to larger measurement errors. Type I are more accurate than type II and type III landmarks because they can be located more easily and do not depend on orientation, so that the margin of error is usually smaller (Bookstein 1991).

Finally, constructed or pseudolandmarks are considered as mathematical points located along a contour, between two anatomical or mathematical landmarks (Dryden and Mardia 1998, Lele and Richtsmeier 2001). These landmarks are derived by geometric construction from the arrangement of neighbouring parts and they are often used to cover those regions that do not present anatomical landmarks, as for example the cranial vault.

The forms under analysis can be recorded with as much precision as desired. The researcher decides the number of landmarks to be digitized. However, sample size can be a limiting factor. In order to guarantee statistical significance, sample sizes should exceed the number of variables included in the analysis: for 2D data, the number of individuals should be greater than twice the number of landmarks; whereas for 3D data sample size should be more than three times the number of landmarks. However, the decision about the number of landmarks to be digitized also depends on the available sample size, as well as on the effort and the time required in measuring those landmarks.

Landmark coordinates can be obtained from a wide variety of sources and equipment: 2D landmarks can be registered from digital images such as photographs, flatbed scans and radiographs; whereas 3D landmarks can be recorded with digitizers such as the Microscribe or the Polhemus (a stylus device that registers the coordinates of landmarks manually pointed by the observer), as well as with optical scans that reconstruct the entire surface of an object, or with computed tomographies, which reconstruct both the external and internal features of an organism. The accuracy of 3D landmark recording devices is notably high. The landmark recording method is very useful and can provide rich data sets, because distance, angular and area data can also be obtained from landmark coordinates by applying the basic principles of geometry.

1.4.5 GM TECHNIQUES

There are several GM techniques; here we provide a brief description of them.

1.4.5.1 *Superimposition.*

Once landmarks are digitized, the morphology of the measured objects is captured in the form of coordinate configurations. As explained above, landmark configurations must be converted into shape space data (Rohlf 1996). This is achieved by superimposition techniques that retain shape geometric information and distinguish it from size information (Figure 18).

There are several techniques to perform superimposition of coordinate data, such as the Two-point registration (Bookstein 1991) and the General Resistant Fit superimposition (Rohlf and Slice 1990), but the most widespread and accepted one is the Generalized Least Squares superimposition (also called Generalized Procrustes Analysis, GPA) (Rohlf and Slice 1990, Bookstein

1991). Several studies have reported that GPA is the most efficient procedure to superimpose landmark configurations (Rohlf 2000, Monteiro et al. 2000, Rohlf 2003), although this has been challenged by other authors that support coordinate-free analysis that are invariant to object orientation (Lele and Richtsmeier 1991, Richtsmeier et al. 2002).

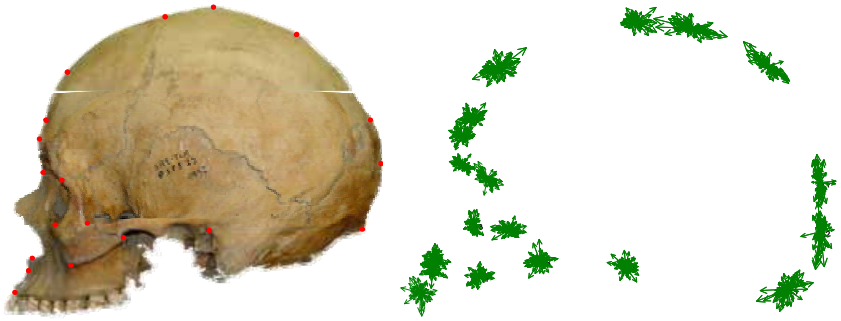

Figure 18. Landmark configuration capturing the form of a human skull and superimposed configurations.

GPA is actually the central procedure in shape analysis and is the technique that has been used in the present thesis. The Procrustes superimposition extracts shape information by standardizing size, position and orientation of form configurations. Moreover, it extracts a measure of size, the centroid size, which is technically defined as the square root of the sum of the squared distances of a set of landmarks from their centroid or centre of gravity (Bookstein 1996b). In other words, the centroid size is a measure of the amount of dispersion of landmarks around the centroid. Centroid size is a useful measure and fulfils the criterion for a size variable. However, it must be reckoned that centroid size estimation depends on the number of landmarks. Therefore, for comparative purposes, it only can be used when comparing configurations of corresponding landmarks.

Procrustes superimposition is a three-step procedure based on the Euclidean similarity transformations (Dryden and Mardia 1998). The first step consists in removing size effects by scaling form configurations to unit centroid size. The second step involves a translation movement that shifts the centroids of all configurations to the origin of coordinates (0,0,0). And third, the configurations are rotated around the centroid until least squares estimates yield the best fit (that is, the minimal sum of squared distances between corresponding landmarks). Furthermore, configurations are allowed to be reflected. Since many configurations are analyzed at a time, GPA is an iterative procedure performing cyclic pair-wise fits of configurations. After Procrustes,

the variation remaining in the landmark coordinates of the superimposed configurations is just due to shape variation. Procrustes coordinates can be used for further statistical analyses and can be used to estimate the mean shape configuration or consensus shape (Dryden and Mardia 1998).

The statistical model more commonly used in GM to describe individuals' variation with regard to a mean or consensus form is expressed in the following way:

$$X_i = (M + E_i)\Gamma_i + \gamma_i$$

where X_i is the landmark coordinate matrix of every i specimen (i=1,2,…,n), M_i is the mean configuration, E_i is the variation of each individual, Γ_i represents rotation and γ_i translation (Bookstein 1986, Goodall 1991, Lele 1993, Dryden and Mardia 1998).

The Euclidean similarity transformations (Dryden and Mardia 1998) applied to form configurations by Procrustes methods convert the landmark configuration of each specimen into points of a shape space called Kendall's space (Kendall 1984). From the original recording space to Kendall's shape space, landmark configurations pass through several morphospaces, each with different statistical characteristics and dimensionalities (Dryden and Mardia 1998). Digitized specimens are landmark configurations that are represented as a $p \times k$ matrix. This matrix contains as many rows as registered landmarks (p) and as many columns as dimensions or number of coordinates (k= 2 or k=3). This represents the first departure from real space to GM morphospaces and specimens are considered to lie in figure space, which has pk dimensions (Dryden and Mardia 1998). By translation, k coordinates of each specimen are fixed and specimens are moved into preform space, which has pk-k dimensions. By rotation, $k(k-1)/2$ dimensions are lost, so that specimens enter the so-called form space, because it still preserves size information. If scale is removed, one further dimension is lost and specimens are finally represented in shape space, which has pk-k- $k(k-1)/2$-1 dimensions. That is, shape space has $2p$-4 dimensions in two-dimensional (2D) analyses and $3p$-7 dimensions in three-dimensional (3D) analyses. Shape space is called Kendall's space because this author defined and developed the statistical characteristics of shape spaces (Kendall 1984), which was one of the main improvements of GM.

Landmark configurations can now be analyzed as points lying in a multidimensional shape space (Rohlf 1996). Kendall's shape space can be considered as a curved surface determined by all possible shape variations within a given configuration of landmarks. The distance between two points in

the shape space is called Procrustes distance and represents the dissimilarity among two shapes. Technically, it is defined as the root sum of squared distances between homologous landmarks when two configurations are each scaled to unit centroid size and Procrustes superimposed. According to Bookstein (1996a), Procrustes distance is the only statistically meaningful shape distance for landmark data.

As Kendall's shape space is rather complex, the most common visualization of it concerns the shape space of triangles (figures defined by just three landmarks, those of the triangle vertices). From the above formula, it is straightforward that the morphospace for triangles is two dimensional (*2x3-4=2* dimensions*)* and is thus a sphere (Figure 19). Within this sphere are represented all the possible shapes of a triangle. Note that the hemispheres are symmetrical and that different kinds of triangles occupy specific locations of the sphere: equilateral triangles are found at the poles, collinear triangles lie at the equator and isosceles triangles are placed at the meridians (Figure 19).

Kendall's space is therefore non-linear and until recently it was considered as spherical (Kendall 1984, 1989, Bookstein 1991, Rohlf 1996). However, it has been shown that Procrustes aligned landmark configurations lie in a hyper-hemispherical variant of Kendall's shape space (Slice 2001). Anyway, the statistical implication of this is that the shape space in non-Euclidean and thus standard multivariate methods can not be applied. In order to solve this pitfall, Procrustes aligned landmark data are projected into a Euclidean tangent space (Figure 19). If form variation is relatively small, this projection does not cause any significant bias in Procrustes data. Therefore, multivariate statistics can be reliably applied on the projected Procrustes landmark coordinates laying on tangent Kent's tangent space (Rohlf 1996, 1999).

There are many tangent spaces to Kendall's shape space but the most optimal projection is achieved when the mean shape is used as tangent point, because distortion is minimized (Figure 19). When all the specimens are projected onto the tangent space, the Procrustes geodesic distances between them are modified. The distortion is positively proportional to the distance from the tangent point. In the tangent shape, distances tend to be smaller than Procrustes distances. If the mean shape is selected as tangent point, then all the points of Kendall's space are closer to the tangent point and distortion is minimal (Figure 19). Studies of variation within the same species or among closely related species are usually unaffected by the projection of Procrustes coordinates to tangent space (Marcus et al. 2000), but this could not stand for

large phylogenetic scale comparative analyses. This bias can be estimated by correlation analysis of Procrustes and tangent distances.

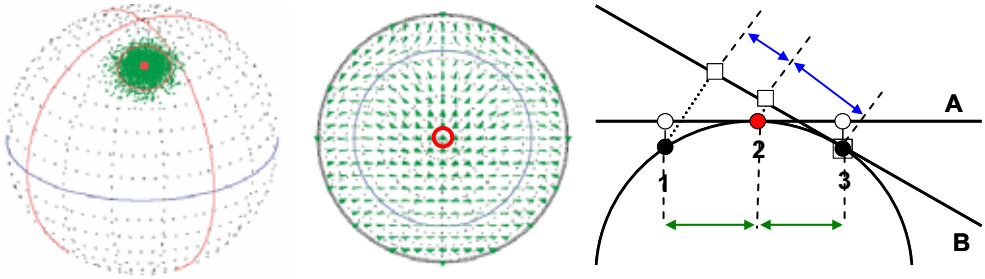

Figure 19. Kendall's shape sphere for triangles. On the left, the shape space of a random sample of triangles is shown and the position of the mean shape is marked with a red circle. On the middle, Kendall's tangent space is shown. Note that the point of tangency (red circle) is located at the centre, representing an equilateral triangle, which would correspond to the North Pole of Kendall's shape space. On the right, two tangent spaces are depicted (A and B). A) When the tangent point is the mean shape (red circle), the distances between point 1, 2 and 3 in the shape space are minimally distorted when they are projected to the tangent space (note in green arrows that the equidistance between them is almost conserved). B) When the tangent point is another point, the distances between these points are considerably distorted (note in blue arrows that the distances are not equidistant anymore). Modified after Monteiro et al. (2000), Rohlf (1999) and Slice (2001).

All the analyses presented in this thesis are based on superimposition methods. However, there are other kinds of GM methods, as deformation methods (Bookstein 1989, 1991) and coordinate-free methods, such as the Euclidean Distance Matrix Analysis (Lele and Richtsmeier 1995, 2001). A brief description of them is provided.

1.4.5.2 *Deformation.*

Deformation methods are based on thin-plate spline (TPS) techniques (Bookstein 1989, 1991) and these are one of the most common used techniques for analysis, comparison and visualization of shape variation. Shape change is visualized as deformation grid splines (Figure 20): two shapes are compared by analyzing the deformation patterns obtained from distortion of the first shape (the reference shape) onto the second one (the target shape). The deformation is composed of affine and nonaffine components (Bookstein 1989, 1991). Nonaffine components require bending energy, whose computation produces the partial warps scores. The partial warps define the position of each individual in the shape space and highlight morphological changes at progressively smaller scales (Bookstein 1996a, Rohlf 1998). They are collected in the so-called weight matrix. Global affine transformations (such as

translation, scale, rotation and shearing) are computed as the uniform component and can also be included into the weight matrix.

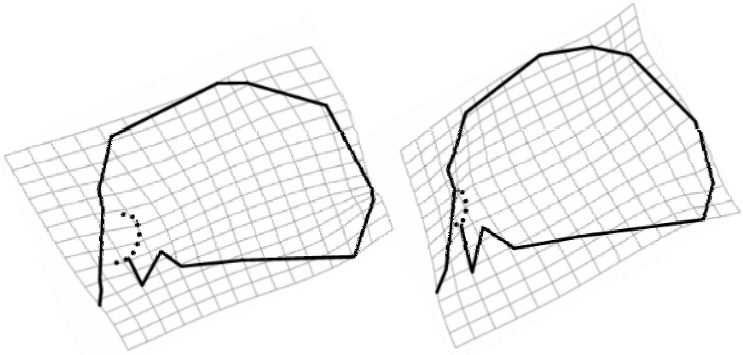

Figure 20. Deformation grids comparing two craniofacial shapes. Modified after Martínez-Abadías et al. (2006).

1.4.5.3 *Euclidean Distance Matrix Analysis.*

EDMA (Lele and Richtsmeier 1995, 2001, Richtsmeier et al. 2002) is a coordinate-system-free approach that is invariant to shape orientation (Lele and Richtsmeier 2001). While EDMA methods also use landmark coordinates as raw data, the form of each individual is represented as the matrix of Euclidean distances between all possible pairs of landmarks, the so-called form matrix. The form matrix is an equivalent representation of the landmark coordinate data, which is invariant to the nuisance parameters of translation, rotation and reflection (Lele and Richtsmeier 2001). The mean shape matrices for each sample can be obtained by standardizing the mean form matrices of each sample by a scaling factor, namely the geometric mean. The scaled interlandmark differences found among populations (Figure 21) can be used to explore localized skull shape changes (Lele and Richtsmeier 1995, 2001, Richtsmeier et al. 2002). This procedure shows which distances are significantly shorter or longer in the two forms that are being compared (Figure 21).

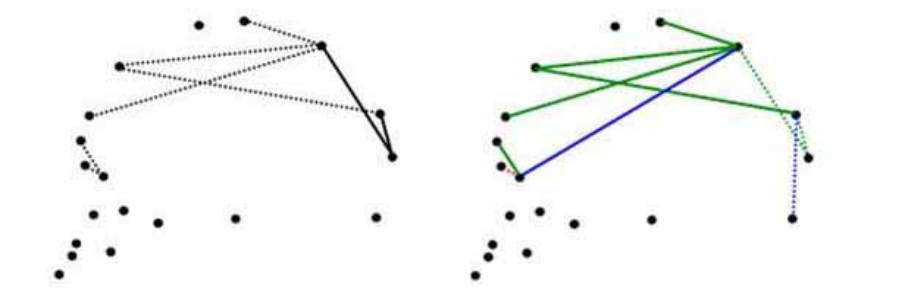

Figure 21. EDMA comparisons of craniofacial shapes. Modified after Martínez-Abadías et al. (2006).

Furthermore, Lele and Cole III (1996) described a procedure to test for significant differences in shape and size, based on the computation of the z-statistic. According to them, the statistical significance of localized shape differences is tested using a Monte Carlo approach, a parametric bootstrap procedure to calculate the 100 (1-α)% confidence interval for each size-corrected linear distance (Lele and Cole III 1996, Lele and Richtsmeier 2001). According to EDMA-II testing, a particular interlandmark distance is considered to be equal in two given samples if the resulting interval contains the value of zero. Otherwise, the equality null hypothesis is rejected and it is assumed that at the α significance level (usually α=0.5), a shape difference exists in that specific region (Lele and Cole III 1996).

1.4.6 MULTIVARIATE STATISTICS

The usefulness of GM for biological studies lies in the fact that it provides a way to accurately capture size-and-shape information from organismal form, as well as a way to statistically address and test hypotheses about morphological variation (Bookstein 1991, Rohlf and Marcus 1993, Dryden and Mardia 1998, Lele and Richtsmeier 2001, Zelditch et al. 2004). There is a growing body of statistical quantitative shape analysis, both for two- and three-dimensional data, which is being applied in very diverse fields of biology. Advances in GM are providing researchers with powerful tools to answer almost any question regarding comparative morphology. GM methods have not only adapted traditional multivariate statistical analysis to be used with landmark data, but have provided new techniques for the analysis and representation of shape.

Currently, there are many software packages that allow these computations: principal components analysis, discriminant analysis such as canonical variates analysis and cluster analysis, partial least squares analysis, ANOVA, MANOVA, regression, matrix and vector correlation, phylogenetic and quantitative genetic analysis. Most of this software is freely available from internet. One of the most useful sites is Dr. Rohlf's *Morphometrics at SUNY Stony Brook* (http://life.bio.sunysb.edu/morph/), which collects any kind of information regarding GM, such as meetings, workshops, bibliography, people and available hardware and software.

For the analysis of 2D data there are many user-friendly programs available to researchers. The most commonly used are the *TPS* programs developed by Dr. James Rohlf (http://life.bio.sunysb.edu/morph/), the *IMP* by Dr. David Sheets (http://www3.canisius.edu/~sheets/morphsoft.html) and the *Morpheus* program implemented by Dr. Dennis Slice

(http://life.bio.sunysb.edu/morph/morpheus/). The development of programs for three-dimensional analysis is much more limited, although everyday are being launched more and more tools for visualization, registration and analysis of 3D landmark data. The most used programs are the modules for 3D analysis of the *IMP* series, the *Morpheus* program and the *Morphologika* program, developed by Dr. Paul O'Higgins and Dr. Nicholas Jones (http://www.york.ac.uk/res/fme/resources/software.htm). EDMA analysis can be performed using the program WinEDMA, developed by Dr. Cole (http://www.getahead.psu.edu/EDMA_new.asp).

The specific analyses performed in the present thesis are described in full detail at the Material and Methods section of each of the Results chapter. For all the analyses, a pre-release version of the *MorphoJ* program developed by Dr. Christian Peter Klingenberg (http://www.flywings.org.uk/MorphoJ_page.htm) was used. This is a very complete and user-friendly program that performs almost any kind of GM statistical analysis and provides useful quantitative reports and graphical outputs. So far, this is the only program that provides tools for quantitative genetic analysis. Nevertheless, *MorphoJ* is still under construction and its final version is not yet available.

As it has happened in other fields of biology, anthropology has fully incorporated geometric morphometrics as a standard method for comparative anatomy and descriptive morphology (Slice 2005). GM is widely used to analyze skull morphology, but also to analyze other types of bones (see some examples at Slice (2005)) and structures such as the teeth (Martinón-Torres et al. 2006) or even the brain (Richtsmeier et al. 2006).

1.5 *Quantitative Genetics*

Quantitative characters are traits that exhibit continuous or almost continuous variation and that can be measured on a metric scale (Figure 22). These traits, such as weight, stature, craniometric measures and fitness, are assumed to be controlled by a large number of gene loci with small additive effects. The state of a trait in a population is described by the probability distribution of the trait, usually a Gaussian distribution, which is characterized by the mean value and the variance of the trait (Freeman and Herron 2004).

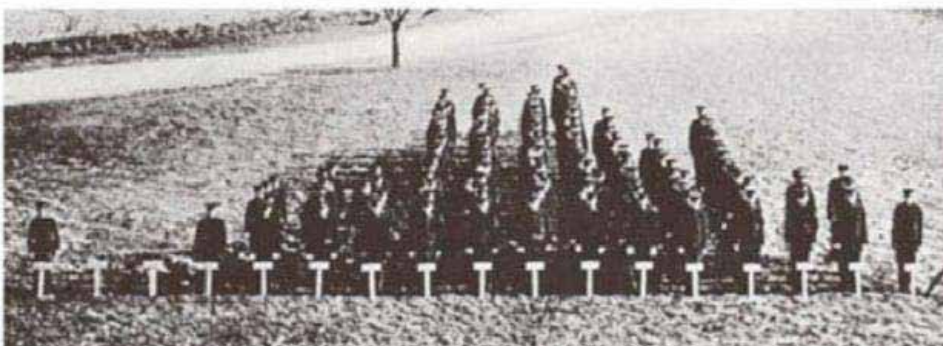

Figure 22. A "living-histogram". Distribution of height in a college class.

In the recent years, quantitative traits such as craniometrical traits have been successfully incorporated in genetic-populational models in order to provide insight into the structure of human populations (Relethford and Lees 1982, Relethford 1994). These studies have provided strong evidence that genetic variance can be inferred from phenotypic variance due to the high correlation between genetic and phenotypic variance-covariance matrices (Cheverud 1988) and the high correspondence between molecular and morphological distance estimates between human populations (Relethford 1994, 2002, González-José et al. 2004). However, little is known about the degree of genetic and non-

genetic influences on the phenotypic expression of more specific, functionally based traits.

Detecting and quantifying the genetic component of craniofacial traits is crucial because evolution operates on the genetic variation of populations. In other words, the evolutionary potential of the different skull regions, as well as their ability to respond to microevolutionary forces, directly rely on the genetic variation underlying the skull morphological and phenotypic patterns. The use of craniometric traits as biological markers for the study of human populations would only be justified if the cranial phenotype had a significant genetic influence. Furthermore, several researchers (Collard and Wood 2000, Lieberman et al. 2000b, Strait 2001, Lieberman et al. 2004) have pointed out the importance of finding "reliable" morphometric characters to make phylogenetic inferences from variation in craniofacial morphology.

A subject matter of quantitative traits is thus the heritability of complex metric traits. The narrow sense heritability is a key parameter in models of evolution of quantitative traits and constitutes a measure of the proportion of variance in a trait explained by genetic transmission (Konigsberg 2000). Moreover, it also shows the environmental variance represented in the phenotypic variance of each population (Varela and Cocilovo 1999). The computation of heritability estimates of cranial structures is so essential to physical anthropology studies because it gives an estimate of cranial plasticity as well as the potential influence of environment and thus natural selection, on particular structures of the skull (Konigsberg 2000, Sparks and Jantz 2002).

1.5.1 HISTORICAL USE OF CRANIOFACIAL TRAITS

Until the development of modern genetics, the anthropological research of past and present human populations was focused on the study of skeletal remains, because this was the only perdurable material that remained from them. Naturalists from the 18th and 19th centuries showed a tremendous interest in the excavation and preservation of human skeletal remains and huge collections accumulated at Natural History Museums from all around the world during this period.

During the 19th and 20th centuries, many physical anthropologists devoted their efforts to support racist or typologist trends of thinking (Deniker 1900, Eickstedt 1934, Biasutti 1941, Coon 1962). However, after

the II World War, most anthropologists and the scientific society in general showed its contempt for racism and Anthropology adopted a populationist perspective. From that point, the typologist paradigm was almost completely abandoned (Montagu 1964, Cavalli-Sforza et al. 1994). With the development of genetic and molecular approaches for studying human populations, some researchers assumed that population genetic analyses could only be performed after molecular markers, such as blood proteins and genetic polymorphisms, which were presumably considered as neutral characters because they were not affected by environment (Cavalli-Sforza and Bodmer 1971). Craniometric analyses were relegated to the paleopathological and bioarchaeological fields, in which craniometric traits were analyzed as markers of local adaptation to detect the effect of environmental factors. In spite of these criticisms, during the second half of the 20th century outstanding physical anthropologists such as William Howells (Howells 1973, 1989) continued to work with craniometric traits to study human populations and discussed their results under the populationist paradigm.

At the end of the 20th century, many evolutionary biologists began to reconsider the scientific use of skeletal remains to unravel human evolutionary paths (Buikstra et al. 1990). From that point, a new paradigm was established: population genetic models were adapted in order to be used after craniometric traits (Relethford and Blangero 1990). Nevertheless, the use of craniometric traits to study the structure and history of human populations is still controversial. It has been focus of continuous debate between researchers that argue against the plasticity of the skull and those who still consider that craniometric traits can not be considered as neutral markers in any case. For instance, González-José (2003) states in his PhD dissertation that "from the empirical point of view, this thesis is a claim of osseous markers as a valid source of information for Population Genetics".

In an archaeological context, craniofacial morphology has extensively been used for the analysis of the structure and history of human populations (González-José et al. 2003, Stojanowski 2004, Stojanowski 2005, Brace et al. 2005, Martínez-Abadías et al. 2006, Stojanowski and Schillaci 2006, González-José et al. 2007). Although it is acknowledged that morphometric traits can be affected by non-genetic factors, there is a growing body of evidence supporting the validity of this methodology. The studies undertaken by Relethford (1994, 2002) and Roseman (2004) show that human craniofacial variation patterns tend to behave as adaptatively

neutral characters. These studies have shown that a multivariant approach to skull samples can be a good fit to an assumption of selective neutrality and that the phenotypic morphological patterns reliably reflect the underlying genetic patterns. For instance, Relethford (2004) found a fit of global craniometric variation to the isolation-by-distance model, assuming a neutral model of quantitative variation. These results show that despite the fact that craniometric variation is affected by environmental influences (both developmental plasticity and climatic adaptation), it reflects the underlying patterns of population structure and history (Relethford 2004). Hence, it is concluded that craniofacial traits can be used to infer the genetic relationships between human populations.

Under several circumstances, craniofacial traits are advantageous over other type of traits. For instance, they are especially useful for the reconstruction of historical events. If samples from different periods and regions are available, one can design and test populational genetical models to detect the influence of microevolutionary agents (Relethford and Blangero 1990), the presence of discontinuities along a diachronic sequence (Konigsberg 1990a, Konigsberg 1990b, Relethford 1991, Steadman 2001, Stojanowski 2005, Brace et al. 2005, Martínez-Abadías et al. 2006, González-José et al. 2007) and/or along a geographic sequence (González-José et al. 2001, 2002, Brace et al. 2005, González-José et al. 2005a, 2007). Moreover, these studies can bring insight into the biological consequences of great technological and cultural transitions in human populations and test if they are correlated with the genetic and phenotypic structure of populations. This is the case of those past or highly admixed populations, because ancient DNA extraction is not always feasible and sometimes genetics is limited to the study of present populations.

1.5.2 THE BOAS DEBATE: IS IT STILL OPEN?

From a theoretical point of view, cranial phenotypes can not be considered as selectively neutral. It is evident that many environmental factors can influence the phenotypic expression of skeletal characters such as craniometrical traits. Therefore, the question is not if the morphology of the skull is subject to environmental influences or not, but if these environmental influences are pervasive over genetic influences. In other words, is the environmental component completely obscuring the genetic component of phenotypic craniofacial expression? Many researchers (mostly geneticists) consider that craniometric characters can not reflect

genetic differences because they are strongly affected by environment (Cavalli-Sforza and Bodmer 1971). It has long been assumed that environmental pressures acting during growth and development were the main force in determining the cranial form (Boas 1912, Cavalli-Sforza and Bodmer 1971). The results obtained from this project will add new arguments to the current debate about the potential adaptation and plasticity of the skull.

Plasticity is due to environmental effects, but it refers to morphological changes occurred during growth and development (Sparks and Jantz 2002). In contrast to adaptive changes, it does not require genetic changes to be expressed. The importance of plasticity in the human skull was settled down by Franz Boas, a North American anthropologist who analyzed the craniofacial form in EEUU by comparing European immigrants with their descendants born in America. According to Boas (1912), the differences in craniofacial morphology of individuals with European ancestry appear as a response to environmental changes. This work had long been cited as evidence for the plastic nature of the human skull until it was recently revisited with more modern statistical techniques (Konigsberg and Ousley 1995, Sparks and Jantz 2002). These authors reassessed the original Boas' data and found that differences between European and American born individuals were non-significant relative to the ethnic (genetic) differences between groups (Sparks and Jantz 2002). Hence, the so claimed plasticity of the skull is no further supported.

It is likely that some particular localized units of covariation, such as the structures related to the respiratory or the masticatory function, are more sensitive to environmental stressors (Franciscus and Long 1991, Hernandez et al. 1997) than other regions such as the base, which may not be under direct environmental influence and thus its genetic component should be relatively greater (Collard and Wood 2000, Collard and O'Higgins 2001). This does not mean that regions such as the nose are not under genetic control, but that the contribution of residual or environmental variation to the total phenotypic variation is expected to be greater than in other regions not directly linked to specific physiological functions or subject to environmental factors such as temperature, humidity, etc. Taken all together, the above evidence suggests that considering the skull as a whole has little sense in terms of heritability and this contradiction must be solved by analyzing craniofacial variation in a more functional and developmental way.

1.5.3 QUANTITATIVE GENETIC MODELS

Quantitative genetics allows the study of complex traits, such as craniometrical ones. Pioneering studies to elucidate the genetic basis of inheritance and the response to selection of a quantitative character were made a century ago (Galton 1889, Pearson 1903, Fisher 1918, Wright 1921). The works of the latter form the basis of classic quantitative genetic theory (see Falconer and MacKay (1996) and Lynch and Walsh (1998)).

The phenotypic variation (V_P) can be decomposed into a genetic (V_G) and a residual (environmental) component (V_E). In its simplest expression,

$$V_P = V_G + V_E$$

Genetic variation can be further decomposed into its additive, dominant and epistatic components (Falconer and MacKay 1996). However, dominant and epistatic components of variance are difficult to measure and in the case of morphological traits they are supposed to be non-significant. Heritability, considered in the narrow sense, expresses the proportion of total phenotypic variance due to additive genetic variance (Falconer and MacKay 1996, Lynch and Walsh 1998).

$$h^2 = \frac{V_G}{V_P}$$

Hence, stating that the craniofacial morphology is just due to environmental effects implies that V_G equals 0 and that V_E is 1. Conversely, many quantitative genetic studies have challenged this opinion and have evidenced a moderate to high significant genetic component of craniometric traits in human populations (Susanne 1975, 1977, Sjøvold 1984, Devor et al. 1986, Devor 1987, Sparks and Jantz 2002, Arya et al. 2002, Carson 2006a, 2006b).

Nevertheless, heritability is not only an intrinsic characteristic of the trait, but also depends on the population from which it is derived (Falconer and MacKay 1996). The population structure and history sets the differences in genetic variance between populations, whereas the influence of the environment and its changing conditions can also modulate the degree of heritability of any metric trait. As a consequence, heritability estimates are not universal, but population-constrained. Heritabilities are neither a measure of the degree of fixed genetic determination (Kohn 1991). There was the misconception that heritability estimates could evidence the causes of differences between populations and this was

particularly used in studies concerning human intelligence (Herrnstein and Murray 1994). However, this idea was strongly challenged and discredited by contemporary authors, such as Stephen Jay Gould in a reviewed edition of his book *The Mismeasure of Man* (Gould 1996).

The need of such heritability estimates has long been claimed by physical anthropologists applying model-bound genetic-population methods to quantitative traits. Relethford and Blangero's (1990) extension of the Harpending & Ward model (Harpending and Ward 1982) requires knowledge of either the additive genetic covariance matrix or the heritabilities of individual traits. Commonly, none of them are known and researchers tend to derive minimum F_{STs}; which is a conservative statistic. Thus, the phenotypic variation is assumed to be solely due to genetic variation, considering $h^2=1$ and distances estimated from the regional centroid are assumed to reflect minimum genetic distances (Relethford and Blangero 1990).

An alternative rough approach is to use estimates of average heritability reported in literature (Relethford 1994), as Devor's (1987) estimate for craniometric traits. Devor (1987) reports an average estimate of 0.55, which was computed from four populations using path analysis. Despite the fact that this estimation is also considered conservative because it was made upon classical craniometric variables measured *in vivo*, it has been widely used. For instance, heritabilities for skeletal measures are thought to be higher (Relethford 1994). Although Relethford & Blangero's model assumes neutrality and the exact choice of parameter values such as heritability is not critical, an estimation of heritability of units of covariance is necessary to better understand levels of morphological intra and inter population variance, as well as the dynamics of the expression of craniofacial structures.

However, rather than estimating an average heritability, greater advance in the comprehension of craniofacial variation will be attained if the skull is decomposed in functional and developmental units. Obtaining such estimates not only implies that model-bound methods as R matrix techniques (Harpending and Ward 1982) will be applied more accurately, but that morphological data will be regarded as complementary to molecular data, since actual genetic and non-genetic influences of cranial form will be determined.

1.5.4 METHODS FOR ESTIMATING HERITABILITY

There are several methods for estimating heritability, but all of them rely on the resemblance between relatives, which relates the phenotype of relatives to their shared genotype. Statistically, it is expressed as the phenotypic covariation between relatives, which is a natural consequence of relatives inheriting copies of the same genes (Lynch and Walsh 1998). From the simplest to the most complex designs, we find three main statistical methods: regression, breeding designs and restricted maximum likelihood (REML) methods. Herein a brief description is provided, but for a thorough revision see Falconer and MacKay (1996), Roff (1997) and Lynch and Walsh (1998).

In the regression method, a comparison between the offspring and their parents is carried out. Usually, the mean offspring value is regressed onto the mid-parent value and the slope of this simple linear regression is equivalent to the h^2 value (Roff 1997). In some cases, it is preferable to make a regression between the mean offspring value and just one parent (i.e. when the phenotypic variance in the trait is different between males and females, or when maternal effects are expected to explain a significant proportion of the phenotypic variation). The use of a single parent involves that the slope of the regression is equal to half the value of heritability and thus the slope must be multiplied by two in order to obtain the true h^2 value (Roff 1997). Due to its statistical simplicity and its low computational requirements, this was the most widespread method during the eighties and early nineties, although it has been reported that this is not the most precise approach (Konigsberg 2000).

The breeding design is statistically more complex and it requires experimental and/or strictly balanced data (Roff 1997). Two basic designs have been developed: the full-sib and the half-sib design. In the full-sib design, families are constituted by full brothers and sisters, without reference to the parents. That is, only individuals who share the same parents (mother and father) are considered as full sibs and thus are included in a family. Conversely, in the half-sib analysis, each male is mated to several dams and brothers and sisters are just half sibs because they only have one parent in common (either the father or the mother). The main limitation of these methods is that they are difficult to apply with natural populations. Furthermore, they have potential important sources of error: dominance variance and common environment can inflate h^2 values. The overestimation of h^2 due to dominance variance is overcome with the half-sib design (Roff 1997). Phenotypic, genetic and environmental covariance

estimates can be estimated by one-way analysis of variance (ANOVA). This approach has also been used extensively used during the nineties until recently.

Nowadays, the most widespread approach to compute heritability estimates is based on restricted maximum likelihood methods (REML). REML analytical methods are advantageous in contrast to parent-offspring regression or sib analyses because they incorporate multigenerational information from unbalanced datasets and hence any breeding design can be accommodated (Roff 1997). Furthermore, they are not bound by assumptions of non-assortative mating, inbreeding or selection (Kruuk 2004). REML methods are usually applied under the animal model, which is a mixed linear model that jointly accounts for fixed and random effects in order to describe the phenotype of each individual. The phenotypic variance is broken down into its components of additive genetic value and other random and fixed effects. The components of variance are estimated by an iterative procedure that maximizes the likelihood of observing the actual data (Lynch and Walsh 1998). The REML analysis provides estimates of the additive genetic variance and the variance of the residual errors, from which the narrow heritability can be estimated. For the estimation of variance components, the general model can be modified in order to account for common environment and maternal effects, as well as other fixed effects such as sex, age and size.

The conceptual basis of this technique is simple, but its implementation is not. Maximum likelihood was introduced as a variance component-estimation method (Hartley and Rao 1967) and restricted maximum likelihood methods for non-balanced data were introduced few years later (Patterson and Thompson 1971) but were not developed until ten years afterwards (Kennedy 1981, Hopper and Mathews 1982, Shaw 1987, Meyer 1989). Computationally, REML is high demanding and this is one of the main reasons why it has not been implemented until recently, with the development of modern computers and sophisticated statistical packages. Roff (1997) advised that "the present difficulties of using such techniques strongly favour the adoption of simple experimental designs".

Despite the statistical differences between these methods, all of them rely on the relationships among individuals and are limited by sample size. In order to obtain precise heritability estimates (with minimum standard errors), large sample sizes are required. However, these are seldom available. Lack of sufficient breeding information can confound additive

genetic variance with phenotypic variance from other sources (McGuigan 2006). Animal models are more powerful tools to distinguish and separate non-additive genetic variance because they account for different classes of relatives. By introducing further random and fixed effects, animal models also provide a means to account for common environment, which is prevalent in some situations and can substantially inflate the additive genetic variance (Lynch and Walsh 1998, Kruuk 2004).

1.5.5 PREVIOUS HERITABILITY STUDIES

One of the main problems that researchers encounter when investigating the heritability of cranial measurements in humans is that suitable, large and pedigree-structured skull series are almost non-existent. As a consequence, heritability is either computed from information collected on family studies of living individuals (Devor et al. 1986, Nikolova 1996), or on twin pairs (Nakata et al. 1974, Sharma and Susanne 1991, Sharma 1998), or is limited to data reported by familial regression. As stated by Konigsberg (2000), these methods are quite inefficient to estimate genetic parameters in natural populations. Moreover, it also has been reported that the inclusion of twins in a family study inflates heritability to some degree (Devor et al. 1986). This project brings the unusual opportunity of studying heritability of cranial structures in a unique collection of skulls in which reconstruction of several nuclear families is possible (Sjøvold 1984). Thus, maximum likelihood methods can be applied. As reported above, this method has an advantage over methods of parent-offspring regression because it uses all kinship information within the pedigrees simultaneously instead of a series of pairwise regressions (Sparks and Jantz 2002).

Previous research devoted to the genetic and environmental components of phenotypic variation was mainly focused on the transmissibility and/or heritability of classical craniometric variables (Cheverud and Buikstra 1982, Devor et al. 1986, Devor 1987, Konigsberg and Ousley 1995). The first studies regarding the heritability of anthropological characters date back to the first decades of the 20th century. In the early twenties, it was proposed a method to estimate heritability based on the comparison of intrapair differences in mono- and dizygotic twins (Dahlberg 1926), what he termed the F-ratio. This approach was extensively used during the fifties and sixties (see Vanderberg (1962) and (Hiernaux 1963) for a revision of studies estimating heritability of anthropometric characters from twin data). Vanderberg

(1962) compared heredity estimates obtained from data of six different studies and noted that despite overall good agreement, there were some discrepancies between results. The author argued that the reasons for these differences were mainly methodological and did not consider the fact that different populations are subject to different environments and have different population histories. These may also yield to differences in genetics and thus in heritability estimates.

Anthropometric character	**Howells measure**	**Heritability (SE)**				
		Devor et al., (1986)	Susanne (1977)	Sparks & Jantz (2002)	Arya et al. (2002)	Carson (2006a)
Head circumference		0.491 (0.069)				
Bizygomatic diameter	ZYB	0.399 (0.065)	0.606	0.49	0.605 (0.045)	0.257 (0.178)
Head breadth	XCB	0.574 (0.069)	0.614	0.61	0.447 (0.051)	0.233 (0.115)
Min frontal diameter		0.282 (0.070)				
Head length	GOL	0.435 (0.072)	0.554	0.55	0.413 (0.051)	0.363 (0.116)
Bigonial diameter		0.496 (0.057)	0.662			
Upper facial height		0.568 (0.059)				
Nasal height	NLH	0.512 (0.060)	0.391		0.417 (0.051)	0.729 (0.153)
Morph facial height		0.397 (0.062)	0.650		0.414 (0.053)	
Nasal breadth	NLB	0.382 (0.072)	0.639		0.498 (0.049)	0.007 (0.122)
Head height	BBH		0.715			
Frontal breadth			0.667			
Ext biocular breadth			0.661			
Nasion-gnathion height			0.581			
Nasal depth			0.548			

Table 2. Comparison of heritability estimates of several anthropometric measures.

Susanne (1975, 1977) performed several studies during the seventies based on living humans and familiar relationships. Susanne (1977) used a sample that included more that hundred families and applied the method of Fisher (1918) for the calculation of the heritability coefficients. His results showed that body measurements had the highest values of heritability (especially those longitudinal, such as stature, sternal height and arm length); whereas face and head measurements had lower but still moderate to high heritability estimates. The heritabilities obtained by Susanne (1977) were rather high (from 0.391 of nose height to 0.715 of head height), but the same author acknowledged that these estimates could be inflated by assortative mating and common environment of relatives. Furthermore, he acknowledged that some of the results could be affected

for some measurement error as well as by the presence of muscular and adipose tissues.

During the eighties, some researches focused on the transmissible and non-transmissible components of anthropometric variation and used the method of path analysis developed by Wright (1921) to distinguish between the genetic and environmental sources of familial resemblance (Poosha et al. 1984, Byard et al. 1984, 1985, Devor et al. 1986, Devor 1987). Devor et al. (1986) reported that estimates of transmissibility for craniofacial measures were intermediate (from 0.382 of nasal breadth to 0.574 of head breadth). In comparison to previous studies using twin data (Nakata et al. 1974), these estimates were lower. In this early study, it was already pointed out the importance of some developmental mechanisms, as well as the hierarchical organization between functional complexes, determining the genetic and nongenetic factors influencing skull size and shape. Devor et al. (1986) concluded that traits related to general size factors had greater transmissibilities that those associated to group and special size factors.

During the nineties some studies analyzed the effect of sex, parental effects and common environment in the genetic variance of craniometric traits using twin data (Sharma and Susanne 1991, Sharma 1998). These authors found that males had higher genetic variance ratios than females. Moreover, they found that facial traits were under higher maternal effect, whereas cranial traits were under more paternal effect. They also pointed out that environmental covariance was higher in monozygotic than in dizygotic twins and thus considered that heritability estimates could be inflated in monozygotic twins. Finally, Sharma and Susanne (1991) reported that these patterns were consistent in two different human populations (Indians and Belgians), but that the heritability estimates were different among them.

More recent studies used restricted maximum likelihood methods to compute heritability estimates of craniometric traits (Sparks and Jantz 2002, Arya et al. 2002, Carson 2006a). Arya et al. (2002) analyzed the effects of caste membership in an Indian population and found that both socioeconomic and nutritional status significantly affect the heritability of some traits, increasing the environmental contribution to phenotypic variation. In this study, the heritabilities tended to be lower than those reported in previous literature, showing low to high degree of genetic component. Their results contradicted the claim that craniofacial measures

have lower heritabilities than linear body measures (Arya et al. 2002): the highest heritabilities were found at bizygomatic breadth, nasal breadth and head breadth.

The Hallstatt skull collection has been previously analyzed by other authors (Sjøvold 1984, Carson 2006a). The work by Sjøvold (1984) was one of the first surveys to heritability on a human skull pedigreed series and heritability of metric and non-metric traits were estimated using the regression analysis. Contemporary studies on human craniofacial dimensions were based on twin data and living humans (Saunders et al. 1980, Devor et al. 1986, Boraas et al. 1988, Sharma and Susanne 1991). Sjøvold (1984) concluded that most of Howell's measurements were significantly hereditary and showed that the structures showing the highest heritabilities were those connected to the size of the brain, the orbits, the nose and the masticatory apparatus.

The study by Carson (2006a) was based in a restricted maximum likelihood estimation method (REML) and presented alternative estimates to that of Sjøvold's (1984). The main conclusion of this study was that Howell's measurements show low to moderate narrow sense heritabilities, and reported that the breadth and facial dimensions were the less heritable. For several measurements, both studies reported different results and came into somewhat different conclusions. According to Carson (2006a), these differences stem from the different statistical techniques used for the heritability estimation. She argued that REML methods are more accurate to estimate heritability from complex, non-balanced pedigree models than the regression method used by Sjøvold (1984). Here, the univariate heritabilities of cranial dimensions have been reassessed with REML methods (Chapter 3).

In a recent paper, Johannsdottir et al. (2005) employed the regression method between parents and offspring to estimate the heritability of maxillofacial and dentoalveolar traits from lateral cephalograms of an Icelandic population. Unfortunately, there is no direct correspondence between craniofacial traits and cephalometric parameters, but their results point that the positions of the lower jaw, the anterior and posterior face heights, as well as the basicranium dimensions show the highest heritabilities, while the dental variables show the lowest (Johannsdottir et al. 2005). Another of the main conclusions of this study was that maternal effects did not affect their results (Johannsdottir et al. 2005).

As reported, a wide range of studies have estimated the heritability of human craniofacial traits, but the comparison of results is controversial since they have been computed upon different kinds of samples of different origins (living humans or skeletal remains from different geographical regions), accounting for different familiar relationships (twins, nuclear or extended families), using different statistical methods (regression, ANOVA, path analysis, REML) and considering different sources of non-genetic variation (Table 2).

1.5.6 WHICH TRAITS ARE HERITABLE?

The heritability of any trait can be derived when relationships among individuals from a given sample are known (twin, sibships, pedigree studies). Nevertheless, the main problem related to heritability estimations is the trait itself. Even when the analytical tools and the experimental design of those studies are well established, a serious pitfall is that raw data usually consists of classical linear measurements. Traditional craniometric traits defined by Martin (Martin and Saller 1957) and Howells (Howells 1973) have been thoroughly used in this kind of studies, although they are arbitrary measures which have neither functional nor developmental sense (e.g. a classical Howell's variable, GOL, the glabella-opisthocranion length, covers several cranial regions with different functional and developmental constraints, such as the face and the braincase). These variables are good tools from the taxonomic point of view, since classifications of human populations based on this kind of traits tend to accurately reflect the historical and structural aspects of populations (Relethford 1994, Relethford and Harpending 1994, Relethford 2002). However, it is very difficult to explore the mechanisms of expression of such variables, since there are not tied either to functional or developmental processes.

If we take into account morphological integration (Olson and Miller 1958, Cheverud 1982, 1984, 1995), which assumes that traits involved in a common function or developmental process are distributed under the same selection pressures and are thus coinherited, it would be more appropriate to consider the heritability of units of covariance in cranial structures rather than linear distances. It seems preferable to group craniofacial measurements in functionally or developmental meaningful ways than to use many unrelated variables. The calculation of heritability of cranial modules would represent a more accurate estimate.

Another problem with the use of craniometric measures is that the skull shape is represented by a collection of single univariate linear and angular measurements that are correlated among them (that is, they are not independent). However, shape is inherently multivariate (Klingenberg and Monteiro 2005) and it makes little sense to decompose shape in such a way when in fact there is available a set of statistical techniques that allow the multivariate analysis of shape, namely geometric morphometrics. Despite this prevalence of geometric morphometrics over traditional methods, very few studies have applied geometric morphometrics to the analysis of the genetic variation of shapes. The first attempt was performed few years ago by Klingenberg and Leamy (2001), who combined the methods of landmark-based morphometrics and quantitative genetics to analyse the heritability of mouse mandible size and shape. They used REML methods but found that the high dimensionality of data was a strong limitation, because software packages were unable to process so many variables at the same time (Klingenberg and Leamy 2001). Currently, these difficulties are just partially overcome. This procedure has been applied in this thesis to separate the genetic and environmental components of phenotypic variation of the human skull (Chapter 4).

Klingenberg and Leamy (2001) emphasized that the concept of heritability, as described in the previous sections, has no direct equivalent in the multivariate context because phenotypic and genetic variations are spread through the dimensions of shape space and cannot be summarized in a single value. Instead, one can compare the phenotypic and genetic covariance matrices (*P* and *G*) and assess their similarity. They interpret such similarity as evidence that both genetic and environmental variation is expressed phenotypically through the same processes (Klingenberg and Leamy 2001). Moreover, these authors argue that through this multivariate approach shape is not dissected in different parts, but it allows detecting differential "behaviours" of morphological regions within the global shape analyzed. In another paper, Klingenberg (2003b) further states that 'the very strength of geometric morphometrics is that the analyses can account explicitly for the spatial heterogeneity that is associated with the anatomy and the ontogenetic origins of biological structures. Although it is mathematically possible to compute a "global" heritability estimate by averaging across all dimensions of shape space, such an overall measure must ignore the spatial structure of variation, and is therefore fundamentally at odds with the goals of geometric morphometrics'. This procedure (Klingenberg and Leamy 2001) brings the opportunity to give

deep insight into the genetic basis of the architecture of biological shapes. However, despite its usefulness, very few researchers have applied it with other kinds of structures and/or organisms.

Monteiro et al. (2002) proposed an alternative approach to combine the methods of geometric morphometrics and quantitative genetics. This was based on the use of Procrustes distances within and among families to estimate shape heritability of honeybee's wing. Procrustes distances are a measure of similarity/difference between pairs of landmark configurations and these authors argued that this approach allowed a multivariate generalization of heritability estimates of shape while retaining its univariate simplicity (Monteiro et al. 2002). Nevertheless, Klingenberg (2003b) challenged this view and highlighted the pitfalls of such an assessment: first, the assumption of the isotropic model, which is often unrealistic; second, the inappropriateness of the distance measure to evaluate the heritability of shape; and third, the need of large and extremely balanced experimental designs. The main handicap of Procrustes distances is that they just account for the magnitude of shape differences and disregard their direction, which is essential to predict the response to selection.

In a recent paper, Klingenberg and Monteiro (2005) further developed this point and explained how to use and how to interpret these measures. The univariate approach of Monteiro et al. (2002) is useful because it is more straightforward than that of Klingenberg and Leamy (2001), but on the other hand is disadvantageous because its limited use: the univariate approach compares if two groups have similar levels of shape heritability, but can not detect why they are evolving in similar ways. Myers et al. (2006) performed both approaches to analyze the quantitative genetics of plastron shape in slider turtles and concluded that the multivariate approach is much preferable: the univariate approach was unable to describe how shape was evolving. In their analysis, they compared the heritability pattern of turtles from two different nesting areas and found that despite both nesting populations showed similar magnitudes of heritability of plastron shape, the direction of shape evolution was completely different (Myers et al. 2006). The detection of such a difference could only be achieved with the assessment of the relative shifting of landmarks, from a multivariate management and treatment of shape.

1.5.7 SELECTION & EVOLVABILITY

Quantitative genetics is essential because as well as providing a method to separate the genetic from the environmental component of phenotypic variation, it also allows to predict the response to selection (Falconer and MacKay 1996). These methods have long been used by animal breeders in selection programs in order to improve animal production, and more and more they are being applied by evolutionary biologists.

The genetic properties of a population are the product of natural selection in the past, together with mutation and random drift (Falconer and MacKay 1996). Genetic variation is reduced after stabilizing selection and genetic drift, whereas is increased by admixture and mutation. In the absence of selection, mutation can generate and maintain a large amount of genetic variation except in very small populations (Falconer and MacKay 1996). Whether mutation is enough to ensure evolvability or not is a current subject of discussion (Hansen and Houle 2004).

Natural selection operates through the available genetic variation and causes changes in the gene frequencies (an increase in those alleles positively selected and a decrease in those selected against or negatively). This occurs through differences of fertility among the parents, or of viability among the offspring. There are several types of selection (Figure 23): stabilizing selection, disruptive selection, directional selection and balancing selection (Freeman and Herron 2004). Herein we are interested in the different effects that selection may produce on phenotypes. Stabilizing selection is thought to be the most common mechanism of natural selection, which favours individuals with intermediate characteristics by decreasing genetic variation and stabilizing on a "mean" trait value. Disruptive selection has exactly the opposite effect: individuals with extreme values at both ends of the distribution are selected for. In some cases, this type of selection may be responsible for speciation (i.e. if individuals from each extreme become geographically isolated). Directional selection can produce a similar effect, but the difference with disruptive selection is that only one end of the variational spectrum is selected for (Freeman and Herron 2004). Directional selection favours a single allele, whereas balancing selection is the force that maintains genetic polymorphisms (multiple alleles) within a population.

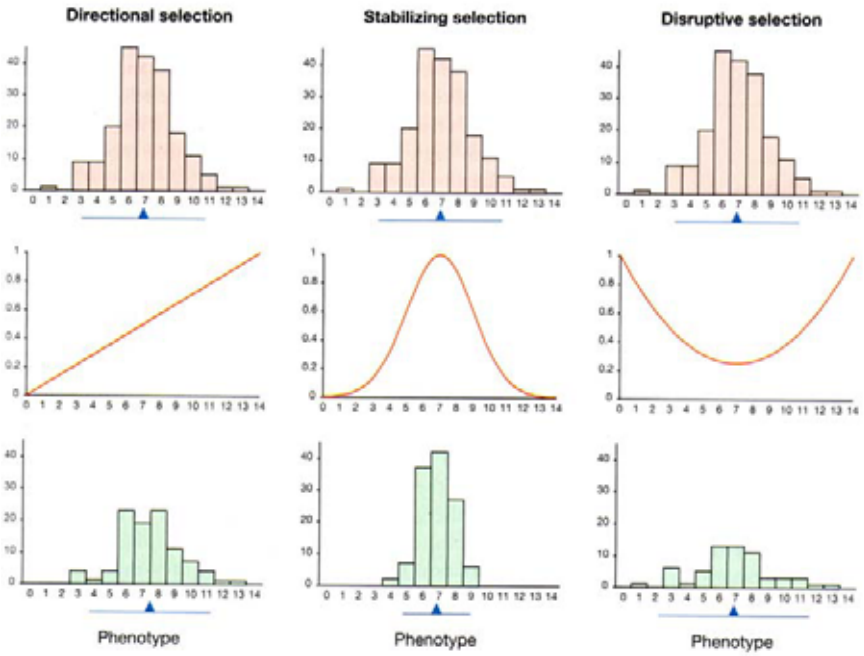

Figure 23. Three modes of selection. The upper histrogram shows the distribution of a given trait before selection; the graph in the middle plot the probability of survival (a mesure of fitness) as a function of the phenotype; the graph at the bottom shows the distribution of the trait after selection. The blue triangle under each histogram shows the mean value of the population, whereas the blue bar indicates the variation ± 2 standard deviations from the mean. After Freeman and Herron (2004).

Directional and stabilizing selection tend to reduce the amount of variation in a population, while disruptive selection tends to increase it (Freeman and Herron 2004). In directional selection, fitness changes along with changes of the selected trait value (increasing or decreasing), which also produce shifts of the mean trait value. Phenotypically, these genetic changes may be detected as changes in the population mean between generations, when the parental and the descendant/offspring populations are compared (Falconer and MacKay 1996). Under directional selection, the mean value of the population shifts towards one end of the trait distribution at each generation. This differential, that is, the difference between the offspring mean and the parental mean before selection, is the so-called response to selection (*R*). The animal breeder's equation relates *R* with the heritability of a quantitative trait by the expression $R=h^2S$, where *S* is the selection differential, which is the difference of the population mean and the parental phenotypic mean after selection (Falconer and MacKay 1996).

The multivariate version of the breeder's equation (Lande 1979) applies the same scheme as the univariate version to predict changes and

selection on shape, but considers covariation within traits. According to Lande (1979),

$$\Delta\mu = GP^{-1}s$$

where Δμ is the response to selection (that is, the vector of differences in trait means between generations), *G* is the additive genetic covariance matrix, *P* is the phenotypic covariance matrix and *s* is the selection differential (vector of differences in trait means in the parental generation before and after selection). The expression $P^{-1}s$ is equivalent to β, the vector of selection gradient, which can also be estimated as the vector of partial regression coefficients of relative fitness on character states. The *G* and *P* matrices are square symmetric matrices with as many rows and columns as the number of traits considered. The diagonal entries are the variances of the traits, while the off-diagonal elements give the covariances between traits.

The main problem of this approach is the high dimensionality of the data: there is a "trade-off" between the number of traits considered and the capability to interpret results. In order to capture shape with more precision, researchers usually tend to increase the number of traits measured, but this has the "negative" effect of increasing the size and dimensionality of variance-covariance matrices, because it complicates the computations and because covariation patterns are difficult to interpret simply by looking at *G*. According to McGuigan (2006), the use of eigenanalysis (e.g. principal component analysis) is advisable in order to reduce the dimensionality of data and to facilitate the interpretation of main patterns of covariation and main directions of shape change.

Although the genetic variance-covariance matrix (*G*) is a statistical abstraction (estimated from the phenotype and the expected resemblance between relatives, but without direct observation of the number of contributing loci and alleles, modes of gene action or individual alleles effects), it is expected to contain information about shared functional, developmental and genetic processes between interacting characters (McGuigan 2006). It is widely accepted that *G* evolves and that the evolution of *G* can drive macroevolutionary and microevolutionary patterns and determine its phenotypic outcome (Steppan et al. 2002). However, very little is known about the exact mechanisms by which *G* evolves. Questions about the rate and processes operating in *G* evolution remain unsolved. Quantitative genetics is one of the most promising fields

of evolutionary biology because it provides the tools for predicting evolution in phylogenetic contexts.

Theoretically, in long-term evolution, *G* can keep constant, proportional or change differently but in a predictable manner (Steppan et al. 2002). It is often considered that genetic drift produces proportional changes in *G* (Roff 2000), while natural selection causes non-proportional changes. However, this view has been challenged by several authors, who suggested that in the wild proportionality of *G* is not a useful criterion for distinguishing the action of drift from that of selection, because under certain circumstances genetic drift can also cause non-proportionality (for a revision see (McGuigan 2006)). The direction and rate of evolution depend on the intrinsic characteristics of *G* along with the adaptive landscape, which relates the forces of natural selection upon a set of traits with differential fitness: it considers which combination of traits would provide the individual/population with greater reproductive success.

If *G* is stable, it can be used to predict the evolutionary potential of a population or to reconstruct the form of selection that has led to divergence among populations (Steppan et al. 2002). Persistent multivariate stabilizing selection may yield to a stable *G* that is aligned with the adaptive landscape (McGuigan 2006). However, the relationship between G and the type of selection is not straightforward, since the sensitivity of G to selection might depend on whether selection affects just the multivariate mean or if there is selection for phenotypic covariance (McGuigan 2006). Conversely, if *G* is not stable and has changed through time, it can be tested the likelihood of specific adaptations being responsible for such changes. According to McGuigan (2006), adaptive variation both reflects historical evolution and determines the population's future phenotypic response to evolutionary processes. *G* can be regarded as a tool for retrospective analysis: the analysis of *G* can identify evolutionary constraints and differences among populations in their potential to evolve and specifically predict the direction and rate of phenotypic divergence (adaptive or neutral) (Lande 1979, Cheverud 1984, McGuigan 2006).

Quantitative traits usually have abundant genetic variation and *a priori* they have high capacity to evolve. However, it is not only quantity of variation what matters, but also quality of variation. According to Hansen and Houle (2004), despite high amounts of genetic variation, genetic variation can be constrained by integration of characters (pleiotropic constraints) or by integration among genes (epistatic constraints). Merilä

and Björklund (2004) state that 'organisms are capable of adapting to most challenges posed by their environment given sufficient time and genetic variability, but the possible solutions will always be constrained to some degree by their history'. In fact, this means that the potential evolvability of any trait is in practice reduced.

This causes the paradox of stasis: if quantitative traits supposedly have enough additive genetic variation and potential to evolve, why so many traits are maintained through evolution and long-term macroevolutionary scales? Traditionally, stabilizing selection was considered as the main cause of this conservationism (Hansen and Houle 2004). However, current thinking points out that stasis may stem from more complex processes, which should explain how a trait selective optimum is conserved, especially if we take into account that fitness functions of most quantitative traits depend on multiple factors interrelated between, instead of on a single selective factor.

In order to provide such an explanation, Hansen and Houle (2004) used the concept of constraint, which is defined as any mechanism that may limit or bias the response to selection. They distinguished between variational and selective constraints. Variational constraints are considered as a consequence of limitation in the variability of the characters, whereas selective constraints result from "conflicting selection pressures" (that is, when different selection factors directly or indirectly favour the evolution of a trait in opposite directions). Constraints may participate in stasis and may also be responsible for the evolutionary failure of some "natural impossible forms" (Merilä and Björklund 2004). Following the methodology proposed by Klingenberg and Leamy (2001), it can be detected which directions of morphological shape change have no genetic variation at all and which are correlated among them. This approach has been applied in the present thesis (Chapter 4).

According to Wagner and Altenberg (1996), the evolvability of a trait depends more on its variability (the capacity of traits to vary) than on the standing level of variation. The variability of a trait is a direct consequence of its genotype-phenotype map, what Hansen and Houle (2004) consider as the functional architecture: the collection of pathways that lead from the genes to the character. Regarding this point, they further introduce the concept of conditional evolvability, which is defined as the evolvability of a character y in the event that a set of constraining characters x is not allowed to change. Hansen and Houle (2004) propose a means to assess

conditional evolvability, which consists in estimating the conditional genetic variance, by regressing the genetic value of *y* on the genetic value of *x*, and obtaining the residual variance. Therefore, this is an estimate of conditional evolvability that is independent from the strength of stabilizing selection on *x* (Hansen et al. 2003).

In the context of the evolution of skull morphology, conditional evolvability can also be applied: as different skull regions are not independent, morphological traits do not vary and evolve separately, but in a coordinated way that is compromised by pleiotropy with other characters. Pleiotropic effects and constraints are barriers to evolutionary change and the short or long term paucity of changes could be interpreted as traits being under stabilizing selection, but this may be just an appearance: traits don't change because they cannot, not because they are selected for reaching and maintaining a selective optimum. Furthermore, as a structure is more complex, so is the developmental network that is responsible for it and hence its key traits are more and more entrenched and can even lose their variational freedom.

Another potential cause influencing the evolvability of a trait is epistasis, where interaction between genes can cause a cascade of effects within the genetic pool when a single or more alleles change at a given locus or loci. Epistasis might have opposite effects on evolvability (Hansen and Houle 2004): it can either restrict it (when there is negative feedback between genes), or enhance it (when there is positive feedback between genes and the gene effects are intensified). Therefore, the functional architecture relies both on epistatic and pleiotropic effects and both can constrain the evolvability of characters. The force by which this functional architecture might evolve is selection, either by correlated stabilizing selection (Olson and Miller 1958, Cheverud 1984, 2001) or by correlated directional selection (Wagner 1996).

The genotype-phenotype map is essential at this point, and unfortunately very few things have been unravelled so far from the human skull genotype-phenotype map. There is certain knowledge about scattered genes participating in skull and brain development, but we have not yet reconstructed the whole developmental pathways linking the genetic background with its phenotypic expression. Several researchers as Dr. Benedikt Hallgrímsson (University of Calgary) and Dr. Daniel Lieberman (Harvard University) are dealing with this line of research and their contributions are especially important for this field. The contribution of

this thesis is somehow limited on this point, because any direct evidence of the genes involved in the phenotypic skull morphology is lacking, but at least some information is provided regarding the genetic variation, variability, as well as potential and conditional evolvability of craniofacial traits (Chapters 3 to 6).

As predicted by theoretical genetic quantitative models (Lande 1979), the rate and direction of evolution strongly depend on the genetic variance covariance matrix (*G*), which is morphologically expressed as phenotypic integration. In this matrix are "hidden" the genetic constraints that modulate the phenotypic response to selection: either through low levels of genetic variation, or through genetic trade-offs among components of fitness (Merilä and Björklund 2004). In comparison to the univariate model, the multivariate model is more appropriate because it considers all the traits simultaneously, taking into account their genetic variation but also the genetic covariation among them. Therefore, interdependence, constraints and integration are taken into account. It should be emphasized the relevance of covariation between traits: if traits were completely independent and covariation was non-existent, the response to selection ($\Delta\mu$) would be mainly determined by the direction of the selection gradient (β); otherwise, if traits were highly interdependent and covariation between traits was significant, the response to selection ($\Delta\mu$) would be driven by G (specifically by the direction of the eigenvector associated with the largest eigenvalue, g_{max}) rather than β (Merilä and Björklund 2004).

The distribution of eigenvalues of *G* can result very insightful: if all eigenvalues have the same weight, evidences weak correlations between traits; otherwise, if one eigenvalue is much larger than the remaining ones, it means that covariances are high (and as a consequence, the adaptive peak may never be reached). Furthermore, this indicates that the rest of eigenvalues are low or even zero and these represent "forbidden" evolutionary trajectories (Kirkpatrick and Lofsvold 1992). Therefore, the dimensionality of *G* provides an estimate of the number of independent traits contained in *G* and points out which regions of phenotypic space are evolutionary accessible and which are not (McGuigan 2006). If *G* has fewer dimensions than traits, there are phenotypes (trait combinations) that cannot evolve in the population because they have no additive genetic variation.

However, as explained above, integration between traits not only restricts evolution, but also can facilitate further adaptation. When

organismal structure is modularized, mosaic evolution is enhanced and directional selection can favour rearrangements of these complexes without large cascading effects (Merilä and Björklund 2004). A couple of predictions are derived from this principle: first, it is expected that traits that are highly integrated within populations will be so also across populations; and second, that functionally highly integrated traits should be less affected by environmental and genetic perturbations.

In sum, it should be considered that the nature of integration (and its consequences on evolvability) does not only depend on the trait itself, neither on the population, but that it is influenced by the prevailing environmental conditions and the microevolutionary forces that are acting on a given population at a given period of time. In other words, it should be considered that integration patterns, as well as genetic variation and heritability, can fluctuate and vary depending on the past history and current conditions of the populations.

There are very few studies that have jointly encompassed the subjects of heritability, evolvability and morphological integration. Cheverud (1996b) performed such an attempt and compared these patterns in two species of New World monkeys, the cotton-top and saddle-back tamarins (*Saguinus oedipus* and *S. fuscicollis*). This study showed that patterns of phenotypic, genetic and environmental variation and correlation were very similar across species and among the types of variance within species. Craniometric traits showed low to average levels of heritability (from 0.08 to 0.87 and an average value of 0.40-0.45 in both species). Correlation patterns evidenced that in both species developmental related traits were more integrated than developmental independent traits: the highest integration was found within cranial vault traits and characters measuring the oral apparatus. Cheverud (1996b) concluded that the relative constancy in patterns of variation morphological resulted from the relative constancy of the developmental patterns responsible for that morphological outcome among closely related species.

When comparing the evolvability of craniometric traits in each species (Cheverud 1996b), as measured by the genetic coefficient of variation (Houle 1992), results pointed out consistent patterns among species. Taking everything into account, it seems that tamarins share common patterns of morphological variation. However, there are obvious differences between them that have caused species diversification and that could be explained by differential selection. The response to selection

analysis highlighted that selection in the cotton-top tamarin could have resulted in an increase in the area of attachment for the anterior temporalis muscle, which could have improved incisive food preparation and increased efficiency of mastication and increased facial prognathism. This holistic approach is extremely interesting, but it has not yet been applied in humans. This is one of the main goals of this thesis (Chapters 3 to 5).

There have been several attempts to detect and quantify the magnitude of natural selection on primate and human cranial morphology (Marroig et al. 2004, Ackermann and Cheverud 2004, Roseman 2004, Marroig and Cheverud 2004, Roseman and Weaver 2004, Weaver et al. 2007). These studies have provided indirect evidence that most evolutionary changes in modern human craniofacial form are the result of genetic drift rather than adaptive selection. Exceptions to this general pattern concern some regionalized adaptations (mainly nasal) to extreme cold weather (González-José 2003, Roseman 2004) and a decrease in mechanical loadings at the masticatory apparatus with the consumption of softer and more processed foods (Larsen 1997). At a higher taxonomic level, Marroig and Cheverud (2004) found that natural selection was the main force of evolutionary diversification of New World Monkeys, at least above the level of genus, although genetic drift may account for differences between some species. These results indicate that not all speciation events are linked to adaptation. In humans, Ackermann and Cheverud (2004b) reported that within *Homo,* genetic drift was probably the main force responsible for facial diversification, but acknowledged that selection most likely shaped hominin facial morphology in the late Pliocene. This means that the split between *Australopithecus* and *Homo* involved adaptive selection and divergence, but that afterwards random processes yielded to current modern human craniofacial diversity (Ackermann and Cheverud 2004b).

Moreover, the same authors and others have been concerned about the stability of the phenotypic variance/covariance matrix *(P)* (Marroig and Cheverud 2001, Ackermann 2002, González-José et al. 2004), assuming that this is proportional to the additive genetic variance/covariance matrix *(G)*. The common scope of these studies was to test functional and developmental integration hypotheses and to detect whether integration patterns were stable or not within platyrrhini (Marroig and Cheverud 2001), hominids (Ackermann 2002) and modern human populations (González-José et al. 2004). These three studies supported the functional/developmental integration hypothesis but results pointed to

different conclusions, even though they applied the same methodology to compare integration patterns among groups, namely Mantel correlation tests. Steppan et al. (2002) discussed the weaknesses of this and other methods for matrix comparison and concluded that more complex and powerful analytical methods were needed. Although Steppan et al. (2002) considered that CPCA (common principal components analysis) also have some restrictions due to orthogonality, it is the most common applied method (McGuigan 2006): it determines whether eigenvectors and associated eigenvalues differ among matrices.

The works by Marroig and Cheverud (2001) and González-José et al. (2004) showed that patterns of morphological integration in platyrrihini and in modern humans were quite stable and homogeneous, whereas those of hominids were not (Ackermann 2002). This author reported that gorillas, chimpanzees and humans had divergent patterns of variation and that this difference clearly corresponded to the phylogenetic relationships among them. Instead, González-José et al. (2004) showed that the correlation or the variance/covariance structure was not associated either with molecular or morphological distance matrices among modern human populations and concluded that the stability of integration patterns was independent of the history and structure of populations. González-José et al. (2004) argued that a feasible explanation to this pitfall is that integration patterns might be constrained to the intraspecific value and that, at the skull level, speciation events may have involved large rearrangements of integration patterns in order to allow the evolution of the different skull regions.

2 *Objectives*

Objectives

This PhD deals with the quantitative genetics of the human skull. The main goal is to perform a functional and developmental approach to estimate the genetic variation underlying the cranial phenotype. This is an integrative attempt to shed light on the evolutionary patterns of the human skull and to assess the ability of craniofacial structures to reveal genetic patterns. From this general scope, arise the following specific objectives:

1. Combine the methods of quantitative genetics and geometric morphometrics in order to explore the genetic and environmental components of variation underlying the human skull phenotype.
2. Quantify the genetic and the phenotypic patterns of variation and covariation of cranial shape, both at a global and a regional level, to account for the complex functional and developmental patterns of the human skull. This will be done using two different types of craniometric traits:
    a. Univariate traits: Traditional craniometric traits such as linear distances between two osteological landmarks that will be assigned to each of the main regions of the skull (namely the face, the neurocranium and the basicranium)
    b. Multivariate traits: Three-dimensional shape reconstructions of each of these regions, as well as a global configuration of landmarks that will represent the entire skull shape.
3. Analyze the patterns of morphological integration among cranial regions, both at the genetic and the phenotypic level.
4. Assess the evolvability of the human skull and identify potential evolutionary constraints to morphological change.

5. Simulate the evolution of the four derived characters of the skull of modern humans: an advanced position of the foramen magnum, a globular and expanded cranial vault, a retracted face and strong cranial base flexion.
6. Obtain direct estimates of natural selection in the size and shape of the human skull, combining fitness measures and morphological data, and compare them to secular trends.

These goals have been achieved through the analysis of the unique collection of skulls with associated genealogical information from Hallstatt (Austria). These analyses integrate the disciplines of quantitative genetics, geometric morphometrics and biological anthropology and the results obtained are discussed in four manuscripts that are prepared for submission.

The first manuscript (Chapter 3) explores the genetic and phenotypic patterns of variation and covariation of cranial shape using the univariate approach (human cranial dimensions); whereas the second one (Chapter 4) uses the multivariate approach (3D shape reconstructions). Both studies consider the main functional and developmental regions of the skull and shed light into the integrated genetic architecture of the human skull.

The third manuscript (Chapter 5) is a study of the quantitative genetics of geometric shape in humans. The evolutionary constraints of cranial shape have been explored by simulating different selection regimes modelled after the key cranial characters of modern humans.

Finally, the fourth manuscript (Chapter 6) explores the role of natural selection in the evolution of the human skull. It measures the action of direct and indirect components of selection in skull size and shape by assessing how life-history and reproductive fitness measures relate to craniofacial variation.

3 *Results I*

3.1 *Heritability of human cranial dimensions: comparing the evolvability of different skull regions*

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Heretabilitat de les dimensions del crani humà:

comparació de la capacitat evolutiva de les diferents regions cranials

Determinar la variació genètica de la forma del crani humà és una qüestió clau pels estudis d'antropologia. Tradicionalment, s'estimava calculant l'heretabilitat de caràcters craniomètrics clàssics, com ara distàncies linears i angulars del crani. Aquests estudis van demostrar que la variació genètica d'aquests trets és moderada o elevada i que, per tant, poden proporcionar una senyal filogenètica adequada. No obstant, en aquests estudis no es va considerar la complexitat funcional i del desenvolupament de la morfologia cranial humana. Tampoc es van tenir en compte els patrons d'integració morfològica, que per una altra banda són dominants en el crani humà. L'objectiu d'aquest estudi és reanalitzar els patrons de variació de 58 distàncies cranials, considerant les principals regions funcionals i del desenvolupament cranial i estimant els patrons de correlació genètica i fenotípica entre aquests trets craniomètrics.

Concretament, es van testar quatre hipòtesis: H1) no existeixen diferències significatives entre els nivells d'heretabilitat de les dimensions facials, neurocranials i basals; H2) els patrons de correlació fenotípica (P) no reflecteixen els patrons de correlació genètica (G); H3) els patrons de correlació observats encaixen amb els patrons de correlació esperats segons les hipòtesis clàssiques d'integració del crani humà; i H4) el patró d'integració està dominat per la covariació entre les amplades màximes de les principals regions del desenvolupament del crani (cara, neurocrani i basicrani).

La mostra analitzada prové de l'una col·lecció de cranis decorats de Hallstatt (Àustria), per als quals s'han identificat les seves relacions familiars. Això ha estat possible gràcies als arxius parroquials de naixements, matrimonis i defuncions (1602-1900), que han permès reconstruir les genealogies de les famílies del poble (incloent un total de 18.134 individus). S'han analitzat 355 cranis d'individus adults d'ambdós sexes, dels quals 317 queien dintre de les genealogies. Mitjançant un digitalitzador Microscribe es van registrar les coordenades tridimensionals de 65 punts craniomètrics, a partir de les quals es van estimar 58 mesures lineals. Cadascuna d'aquestes distàncies es va assignar a una regió funcional o del desenvolupament del crani humà. Utilitzant mètodes de màxima versemblança es va calcular l'heretabilitat d'aquestes distàncies i posteriorment es van estimar els patrons de correlació genètica i fenotípica.

Els resultats van mostrar que la quantitat de variació genètica present al crani humà és considerable i que no hi ha diferències significatives entre els nivells de variació genètica de les diferents regions del crani. Les dimensions de la cara, del neurocrani i del basicrani presenten una heretabilitat mitjana de 0.23 i els tests estadístics indiquen que no hi ha diferències significatives entre elles. Per tant, no podem rebutjar la hipòtesi nul·la H1.

La correlació entre les matrius G i P va ser elevada (r=0.74) i molt significativa. Per tant, rebutgem la hipòtesi nul·la H2. Aquest resultat mostra que la matriu de covariació fenotípica pot ser un bon indicador de la matriu de covariació genètica quan no es té disponible informació genealògica associada. Malgrat aquesta similitud entre les matrius, quan s'observen amb deteniment els patrons de correlació s'observa que els patrons genètics són més complexos i estan més jerarquitzats que els fenotípics.

Quan es van testar les hipòtesis d'integració morfològica, es va trobar que els patrons de correlació genètica entre les mesures facials i neurocranials no compleixen la predicció de les hipòtesis clàssiques: detectem que l'amplada màxima del neurocrani està positivament correlada amb l'amplada màxima de la cara, però no detectem cap correlació negativa entre l'amplada màxima del neurocrani i l'alçada facial, ni amb la longitud ni l'alçada neurocranial. Així, rebutgem la hipòtesi nul·la H3 i concloem que la classificació clàssica entre cranis braquicèfals i dolicocèfals no es sustenta en cap base genètica ni del desenvolupament i no reflectiria, per tant, l'arquitectura genètica del crani humà.

Finalment, es va trobar que els patrons d'integració genètica estan dominats per la covariació entre les mesures d'amplada de la base del crani, el

neurocrani i la cara. Per tant, no podem rebutjar la hipòtesi nul·la H4 i confirmem evidències prèvies trobades en ratolins.

Aquest estudi assenyala, en definitiva, que la capacitat evolutiva del crani humà està limitada per una forta integració, tant a nivell genètic com fenotípic, i que les diferents regions del crani no presenten en sí diferències en els seus nivells de determinació genètica.

Heritability of human cranial dimensions: comparing the evolvability of different skull regions

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ABSTRACT Quantitative craniometrical traits have been successfully incorporated into population genetic methods in order to provide insight into human population's structure. However, little is known about the degree of genetic and non-genetic influences on the phenotypic expression of functionally based traits. Many studies have assessed the heritability of craniofacial traits, but complex patterns of correlation among traits have always been disregarded. This may represent a serious pitfall since the human skull is strongly integrated. Here we reconsider the evolutionary potential of craniometric traits assessing their heritability values as well as their patterns of genetic and phenotypic correlation using a large pedigree-structured skull series from Hallstatt (Austria). The sample includes 355 complete adult skulls that have been analyzed by means of 3D geometric morphometric techniques. Heritability estimates for 58 cranial linear distances were computed using restricted maximum likelihood methods. These distances were assigned to the main functional and developmental regions of the skull. Results showed that the human skull has substantial amounts of genetic variation, and a *t*-test showed that there are no statistically significant differences among the heritabilities of facial, neurocranial and basal dimensions. However, skull evolvability is limited by complex patterns of genetic correlation. Phenotypic and genetic patterns of correlation are consistent and do not support traditional hypothesis of integration of the human shape, showing that the classification between brachy- and dolicephalic skulls is not grounded on the genetic level. Here we support previous findings in the mice cranium and provide empirical evidence that covariation between the maximum widths of the main developmental regions of the skull is also the dominant factor of integration in the human skull.

KEYWORDS Human skull, heritability, evolvability, quantitative genetics, geometric morphometrics,.

INTRODUCTION

The human skull is an important source of information for phylogenetic and population genetic studies (Strait 2001, González-José et al. 2003, Ackermann and Cheverud 2004a, 2004b). The complex morphology of the skull is usually decomposed in a series of craniometric measurements and it has been demonstrated that moderate amounts of genetic heritable variation are underlying these traits (Sjøvold 1984, Sparks and Jantz 2002, Carson 2006a). To some extent, this suggests that the skull morphology has substantial potential to evolve and that craniometric characters provide consistent phylogenetic signals. Nevertheless, most studies

have disregarded the integrated nature of the skull. Morphological integration in the human skull (Lieberman et al. 2000a, 2000b, McCarthy and Lieberman 2001, Bookstein et al. 2003, González-José et al. 2004, Bastir et al. 2004, Bastir and Rosas 2004, 2005, 2006), can constrain the evolvability of traits (Merilä and Björklund 2004) and bias the results of phylogenetic analysis (Strait et al. 2007, Lockwood 2007).

Although the most appropriate approach to address this issue is to account for genetic and phenotypic covariation patterns of multivariate skull shape (Klingenberg and Leamy 2001, Klingenberg 2004, 2005), an alternative approach is to assess both the patterns of genetic variation and correlation of univariate craniometric measurements. Here we explore the genetic architecture underlying the skull following this latter approach, which is relevant for evolutionary biology because craniometric traits are still in full-use. For instance, recent studies have extensively applied population genetics-based models departing from classical measurements (Roseman 2004, Neves and Hubbe 2005, Schillaci and Stojanowski 2005, Harvati and Weaver 2006). Our goal is to reconsider the evolutionary potential of craniometric traits accounting both for their heritabilities and for the patterns of genetic and phenotypic correlation among them. Furthermore, we will test hypotheses of cranial integration formulated after this kind of traits (Enlow and Hans 1996, Hallgrímsson et al. 2007).

Genetic variation in the human skull

The estimation of the genetic and non-genetic components underlying the phenotypic variation of the human skull has long been a main focus of anthropological research (Boas 1912, Kohn 1991, Varela and Cocilovo 1999, Konigsberg 2000). The first studies addressing this issue date back to the first decades of the 20th century (Dahlberg 1926), but the interest increased at the end of the century because evolutionary biologists reconsidered the use of skeletal remains to unravel human microevolutionary paths (Relethford and Lees 1982, Relethford 1994). This new paradigm was built upon the growing evidence that human patterns of craniofacial variation reflected the underlying genetic patterns of variation (Cheverud 1988, Buikstra et al. 1990). Craniometric traits were thus regarded as useful tools to study the structure and history of human populations (Relethford and Lees 1982) and population genetic models were adapted in order to be used after craniometric traits (Relethford and Blangero 1990, Relethford 2002, 2004). The heritability of complex metric traits, considered in the narrow sense, expresses the proportion of total phenotypic variance due to additive genetic variance (Falconer and MacKay 1996). Heritability provides a measure of the proportion of variance in a trait explained by genetic transmission and is therefore a key parameter in models of evolution of quantitative traits (Konigsberg 2000).

A wide range of studies have estimated the heritability of craniofacial traits (Vandenberg 1962, Hiernaux 1963, Nakata et al. 1974, Susanne 1975, 1977, Sjøvold 1984, Devor et al. 1986, Sharma 1987, Sharma and Susanne 1991, Konigsberg and Ousley 1995, Nikolova 1996, Sharma 1998, Sparks and Jantz 2002, Arya et al. 2002, Johannsdottir et al. 2005, Carson 2006a). The general conclusion is that human craniofacial traits have moderate to high degrees of genetic variation. However, the comparison of results from different studies is controversial since they

have been computed upon very different kinds of samples (living humans or skeletal remains) from different geographical regions, accounting for different familiar relationships (twins, nuclear or extended families) and using different statistical methods (regression, ANOVA, path analysis or REML, restricted maximum likelihood analysis). REML methods are considered as the most efficient method to estimate genetic parameters in natural populations (Konigsberg 2000). However, they have not been used until recently because they are computationally high demanding (Roff 1997).

Moreover, one of the main problems concerning the heritability estimation of cranial measurements in humans is that suitable, large and pedigree-structured skull series are almost non-existent. Such a collection of skulls with genealogical associated data exists in Hallstatt (Austria) and has been previously studied in order to measure the heritability of metric and non-metric cranial traits (Sjøvold 1984, Carson 2006a, 2006b). The work by Sjøvold (1984) was one of the first surveys to heritability on a human skull pedigreed series and the heritabilities of cranial traits were estimated using regression analysis. Sjøvold (1984) concluded that most of Howell's measurements were significantly hereditary and suggested that the structures showing the highest heritabilities were those connected to the size of the brain, the orbits, the nose and the masticatory apparatus. In a recent study, Carson (2006a) used a REML method to provide alternative estimates of the heritability of Howell's measurements. The main conclusion of this study was in agreement to Sjøvold's study and reported that craniometric traits show low to moderate narrow sense heritabilities. However, Carson (2006a) pointed out some differences and concluded that facial dimensions and cranial breadth measures are the less heritable characters of the skull. According to Carson (2006a), these differences stem from the different statistical techniques used for the heritability estimation.

The patterns of genetic variation of craniometric traits have thus been analyzed previously, but the patterns of genetic correlation among them are completely unexplored. This issue is of crucial importance because morphological integration is pervasive in the human skull (Lieberman et al. 2000a, 2000b, Bookstein et al. 2003, González-José et al. 2004, Bastir et al. 2004, Bastir and Rosas 2004, 2005, 2006) and integration between characters can limit the evolvability of traits and determine their evolutionary response (McGuigan 2006).

Morphological integration in the human skull

Integration is expressed through covariation between traits and it plays a key role in the evolution of complex morphological structures such as the human skull, since it can enhance or constrain the evolution of its morphology towards certain directions of shape change (Klingenberg 2004, 2005). Morphological integration assumes that functionally and/or developmentally related traits will be coinherited and will produce coordinate responses to evolution (Olson and Miller 1958, Cheverud 1982, 1984, 1995, 1996a).

The human skull comprises three regions with different developmental origins and functional requirements (Carlson 1999): the cranial base, the cranial vault and the face. The cranial base is formed from endochondral bone that arises from a cartilaginous precursor originated from mesoderm (Mooney et al. 2002). The base supports the inferior parts of the

brain as well as the pons, the medulla oblongata and the brain stem (Richtsmeier 2002). The cranial vault is formed from membranous bone of paraxial mesodermal and neural crest origin and it gives room and protects the cerebral hemispheres and the cerebellum (Sperber 2001). The facial skeleton ossifies intramembranously from neural crest precursors (Sperber 2002) and it surrounds the pharynx as well as the oral, respiratory and orbital cavities, supporting the functions of feeding, breathing and vision. The cranial base is the most ancient structure and it represents a vestigial structure of the vertebrate skull that has been highly preserved through phylogeny (Carlson 1999). Therefore, it is considered that the cranial base is under stronger genetic control than the cranial vault and the face (Schilling and Thorogood 2000, Sperber 2001). Moreover, it is assumed that the face is the skull region that is more sensitive to nongenetic factors because it plays a key role in foraging and adaptation to environment and because facial growth is more extended into the postnatal period (Siebert and Swindler 2002).

The level of integration between these skull regions is a matter of current investigation. Most studies of morphological integration in the skull of mammals (Hallgrímsson et al. 2004, 2006, Goswami 2006, 2007), non-human primates (Cheverud 1982, 1995, Marroig and Cheverud 2001, Hallgrímsson et al. 2004, Ackermann and Cheverud 2004b) and humans (Lieberman et al. 2000a, 2000b, McCarthy and Lieberman 2001, Bookstein et al. 2003, González-José et al. 2004, Bastir et al. 2004, Bastir and Rosas 2004, 2005, 2006) have considered integration at the phenotypic level. However, researchers have not identified yet which phenotypic units reflect morphogenetic units (Lieberman et al. 2004) and little is know about the genetic integration and constraint among the functional and developmental regions of the skull.

The first studies of cranial integration in primates were developed by Cheverud (1982, 1995) and evidenced that functionally and developmentally related traits were in fact integrated. These findings provided support to the Functional Matrix Hypothesis (Moss and Young 1960), which expects that covariation within functional units is stronger than covariation within individual bones or osseous subdivisions with different developmental/tissue origins. Afterwards, Hallgrímsson et al. (2004) reported that this functional/developmental pattern of craniofacial integration was consistent in rhesus macaques but not in mice. More recent studies of modularity in mammals (Goswami 2006, 2007) and primates (Ackerman and Cheverud 2004b) have identified six phenotypic cranial modules, corresponding to four functional regions of the face (namely the oro-nasal, the molar, the orbital and the zygomatic-pterygoid regions), one neurocranial region (the vault) and one basicranial region (the basicranium). The patterns of covariation within and among regions indicated that the face (the oro-nasal and the molar regions) and the cranial base were the highest integrated structures of the skull, whereas the cranial vault showed differing levels of integration across taxa. According to Ackermann and Cheverud (2004b), the zygomatic region is one of the main sources of facial integration in African apes and humans. Furthermore, they report that the loose integration of the cranial vault provided the skull with more capability to evolve in response to encephalization.

Other studies (Lieberman et al. 2000a, 2000b, Bastir and Rosas 2004) support the existence of two modules in the human skull, namely the face and the braincase. Lieberman et al. (2000a, b) consider that the basicranium and the neurocranium form a highly integrated morphological unit, the neuro-basicranial complex, which is partially independent from the

face. However, Bastir et al. (2006) highlighted that the cranial base can not be interpreted as an integrated unit, at least at the ontogenetic level, since midline and lateral basicranial structures show different growth patterns. Further differences in growth may also explain the lack of integration between the braincase and the face: whereas the basicranium and the neurocranium grow jointly following a rapid neural trajectory (Bastir et al. 2006), facial growth extends more into the postnatal period and is more influenced by environmental factors (especially mechanical loadings). According to this, the face would be more prone to plastic responses (Kohn 1991, Strand Vidarsdóttir et al. 2002, Bastir and Rosas 2004) and it has been suggested that from the phylogenetic point of view facial traits would not be as informative as neuro- and basicranial traits, which are more conservative and would reflect more reliably the underlying genetic patterns (Collard and Wood 2000, Collard and O'Higgins 2001).

In the primate skull, the cranial base appears to have a key integrative role (Lieberman et al. 2000a, 2000b, Bookstein et al. 2003, Zollikofer and Ponce de León 2004). Anatomically, it is a hinge-structure between the face and the cranial vault and developmental and growth studies support this view. Enlow and Hans (1996) suggested that the craniofacial architecture is based on a system of hierarchical modules organized into several craniofacial levels, in which the basicranium responds to modifications of the brain and translates them epigenetically into changes of facial proportions along a cerebro-mandibular gradient. Therefore, the base is the structural foundation that sets out the spatial development of the face and to some extent regulates the overall cranial development via integration with the brain and the cranial vault. Regarding human craniofacial variation, Enlow and Hans (1996) considered that there are two extreme headform types along a continuous spectrum: first, the dolicocephalic type, which is characterized by a long and narrow skull associated to a flat base and a supero-inferiorly longer face; and second, the brachycephalic form, in which a short and broad skull is associated to a more flexed cranial base and the face reveals a decreased anterior facial height and increased facial breadths. However, this traditional hypothesis of integration is not supported by developmental models of craniofacial biology (Lieberman et al. 2000a, Bastir and Rosas 2004).

Recent experimental research using mice as animal models (Hallgrímsson et al. 2007) suggests that integration in the mammal skull is highly structured following a hierarchical scheme that is dominated by strong covariation between the widths of the neurocranium and the basicranium and also with that of face, but to a lesser extent. This study has further emphasized the stronger integration of the neurocranium and the basicranium with respect to the face, which is more independent but still covaries with the braincase (Hallgrímsson et al. 2007). After analyzing the influence of epigenetic factors in craniofacial variation, the authors conclude that phenotypic variation arises from few key developmental processes (such as brain growth) that channel the underlying genetic variation towards certain phenotypic expressions that maintain an integrated functional skull.

At the present study we reanalyze the pedigreed skull collection from Hallstatt (Austria) in order to explore the genetic patterns of variation determining the phenotypic expression of the skull and to assess the levels of correlation among craniometric characters. This will allow us to account for both the heritable and the integration patterns of the human skull. Here we test several hypotheses regarding these issues.

Hypotheses

Hypothesis 1 (H1) examines the heritability patterns of facial, neurocranial and basicranial dimensions and tests if there are differences in the amounts of genetic variation of these regions. The null hypothesis states that there are no significant differences among the heritability of each region, whereas rejection of the null hypothesis indicates differential genetic contribution to the phenotype of each region, suggesting that they are subject to different evolvabilities and levels of plasticity.

Hypothesis 2 (H2) explores genetic and phenotypic patterns of correlation of specific suites of craniofacial traits within and among major and minor developmental/ functional regions of the skull. The null hypothesis implies no correlation between the genetic (G) and phenotypic (P) matrices; that is, the patterns of phenotypic correlation do not reflect the genetic ones and show different strengths of morphological integration. The null hypothesis is rejected if the correlation of G and P is high and significant, which would suggest that genetic and environmental effects on development produce similar patterns of phenotypic variation. Thus, in those cases where G is not available, P could be used as a good proxy to G in population quantitative genetic models (Cheverud 1988).

Hypothesis 3 (H3) tests the traditional hypothesis of integration of the human skull (Enlow and Hans 1996). Under this hypothesis, maximum cranial breadth should be positively correlated with facial breadth and negatively correlated with facial height, neurocranial length and neurocranial height. The null hypothesis is rejected if the observed patterns of correlation between these pairs of distances do not fit the expected patterns of integration.

Hypothesis 4 (H4) tests if the overall pattern of genetic integration in the human skull is dominated by the covariation between the maximum widths of the major developmental regions, namely the face, the neurocranium and the basicranium. This hypothesis was put forward by Hallgrímsson et al. (2007), who investigated the influence of epigenetic factors in the patterns of morphological integration of mice skull. The null hypothesis expects that the genetic correlations between facial, neurocranial and basicranial width are high and significant.

MATERIALS AND METHODS

The sample examined here derives from the Hallstatt skull collection, which is a large sample of human skulls with identified familiar relationships. It provides the unusual opportunity to perform quantitative genetic analysis in a human skeletal sample. This unique collection is made up of more than 700 decorated skulls that have been accumulating in the charnel house of Hallstatt from the beginnings of the 18th century. It stems from a local tradition to honour predecessors (Burgstaller 1961). Upon request of the families, the gravedigger recovered the skeletal remains of their relatives, decorated their skull with floral paintings and wrote the name of the individual on them (Fig. 1). This custom was widespread in Austrian and German regions surrounding the Alps (Sauser 1952), but Hallstatt is the only place where it has provided such a large skull series and has endured for so long, since the last

skull was incorporated in 1986. The series covers a temporal span of more than 250 years, but most of the identified skulls date back from the 19th century.

Skull identification and genealogy reconstruction

The name and type of decoration of the skulls allowed us to identify at the parish demographical records almost 60% of the individuals. To reconstruct the genealogies of the Hallstatt population, we compiled the complete parish records of births, deaths and marriages from 1602 to 1900, which included 18,134 individuals. The most complete families range back up to seven generations, including all kind of familiar relationships from first to fourth degree of relationship (Fig. 1). Most of the identified skulls are preserved at the charnel house in Hallstatt (n=374), but a few of them are on loan at several Austrian Museums: the Musealverein in Hallstatt (n=3), the Naturhistorisches Museum Wien (n=17), the Österreichisches Museum für Volkskunde in Vienna (n=1), and the Anatomisches Institut in Innsbruck (n=11). From the first surveys carried out by Sjøvold during the eighties (Sjøvold 1984), 25 identified skulls have disappeared from the charnel house and the names of several individuals have been changed because of recent renewed decoration (Fig. 1).

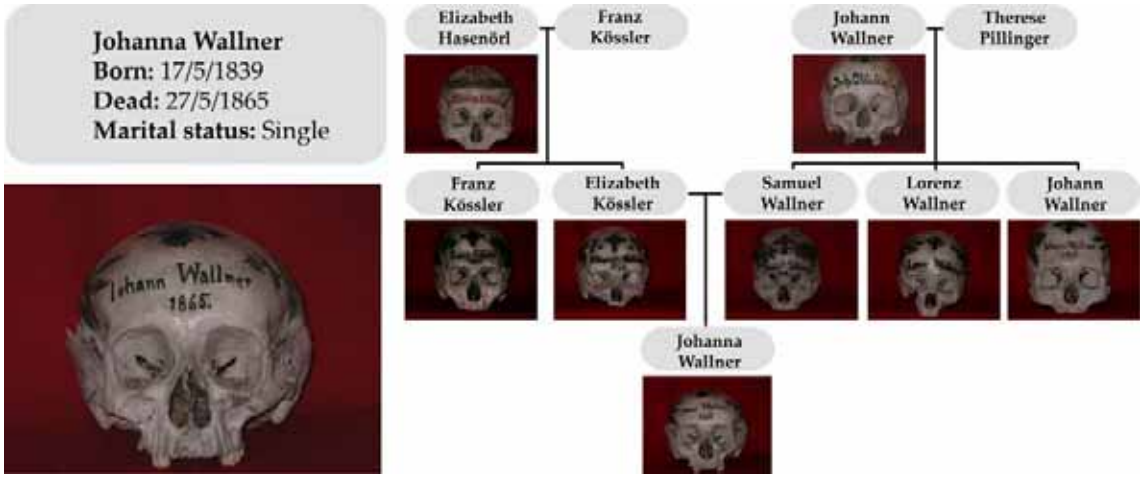

Fig. 1. Example of genealogy of one decorated skull from the Hallstatt's collection. Genealogical trees can be reconstructed from the parish demographical records. Here the genealogy of Johanna Wallner is shown: parents, uncles and grandparents have been identified. In this skull the original name was changed after repainting of the fading decoration: on the skull it is written Johann Wallner, but Sjøvold's photographic records dating back from the eighties revealed that the original name was Johanna. In fact, discriminant analysis on sex confirmed that the skull belonged to a female and no matches were found at the demographical records when the "new" name was considered.

Morphometric analyses

In this study, we analyzed a sample of 355 adult complete skulls from both sexes (40.6% females and 59.4% males), from which 317 fall into the extended, multigenerational genealogies. To avoid sample bias, subadult (n=35) and fragmentary individuals (n=16) were excluded from the total sample (n=406). Adulthood was assessed by skeletal criteria, as determined by a fully

closed spheno-occipital synchondrosis. A set of 65 anatomical landmarks (Table 1) was recorded on each skull with a 3D digitizer (Microscribe, Inc). Five landmarks from the alveolar region (prosthion, inner prosthion, ectomolare right and left, and palate) were removed because they were missing in more than 50% of the cases due to tooth loss and high levels of alveolar bone resorption.

Table 1. List of digitized landmarks. Codes and definitions used are provided (r right; l left).

Code	*Landmark*	*Definition*
aam l	Anterior auditory meatus	Most anterior point at the external auditory meatus
al r/l	Alare	The most lateral point on the margin of the nasal aperture
alv l	Alveolar point	Posterior limit of the maxillary alveolar arch at the pterygo-alveolar suture
ast r/l	Asterion	The point where the lamboidal, parietomastoid, and occipitomastoid sutures
b	Bregma	The ectocranial point where the coronal and sagittal sutures intersect
ba	Basion	The midline point on the anterior margin of the foramen magnum
ek r/l	Ectoconchion	The most lateral and posterior point on the orbital margin
eu r/l	Euryon	The point of greatest breadth of the brain case perpendicular to the sagittal plane
fmo r/l	Frontomalare orbitale	The point where the frontozygomatic suture intersects the orbital margin
fmt l	Frontomalare temporale	Is the point where the frontozygomatic suture crosses the temporal line
ft r/l	Frontotemporale	The point where the temporal line gets its most anteromedial position
g	Glabella	The most anterior midline point on the frontal bone, above the frontonasal
gle l	Glenoid fossa	The most posterior point on the margin of the glenoid fossa
ho	Hormion	The most posterior midline point on the vomer
i	Inion	An ectocranial midline point at the base of the external occipital protuberance
iam l	Inferior auditory meatus	Most inferior point at the external auditory meatus
izt l	Inferior zygo-temporal	Inferior point at the suture between temporal and zygomatic bones
ju r/l	Jugale	Depth point of the notch between the temporal and frontal processes of the
l	Lambda	Midline point of the intersection of the sagittal and lamboidal sutures
m	Metopion	Midline point where the elevation above the chord from n to b is greatest
mf r/l	Maxillofrontale	The point where the anterior lacrimal crest meets the frontomaxillary suture
ms l	Mastoidale	The most inferior point on the mastoid process
mw r/l	MW	Tip of the process at the infratemporal crest
n	Nasion	The midline point where the two nasal bones and the frontal intersect
nar r/l	Nariale	The most inferior point on the nasal aperture
o	Opisthion	The midline point at the posterior margin of the foramen magnum
oc l	Optic canal	Most superior, medial, and anterior points of the optic canal
op	Opisthocranion	The posterior-most point of the skull in the medial sagittal plane.
or r/l	Orbitale	The lowest point on the orbital margin
pam l	Posterior auditory meatus	Most posterior point at the external auditory meatus
pns	Posterior nasal spine	Vomer-palatin junction
po l	Porion	The uppermost point on the margin of the external auditory meatus
pt r/l	Pterion	The point where the frontal, parietal, temporal and sphenoides bones meet
ra r/l	Radicular	Lateral point on zygomatic process of the temporal bone at the postglenoid
ss	Subspinale	The deepest point seen in the profile below the anterior nasal spine
stf l	Stylomastoid foramen	Stylomastoid foramen
szt l	Superior zygo-temporal	Superior point at the suture between temporal and zygomatic bones
v	Vertex	Midsagittal superior point of the cranium when the skull is in Frankfort
zy r/l	Zygion	The point of maximum lateral extent on the surface of the zygomatic arch
zym r/l	Zygomaxillare	The most inferior point on the zygomaticomaxillarysuture
zym:a r/l	Zygomaxillare anterior	The most anterior point on the zygomaticomaxillary suture
zyo r/l	Zygoorbitale	The point where the orbital rim intersects the zygomaticomaxillary suture

Measurement error was evaluated by a repeated recording of a subsample of 91 individuals that were each digitized twice. Analysis of shape variation showed that repeatability ranged over 90%. Outlier points were detected by means of Box and Whisker plots assuming an outlier coefficient of 1.5. These points were deleted and considered as missing data. The overall percentage of missing values was of 2.18% and these were replaced either by multivariate regression or by coordinate reflection when the missing landmark had a symmetric counterpart. Finally, to validate the identification made by the gravedigger who decorated the skulls, we confirmed their sex assignment performing discriminant function analyses. Results show that 8 skulls have an overall posterior probability higher than 0.85 of being the opposite sex. These individuals were considered as misidentifications and did not account for the estimation of the genetic parameters.

We estimated 58 linear inter-landmark distances from the three-dimensional landmark coordinates. Of these, 24 correspond to Howell's measurements (Howells 1973) or are close approximations to them (i.e., the prosthion is substituted by the subspinale). The distances were assigned to the three major regions of skull, which have different developmental origins: the face, the neurocranium and the basicranium (Cheverud 1995, Hallgrímsson et al. 2004, 2007). Distances within the face were also assigned into minor functional regions, such as the nasal, the orbital and the zygomatic regions (González-José et al. 2005, Sardi and Ramírez-Rozzi 2007). Those distances covering several regions were grouped into another category, the inter-regional dimensions.

Quantitative genetic analyses

Restricted maximum likelihood methods (REML) were used to estimate the heritability of each distance. REML methods are usually applied under the 'animal model', which is a mixed linear model that jointly accounts for fixed and random effects in order to describe the phenotype of each individual (Lynch and Walsh 1998). The phenotypic variance is broken down into its components of additive genetic value and other random environmental and fixed effects. The components of variance are estimated by an iterative procedure that maximizes the likelihood of observing the actual data (Lynch and Walsh 1998). REML analytical methods are advantageous in contrast to parent-offspring regression or sib analyses because they incorporate multigenerational information from unbalanced datasets. Furthermore, they are not bound by assumptions of non-assortative mating, inbreeding or selection (Kruuk 2004).

We computed the variance components of the traits using the SOLAR 4.0.4 software package (Almasy and Blangero 1998). It provides estimates of the additive genetic variance and the variance of the residual errors, from which the narrow-sense heritability can be estimated (Lynch and Walsh 1998). SOLAR tests the significance of each covariate by separate and computes the amount of variation explained by the significant ones. To guarantee that the continuous metric traits followed a normal distribution, a direct normalization of the trait was performed using an inverse gaussian function before analysis. The model included sex, year of birth and the interaction of sex and year of birth as covariates. Moreover, as 12.4% of the individuals showed slight dysmorphologies possibly related to craniosynostosis, deformation

was also considered as a covariate. This kind of dysmorphology (occipital flattening and prominent forehead) was also reported by in a very similar skull sample from Berg (Austria) (Howells 1989). This author pointed to cradling practices as possible causes of these deformations, but didn't rule out other non-artificial or genetic effects.

To test if there are differences in the amounts of genetic variation at each region (H1), we performed a two tailed *t*-test that compared the average heritability estimations of the three configurations. To analyze the genetic and phenotypic covariation patterns of the skull (H2-H4) we computed the correlation between all the possible pairs of distances of maximum breadth, height and length within and among major and minor developmental/functional regions of the skull. We used SOLAR's bivariate models to estimate the genetic correlation between pairs of distances and the parametric Pearson's correlation to estimate the phenotypic correlation. To test the similarity between the genetic and the phenotypic correlation matrices (H2) we used a matrix correlation (Cheverud 1988) and assessed its significance with a Mantel test (Mantel 1967) after 100,000 permutations of the original matrices. According to Cheverud (1988), the level of heritability influences the similarity between genetic and phenotypic correlation patterns: if heritability is high it increases both the accuracy of the genetic correlation estimates and the similarity of G and P; if it is low or moderate, the accuracy is reduced and similarity of G and P suggests that genetic and epigenetic factors are channelled through the same developmental process. In this latter case, the levels of genetic correlation usually exceed that of phenotypic correlations. To assess the reliability of the genetic correlation estimates, we measured the effective sample size (N_{es}) used in our analyses, as suggested by Cheverud (1988). This is a rough measure of the actual sample size and is derived as the product of the number of families in which is based the estimation of the genetic parameters and the mean heritability of the traits. Previous evidence suggests that an effective sample size of at least 40 should be used to guarantee the reliability of the data (Cheverud 1988).

Finally, to test the integration hypotheses (H3 and H4) we compared the expected patterns of genetic correlations between the involved measurements with the observed ones. The null hypothesis is rejected if the observed patterns of correlation between these pairs of distances do not fit the expected patterns of integration.

RESULTS

The univariate maximum likelihood estimates of heritability of facial, neurocranial and basicranial dimensions are presented in Tables 2, 3, 4 and 5. As a summary of these results, we present in Fig. 2 the five most heritable traits of each region. The obtained heritabilities are comparable between them because the estimation of the phenotypic and genetic variance components was always based on the same number of individuals. Results show that craniofacial traits are low to moderate heritable characteristics. Heritability values ranged from 0.00 to 0.43 and 72.2% of them were significant at the 0.05 level. Regarding the regional patterning of heritabilities, the face is the skull region with a higher number of significantly heritable traits (81%) and the highest mean heritability (0.26), followed by the basicranium (73% and 0.23) and the neurocranium (61.5% and 0.19). The percentage of significant heritability

estimates within the inter-regional dimensions was of 50%. Despite these slight differences, there is no clear-cut among regions and the *t*-test showed that the comparisons of the genetic amounts of variation among regions were not statistically different. The statistical significances of the differences between the average heritability of the three regions were the following: facial *versus* neurocranial (p=0.053); facial *versus* basicranial (p=0.433); and neurocranial *versus* basicranial (p=0.336). Thus, we can not reject H1 null hypothesis.

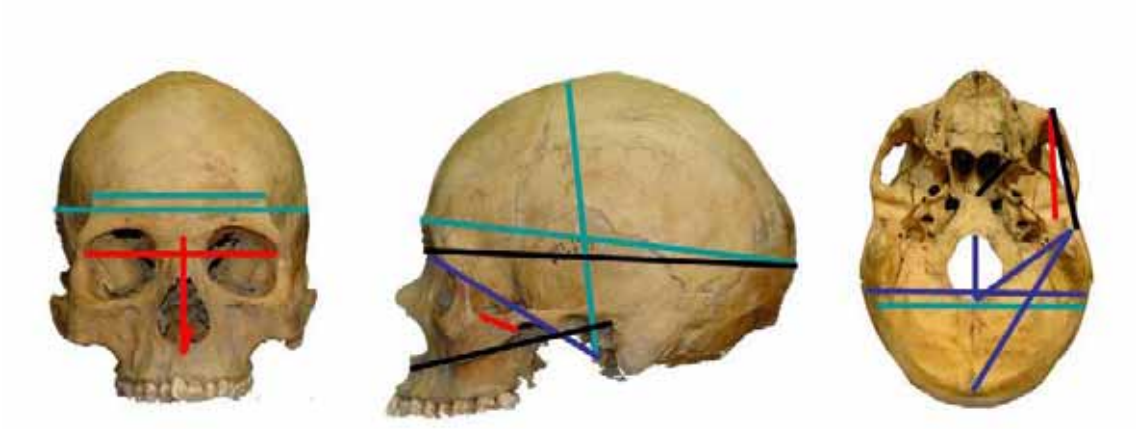

Fig. 2. Frontal, lateral and inferior views of a skull showing the cranial dimensions with higher heritabilities. Colours indicate dimensions from the facial (red), neurocranial (green) and basicranial (blue) regions, whereas inter-regional dimensions are depicted in black.

Regional heritability estimations

Total facial dimensions (maximum facial breadth, length and height) have moderate heritabilities, showing that additive genetic variation accounts for approximately 30% of the phenotypic variation of these traits (Table 2). Minor functional regions within the face show diverse patterns of genetic variation: the orbital and the nasal regions show some of the highest amounts of genetic variance and thus the highest heritabilities of the skull (Fig. 2), whereas the masticatory apparatus tends to show lower estimates (Table 2). The mean heritabilities of the nasal, orbital and zygomatic regions are 0.27, 0.33 and 0.22 respectively. Total breadth, length and height orbital measurements show moderate to high significant heritability estimates. Other breadth measures such as the bi-orbital breadth and the interorbital breadth also show moderate heritabilities. Nasal height and length show high heritability estimates, but nasal breadth shows no additive genetic variance at all (Table 2). The *t*-test comparison for functional facial regions showed that the orbital region is significantly more heritable than the zygomatic (p=0.044). Further comparisons did not any provide significant differences.

Total neurocranial dimensions (Table 3) also have moderate significant heritabilities. The anterior breadth measure and the maximum cranial breadth measure have indeed high estimates (Fig. 2). The rest of neurocranial measurements tend to show low heritability estimates, whereas Howell's chord distances show no genetic variation. All neurocranial breadth measures are significantly heritable, except the distance between pterions. The heritability estimates of the basicranial region (Table 4) were moderate and significant, except

for the distances between the inion and the opisthion, the mastoid height and the otic height. The length of the foramen magnum as well as the auricular breadth showed some of the highest heritability estimates, whereas total cranial base length and height show more moderate estimates (Fig. 2). Finally, inter-regional dimensions show two different patterns (Table 5). Those distances that mostly cover the face show moderate and significant heritabilities, whereas those mostly covering the cranial vault tend to show low and non-significant estimates, although one exception is the distance from the nasion to the opisthocranion (Fig. 2).

Table 2. Facial dimensions: Narrow-sense heritability estimations (h^2) and associated standard errors (SE). Statistical significant estimations (α=0.5) are bolded. The proportion of variation explained by the significant covariates (α=0.1) is also provided. Each measure corresponds to the distance between two landmarks (see Table 1 for definitions) and has been assigned to a minor function region within the face (although some distances may cover several regions).

Distance							Covariates	
	Measure	Landmarks	Howells	h^2	SE	*p*	Variance explained	Significant covariates
Total	height	ss>n	NPH*	**0.34**	0.13	0.002	0.15	sex
	lenght	ss>ba	BPL*	**0.32**	0.12	0.001	0.14	sex
	breadth	zy r>zy l	ZYB	**0.28**	0.13	0.008	0.43	sex, year birth
Orbital	breadth	fmor r>fmo l	FMB	**0.40**	0.13	0.001	0.19	sex
	lenght	or l>oc l		**0.35**	0.14	0.004	0.06	sex
	breadth	ek r>ek l	EKB	**0.34**	0.14	0.005	0.17	sex, year birth
	breadth	mf r>mf l	DKB*	**0.33**	0.13	0.003	0.05	sex
	height	or l>fmo l		**0.29**	0.14	0.015	-	-
	breadth	mf l>ek l	OBB*	**0.28**	0.14	0.013	0.07	sex
Nasal	height	n>nar r/l	NLH	**0.43**	0.13	0.000	0.13	sex
	lenght	ss>pns		**0.38**	0.14	0.001	0.19	sex, year birth
	breadth	al r>al l	NLB	0.00	0.00	0.500	0.05	sex, year birth
Masticatory	height	szt l>izt l		**0.38**	0.13	0.001	0.11	sex, deformation, year birth
	lenght	zym l>gle l		**0.37**	0.12	0.000	0.15	sex, year birth
	height	zym:a l>fmo l		**0.34**	0.13	0.004	0.10	sex
	lenght	izt l>mw l		**0.28**	0.11	0.002	0.15	sex
	height	zym l> or l	WMH	**0.24**	0.12	0.014	0.13	sex
	height	zyo l>fmo l		**0.23**	0.14	0.029	0.32	sex
	lenght	or l>izt l		**0.22**	0.12	0.024	0.17	sex, year birth
	lenght	fmo l>fmt l		**0.22**	0.12	0.020	0.04	sex
	lenght	zym:a l>izt l	IML	**0.22**	0.13	0.037	0.13	sex, deformation, year birth
	lenght	zyo l>izt l	XML	**0.20**	0.11	0.018	0.23	sex, deformation, year birth
	breadth	ju r>ju l	JUB	0.19	0.13	0.071	0.38	sex, year birth
	height	zyo l>zym:a l		0.09	0.10	0.143	0.13	sex
	breadth	zym:ar>zym:a l	ZMB	0.07	0.10	0.232	0.23	sex, year birth
	height	or l>zym:a l		0.03	0.10	0.364	0.13	sex

* These are not the exact measures of Howell's (1973) but a close approximation.

Regarding the covariates included in the analyses (Tables 2, 3, 4 and 5), sexual dimorphism was the most important effect since it affected more than 90% of the measurements, especially the facial ones. The second more important effect was the temporal

span of the sample, which could be reflecting morphological secular trends: year of birth significantly affected facial (41.7%), neurocranial (25%) and basicranial dimensions (40%). Finally, deformation had a smaller effect but significantly affected three facial dimensions, two neurocranial, one basicranial and three inter-regional dimensions. The joint effect of sex and year of birth just influenced one measurement from the cranial base.

Table 3. Neurocranial dimensions: Narrow-sense heritability estimations (h^2) and associated standard errors (SE). For more coding details see Table 2.

Distance							Covariates	
	Measure	Landmarks	Howells	h^2	SE	*p*	Variance explained	Significant covariates
Total	breadth	eu r>eu l	XCB	**0.36**	0.14	0.002	0.17	sex, year birth
	length	g>op	GOL	**0.31**	0.12	0.002	0.18	sex
	height	b>ba	BBH	**0.24**	0.12	0.016	0.18	sex, deformation
Other	breadth	ast r>ast l	ASB	**0.23**	0.14	0.034	0.05	sex
	breadth	ft r>ft l		**0.23**	0.12	0.024	0.07	sex
	length	m>b		**0.22**	0.12	0.020		
	breadth	pt r>pt l		0.21	0.15	0.072	0.13	sex, year birth
	height	g>m		**0.20**	0.12	0.031	0.16	sex, deformation
	length	v>l		**0.19**	0.12	0.043	0.03	sex
	breadth	mw r>mw l	WCB*	0.16	0.11	0.050	0.05	sex
	length	b>l	PAC	0.06	0.10	0.262	0.07	sex
	height	l>op	OCC	0.04	0.12	0.379	0.02	sex, year birth
	length	b>v		0.00	0.00	0.500		

Genetic and phenotypic integration

Our results show that the observed genetic and phenotypic correlation patterns of skull integration are consistent in our sample. The matrix correlation between G and P was high (r=0.74) and the Mantel test revealed that it was highly significant (p<0.000). Thus, we reject the H2 null hypothesis, which expected independence between these matrices. This suggests that P can be used as a good proxy of G. However, a closer look to the correlation matrices (see Appendix) reveals that genetic integration is more constrained to specific dimensions, whereas phenotypic integration is more widespread throughout the skull. Almost all phenotypic correlations were highly statistically significant, even when the correlation was low. Genetic correlations were usually higher than the phenotypic ones but few of them were statistically significant due to large standard errors. This was an expected output since heritabilities were all low to moderate. To confirm that the genetic correlations were well estimated, we computed the effective sample size and we found that it exceeds the minimal threshold value suggested by Cheverud (1988). In fact, the number of families with skull data was of 209, the mean heritability was 0.23 and thus the effective sample size was 47.4. This result confirms that the genetic correlations are reliable and that G and P are similar because both genetic and environmental variation is channelled through the same developmental pathways.

Table 4. Basicranial dimensions: Narrow-sense heritability estimations (h^2) and associated standard errors (SE). For more coding details see Table 2.

Distance							Covariates	
	Measure	Landmarks	Howells	h^2	SE	*p*	Variance explained	Significant covariates
Total	breadth	ba>po l		**0.29**	0.12	0.005	0.20	sex
	length	n>ba	BNL	**0.24**	0.10	0.003	0.17	sex
Other	breadth	ra r>ra l	AUB*	**0.40**	0.12	0.000	0.19	sex
	length	o>ba	FOL	**0.38**	0.13	0.001	0.13	sex
	breadth	i>po l		**0.27**	0.14	0.011	0.17	sex, year birth, sex*yearbirth
	breadth	adml>pam l		**0.23**	0.14	0.030	-	-
	breadth	po l>ho		**0.21**	0.14	0.049	0.22	sex, year birth
	breadth	ba>ho		**0.20**	0.12	0.034	0.04	sex
	length	i>o		0.16	0.12	0.080	0.02	year birth
	height	ms l>stf l		0.15	0.12	0.081	0.15	sex
	height	po l>iam l		0.00	0.12	0.486	0.075	sex, deformation, year birth

The patterns of genetic and phenotypic correlations between facial and neurocranial dimensions do not follow Enlow's expected pattern of craniofacial variation and headform in humans (Enlow and Hans 1996). As predicted by the hypothesis, maximum cranial breadth is positively correlated with facial breadth (r=0.89, p=0.007), but it does not correlate negatively neither with facial height (r=0.47, p=0.11), neurocranial length (r=0.49, p=0.06) nor neurocranial height (r=0.16, p=0.72). Thus, we reject H3 null hypothesis because neither the genetic nor the phenotypic observed patterns of correlation fit the pattern expected by the traditional hypothesis of integration (Enlow and Hans 1996).

Table 5. Inter-regional dimensions: Narrow-sense heritability estimations (h^2) and associated standard errors (SE). For more coding details see Table 2.

Distance							Covariates	
	Measure	Landmarks	Howells	h^2	SE	*p*	Variance explained	Significant covariates
Other	length	zym l>ra l		**0.34**	0.12	0.001	0.19	sex
	length	n>op	NOL	**0.34**	0.13	0.001	0.15	sex
	length	po l>ss		**0.32**	0.12	0.003	0.24	sex, year birth
	breadth	ho>alv l		**0.29**	0.16	0.034	0.30	sex
	height	op>i		0.13	0.12	0.116	0.04	sex, deformation
	length	n>b	FRC	0.11	0.12	0.161	0.14	sex, deformation
	length	po l>n		0.07	0.11	0.267	0.21	sex, year birth
	height	po l>b		0.03	0.12	0.383	0.22	sex, deformation

Finally, our results confirm the hypothesis of strong covariation between the breadth measures of major developmental regions of the skull (Hallgrímsson et al. 2007). The genetic correlations between facial, neurocranial and basicranial breadth measures were high and statistically significant and dominate the patterns of integration of the human skull ($r_{f\text{-}b}$=0.90, *p*=0.014; $r_{n\text{-}b}$=0.93, *p*=0.007; $r_{f\text{-}n}$=0.89, *p*=0.007). Thus, we do not reject H4 null hypothesis and support the hypothesis that this correlation pattern prevails in skull's integration.

DISCUSSION

This study explored the levels of genetic variation and covariation of craniometric traits through a developmental/functional approach in order to assess the evolutionary potential of the human skull. The above results confirm that the human skull has substantial amounts of genetic variation, which confers the skull a high ability to evolve (Tables 2, 3, 4 and 5). However, evolvability is compromised by complex patterns of genetic integration that may constrain the potential evolution of the skull towards certain directions of change (Appendix). That is, free and random evolution of the skull is unlike because of morphological integration, and this suggests that the developmental system plays an important role channelling the paths through which genetic and phenotypic variation can be expressed (Cheverud 1988, Lieberman et al. 2004, as well as Chapters 4 and 5 of this thesis).

It has been suggested that the different cranial regions could be subject to different levels of evolvability and/or plasticity (Kohn 1991, Strand Vidarsdóttir et al. 2002, Bastir and Rosas 2004). We tested this assumption in hypothesis H1 and we didn't find significant differences between the amounts of genetic variation underlying the three major developmental regions of the skull. Craniometric traits from the face, the cranial vault and the base show similar percentages of significant heritability estimations and low to moderate levels of genetic components of variation. This result confirms previous evidence indicating that within the primate skull basicranial, neurocranial and facial dimensions show similar levels of heritability (Cheverud and Buikstra 1982, Sjøvold 1984, Cheverud 1996b). Moreover, there is no evidence suggesting that the face is the most plastic region of the skull. For instance, our results showed that some facial dimensions associated with functional regions (such as the nasal, the orbital and the masticatory regions) have some of the highest heritabilities of the skull (Fig. 2). Characters with no heritability, and which all their variation is due to environmental effects, are not limited to the face but are widespread through the whole skull and can also be found at the neurocranium and the basicranium (Tables 2, 3, 4 and 5).

Our results support the hypothesis that the cranial base is more conservative and may be under slightly stronger genetic control, since most distances within the basicranium show moderate and significant heritabilities and phenotypic and genetic correlations between the width and length of the cranial base are strong (Appendix). Also, we corroborate the hypothesis that the cranial base acts as the "skull's central integrator" (Lieberman et al. 2000a, 2000b, 2002). In fact, the cranial base strongly influences the overall cranial shape, constraining facial breadth, height and length, as well as neurocranial breadth and length. This mechanism would prevent the different regions to evolve independently and would preserve the functional and architectural requirements of the skull.

Hypothesis 2 (H2) tested the similarity between the genetic and the phenotypic correlation matrices and the Mantel test revealed that they are significantly similar. This is important because many studies are using phenotypic data in population genetic models without any knowledge of the genetic architecture of the skull (Steadman 2001, González-José et al. 2003, Roseman 2004, Ackermann and Cheverud 2004a, González-José et al. 2005, Schillaci and Stojanowski 2005, Stojanowski 2005, Martínez-Abadías et al. 2006, Stojanowski and Schillaci

2006, González-José et al. 2007). This was done assuming that the G and P matrices are similar and proportional, a conclusion drawn from Cheverud's (1988) work. This study compared genetic and phenotypic correlation matrices obtained from 23 published studies, which included a wide range of animals (from human to amphipods) and of kinds of traits (from morphological to cognitive). Here we provide empirical data exclusively for human craniometric traits and support the view that G and P display consistent patterns of morphological variation (Cheverud 1988 and Chapter 4). However, the proportionality of G and P is not a straightforward consequence of the similarity between these matrices. In a multivariate approach to skull shape (Chapter 5), quantitative genetic analyses showed that it can not be assumed that G and P are proportional without previous empirical testing.

The pattern of genetic correlations between facial and neurocranial dimensions do not follow Enlow's expected pattern of craniofacial variation and headform in humans (Enlow and Hans 1996). Under this hypothesis, maximum cranial breadth should be positively correlated with facial breadth and negatively correlated with facial height, neurocranial length and neurocranial height. However, we only found a significant correlation between neurocranial and facial breadth, as it had been previously hypothesized (Weidenreich 1941) and supported by studies of artificial cranial deformation (Antón 1989). Therefore, we conclude that the traditional classification between dolico- and brachycephalic skulls does not reflect the genetic architecture of the human skull nor provides any valuable hypothesis of morphological-genetic integration. This is of crucial importance since many bio-anthropological issues are still being synthesized in terms of dolico- versus brachycephalic forms. For instance, the classical study of Boas on European immigrants to US (Boas 1912, Gravlee et al. 2003), studies of morphological variation among ancient and modern Native Americans (Gonzalez et al. 2003, Fiedel 2004) or studies analyzing the relationship among head shape and climate (Beals 1972, Goodman 1995, 1997) still use this terminology to describe human craniofacial variation.

The clearest integrated module is formed by breadth dimensions covering the neurocranium, the basicranium and the face: the overall pattern of integration in the human skull is dominated by the covariation between the maximum widths of the major developmental regions. This pattern was first reported in the mice cranium (Hallgrímsson et al. 2007) and here we extend it to humans. Evolutionary developmental studies use model organisms as mice to identify candidate genes that are involved in the phenotypic expression of skull morphology (Lieberman et al. 2004; Hallgrímsson et al. 2004, 2006, 2007). To extrapolate the results obtained from such organisms to humans it is important to compare them with other primate species. Hallgrímsson et al (2004) compared phenotypic and genetic correlations in macaques and two strains of mice and did not find a consistent pattern of modularity in these groups. Therefore, it is relevant to find the same predicted pattern of integration in humans and mice. This suggests that covariation between cranial widths is an integrated feature that has been conserved across the evolution of the mammalian craniofacial form.

The present study presents similarities but also some differences to previous analyses carried out with the Hallstatt skull collection (Sjøvold 1984, Carson, 2006a). Although they are all grounded on the same population, results are not totally coincident. However, this is not an unexpected output since each study departed from different familiar data, accounted for

different sources of covariation and did not use exactly the same crania. As sample size is limited, standard errors are substantially large (Falconer and MacKay 1996) and slight differences in sample composition, model definition and data treatment can alter the results. Therefore, general trends are more reliable quantitative parameters than the exact value of the heritability estimations. In common, all studies have shown that craniometric traits are low to moderate hereditary characteristics. However, we do not confirm previous evidence suggesting that breadth and facial dimensions are the less heritable characters of the human skull (Carson 2006a). This study reports low to moderate heritability estimates for breadth measures (Tables 2, 3, 4 and 5) and has tested statistically that there are no significant differences in the amount of genetic variation underlying the main developmental regions of the skull. Although we used the same statistical method to estimate heritability (REML), inconsistencies between studies might also arise due to other methodological issues regarding the number of skulls included in the analyses and the complexity of the pedigree structure. In this study, we extended and revised the pedigrees constructed by Sjøvold (1984), checked the identifications made by the gravedigger by sex confirmation, and thanks to Sjøvold's photographic records from the eighties we could identify the original names of the individuals (Fig. 1). In comparison to previous studies, our analysis included a larger skull sample, did not contain missing values and used larger and more complex genealogies since the whole population was reconstructed.

Understanding the patterns of morphological integration among skull regions will improve our ability to make evolutionary and phylogenetic inferences about human evolution. The use of craniodental characters in phylogenetic analyses of primate and hominid evolution is widespread (Strait et al. 1997, Strait and Grine 1999, Strait et al. 2007, Lockwood 2007) and they are essential because cranial remains are one of the main sources of information on extant and fossil species (Ackermann and Cheverud 2004b, Lockwood 2007). Despite skull morphology is affected to some extent by environmental factors and is under less genetic control than molecular characters, it is accepted that craniometric traits are phylogenetically informative characters (Collard and Wood 2007, Lockwood 2007). However, as there is strong evidence that morphological integration plays an important role in evolutionary biology and can bias the results of such cladistic analyses (Strait et al. 2007, Lockwood 2007), further comprehension of how and why morphological complexes arise in the skull is needed.

Our analysis reports that the human skull has substantial amounts of genetic variation that are constrained by integration. Furthermore, it demonstrates that craniometric traits from the face, the neurocranium and the basicranium do not differ in their heritability patterns. We also provide empirical evidence that genetic and phenotypic correlation patterns in the human skull are consistent and show similar morphological variation patterns. Regarding integration, results suggest that traditional integration hypotheses (Enlow and Hans 1996) do not have a genetic basis, but confirm recent modularity patterns found in mice emphasizing strong covariation between relative widths of the neurocranium, the basicranium and the face as the most dominant integration pattern in the mammal skull (Hallgrímsson et al. 2007).

Our results concerning the heritability and correlation patterns between craniometric traits shed light into the genetic architecture of the human skull. Also, they are especially useful to provide an evolutionary context based on quantitative genetics for classic morphometric

studies and databases using univariate measurements. For a greater comprehension of modularity and integration patterns in the skull, future analyses should account for the multivariate nature of shape (Klingenberg 2004 and Chapter 4). This could be done by combining quantitative genetic methods with geometric morphometric tools, as suggested by Klingenberg and Leamy (2001). Then, we would be able to discuss in deeper detail the genetic and modular basis of complex phenotypes.

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APPENDIX

Table A1. Genetic correlations (lower left) and phenotypic correlations (upper right) among cranial distances. For genetic correlations, the associated standard errors (SE) are also provided. Significant correlations at the 0.05 level are bolded. Note that the genetic correlations involving nasal breadth were non computable because its heritability was 0.00.

		Facial			**Neural**			**Basal**		**Nasal**			**Orbital**			**Zygomatic**		
		breadth	height	lenght	breadth	height	lenght	breadth	lenght	breadth	height	lenght	breadth	height	lenght	breadth	height	lenght
Facial	breadth		**0.47**	**0.43**	**0.68**	**0.41**	**0.47**	**0.7**	**0.49**	**0.25**	**0.46**	**0.49**	**0.42**	**0.17**	**0.4**	**0.61**	**0.45**	**0.56**
	height	0.57±0.24		**0.24**	**0.31**	**0.31**	**0.41**	**0.41**	**0.34**	0.05	**0.88**	**0.42**	**0.37**	**0.28**	**0.14**	**0.42**	**0.55**	**0.37**
	lenght	0.42±0.24	0.42±0.26		**0.21**	**0.3**	**0.48**	**0.37**	**0.8**	**0.2**	**0.18**	**0.72**	**0.27**	**-0.11**	**0.41**	**0.43**	0.06	**0.37**
Neural	breadth	**0.89±0.11**	0.47±0.25	0.46±0.24		**0.28**	**0.33**	**0.52**	**0.26**	**0.18**	**0.28**	**0.28**	**0.31**	**0.15**	**0.24**	**0.36**	**0.3**	**0.29**
	height	-0.01±0.45	0.17±0.39	0.43±0.30	0.16±0.40		**0.36**	**0.49**	**0.52**	0	**0.29**	**0.34**	**0.25**	0.04	**0.33**	**0.3**	**0.23**	**0.33**
	lenght	0.56±0.20	**0.69±0.21**	0.50±0.21	0.49±0.20	0.61±0.25		**0.41**	**0.61**	**0.11**	**0.41**	**0.44**	**0.31**	0.07	**0.33**	**0.33**	**0.29**	**0.34**
Basal	breadth	**0.90±0.13**	**0.78±0.26**	**0.62±0.26**	**0.93±0.14**	0.40±0.35	0.50±0.24		**0.5**	**0.14**	**0.38**	**0.42**	**0.25**	**0.1**	**0.34**	**0.45**	**0.3**	**0.41**
	lenght	0.43±0.23	0.45±0.24	**0.96±0.07**	**0.63±0.22**	0.56±0.25	**0.75±0.15**	**0.79±0.23**		**0.13**	**0.33**	**0.59**	**0.36**	-0.01	**0.4**	**0.39**	**0.19**	**0.46**
Nasal	breadth	1	-1	1	-1	1	1	1	1		**0.17**	0.07	**0.21**	0.05	**0.23**	**0.36**	**0.14**	0.06
	height	**0.57±0.23**	**0.94±0.05**	0.23±0.24	0.48±0.23	0.27±0.34	**0.52±0.21**	**0.61±0.26**	0.25±0.23	-1		**0.36**	**0.35**	**0.31**	**0.16**	**0.42**	**0.58**	**0.28**
	lenght	**0.71±0.18**	**0.77±0.16**	**0.86±0.08**	**0.61±0.19**	**0.75±0.23**	**0.60±0.17**	**1**	**0.91±0.10**	1	**0.57±0.19**		**0.28**	-0.02	**0.36**	**0.4**	**0.21**	**0.45**
Orbital	breadth	0.19±0.37	**0.98±0.36**	0.60±0.34	0.35±0.32	0.26±0.47	0.00±0.33	0.27±0.40	0.45±0.30	-1	0.64±0.32	**0.75±0.29**		**0.26**	**0.18**	**0.31**	**0.32**	**0.25**
	height	-0.08±0.30	0.59±0.30	**-0.63±0.22**	0.1±0.32	-0.44±0.38	-0.16±0.30	-0.07±0.35	-0.43±0.26	1	**0.65±0.26**	**-0.62±0.24**	0.31±0.41		-0.05	-0.01	**0.52**	0.08
	lenght	0.00±0.33	0.08±0.31	0.36±0.24	0.45±0.27	**1**	0.30±0.26	0.66±0.29	**0.71±0.22**	1	0.03±0.27	0.23±0.26	-0.03±0.38	-0.41±0.28		**0.41**	**0.15**	**0.25**
Zygomatic	breadth	0.72±0.37	0.17±0.55	0.79±0.41	0.68±0.43	0.53±0.72	0.16±0.51	1	0.73±0.56	-1	0.23±0.48	0.82±0.28	0.87±0.63	-0.22±0.60	0.41±0.54		**0.34**	**0.34**
	height	0.19±0.33	0.82±0.19	0.03±0.31	0.49±0.29	-0.42±0.52	0.12±0.30	0.27±0.32	-0.04±0.31	1	**0.90±0.17**	0.34±0.28	0.12±0.41	**1**	-0.28±0.34	0.02±0.66		**0.34**
	lenght	0.61±0.24	0.30±0.32	0.49±0.25	0.51±0.31	0.03±0.52	**0.70±0.27**	**0.78±0.22**	0.47±0.26	1	0.27±0.30	**0.77±0.15**	-0.11±0.50	-0.60±0.40	0.30±0.34	0.14±0.66	-0.40±0.48	

4 *Results II*

4.1 *Genetic and phenotypic patterns of variation in the human skull*

Neus Martínez-Abadías, Mireia Esparza, Torstein Sjøvold, Rolando González-José, Mauro Santos, Christian Peter Klingenberg & Miquel Hernández.

Patrons de variació genètica i fenotípica del crani humà

La genètica quantitativa és un camp prometedor pels biòlegs evolutius perquè proporciona les eines necessàries per predir l'evolució en un context filogenètic. Així, per entendre l'evolució morfològica del crani humà és important explorar els patrons de variació i covariació genètica que determinen el fenotip craniofacial. Donat que l'evolució opera sobre la variació genètica dels caràcters, la contribució relativa dels factors genètics i ambientals determinaran el potencial del crani per respondre a l'acció de forces evolutives com la selecció natural i la deriva gènica. No obstant, aquest potencial evolutiu es pot veure limitat pels patrons de covariació i d'integració morfològica entre els caràcters cranials.

En aquest estudi es van explorar els patrons de variació genètica i fenotípica del crani humà utilitzant tècniques de genètica quantitativa multivariada i de morfometria geomètrica, de manera que es va conservar la complexitat tridimensional de les estructures craniofacials. Això representa un avantatge substancial respecte altres estudis, que per analitzar el substrat genètic de la morfologia cranial estimaven les heretabilitats univariades d'un conjunt de mesures craniomètriques. Estudis teòrics i empírics han demostrat que l'aproximació multivariada és capaç de detectar patrons evolutius més complexes perquè reflecteix la naturalesa inherentment multivariada de la forma i respecta els complexes patrons funcionals i del desenvolupament del crani humà.

Per realitzar aquestes anàlisis es va utilitzar una mostra de cranis amb informació genealògica associada procedent de Hallstatt (Àustria). Sobre els 390 cranis analitzats, es van registrar les coordenades tridimensionals de 50 punts craniomètrics utilitzant un digitalitzador Microscribe. A partir d'aquests, es van definir quatre subconfiguracions de punts amb l'objectiu d'analitzar els patrons de variació fenotípica i genètica, tant a nivell global com regional. La primera configuració, l'hemicranial, inclou 29 punts distribuïts pel costat esquerre del crani; mentre que les tres altres configuracions, facial, neurocranial i basal, defineixen estructures locals de la cara, del neurocrani i del basicrani (amb 23, 12 i 12 punts respectivament).

Amb cadascuna d'aquestes configuracions, es van realitzar les següents anàlisis. En primer lloc, es va realitzar una sobreimposició Procrustes i a partir de les noves coordenades es va calcular una anàlisi de components principals (PCA) per reduir la dimensionalitat de les dades. A partir de tots els PCs, es va obtenir la matriu de variació-covariació fenotípica i aquesta va ser descomposta en components de variació genètica i ambiental aplicant mètodes de genètica quantitativa. Seguint aquesta metodologia també es va estimar l'heretabilitat de la mida de cada configuració de punts utilitzant la mida centroide com a mesura de grandària. Concretament, es va utilitzar la tècnica de màxima versemblança restringida (REML). Aquesta tècnica segueix un model mixt linear que contempla conjuntament efectes fixes i aleatoris per descriure el fenotip de cada individu, incorporant informació multigeneracional a partir de bases de dades no balancejades. El model que es va aplicar incloïa els PCs com variables dependents, la mida centroide com a covariable, i l'edat, el sexe i l'estat de deformació com a efectes fixes.

Els resultats van mostrar que la quantitat de variació genètica en el crani humà és considerable: aproximadament el 30% de la variació fenotípica és d'origen genètic. La cara, el neurocrani i el basicrani presenten nivells similars de variació genètica, tant a nivell de mida com de forma. Aquesta determinació genètica proporcionaria al crani una capacitat evolutiva substancial, però els resultats van mostrar que els patrons morfològics del crani estan fortament integrats. Les anàlisis realitzades suggereixen que els patrons de variació genètica i fenotípica són similars però no idèntics i van reflectir uns patrons de covariació genètica molt més complexos que els fenotípics, amb una forta estructuració jeràrquica. Per tant, aporten una nova metodologia per identificar estructures modulars i per visualitzar quines morfologies (o direccions de canvi morfològic) no presenten variació genètica i per tant no poden evolucionar.

Aquests resultats tenen implicacions rellevants pels estudis filogenètics perquè evidencien l'existència d'una forta integració morfològica entre les estructures cranials. Així, es posa de manifest que tant a nivell genètic com fenotípic, els caràcters cranials no són independents entre sí, i que per tant s'incompleix l'assumpció bàsica de les anàlisis cladístiques. La solució a aquest problema podria consistir en tractar els caràcters integrats com a complexes filogenètics individuals. Aquí s'ha mostrat com identificar aquests complexes a nivell genètic i fenotípic, però perquè realment es poguessin considerar veritables mòduls filogenètics també s'haurien d'identificar a nivell intra- e interespecífics.

Genetic and phenotypic patterns of variation in the human skull

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ABSTRACT The evolutionary potential of any biological quantitative character relies on the amount of genetic variation. In complex morphological structures, such as the human skull, it also depends on its integrated nature, because association between traits can constrain the potential for change. The human skull is in fact an integrated whole made up of several relatively independent subunits which have different developmental origins and which account for different functional requirements. Hierarchical modularity yields to integration within structures sharing common developmental pathways or functional basis. Therefore, estimation of genetic variation and covariation of such structures is critical to incorporate craniofacial data in models of evolution of quantitative traits. In this study, we analyzed a skull collection from Hallstatt (Austria) with associated genealogical data by means of 3D geometric morphometric techniques and multivariate quantitative genetic analysis. Genetic and phenotypic components of variation of skull size and shape have been estimated applying restricted maximum likelihood methods. We inspected four different configurations of landmarks (hemicranial, facial, neurocranial and basal) to account for both global and regional patterns of variation. Our results show that there is substantial genetic variation in skull size and shape, but also strong integration patterns that are restricting skull evolvability. In fact, it was detected that some shape features cannot evolve because they don't have available genetic variation. The face, the neurocranium and the basicranium show similar amounts of genetic variation and our results show that phenotypic and genetic patterns of variation are similar but not identical. Overall, we discuss that this is a useful alternative approach for searching modules in complex phenotypes that may be relevant for phylogenetic studies.

KEYWORDS Human skull, integration, quantitative genetics, geometric morphometrics, evolvability.

INTRODUCTION

Quantitative genetics is a promising field for evolutionary biologists because it provides the tools to predict evolution in phylogenetic contexts (Steppan et al. 2002). To understand the morphological evolution of the human skull it is important to explore the patterns of genetic variation and covariation underlying the cranial phenotype (Konigsberg 2000, Ackermann and Cheverud 2004, Hallgrímsson et al. 2007). As evolution operates on the available genetic variation underlying traits, the relative contribution of genetic and environmental factors will determine the potential of the skull to respond to evolutionary forces such as natural selection

and genetic drift (Lynch and Walsh 1998, Hansen and Houle 2004, McGuigan 2006). However, this evolutionary potential is constrained by covariation and morphological integration between cranial characters. For instance, it is likely that cranial morphology evolved under selective pressures through the hominid lineage, mainly as adaptive responses to bipedal locomotion, encephalization and dietary changes (Wolpoff 1999), but strong pervasive integration has obscured the actual action of natural selection (Chapter 5). Thus, research on the genetic background underlying main trends of phenotypic variation on the size and shape of the human skull is necessary to further interpret the evolutionary novelties which characterize our lineage. Quantitative genetics provides theoretical and practical tools which can be applied to samples of related individuals in order to estimate the genetic and non genetic components determining the phenotypic variation. Important quantitative genetic parameters can be estimated after the analysis of pedigree-structured samples (Konigsberg 2000), which are crucial for understanding the evolution of complex structures through correlated responses to selection. Here we investigate the quantitative genetic patterns underlying the human skull, taking into account both global and regionalized patterns of morphological variation.

The shape of the human skull is usually represented by sets of linear and angular measurements (Martin and Saller 1957, Howells 1973), and the common practice to assess the amounts of genetic variation underlying skull morphology is to estimate the univariate heritabilities of these craniometrical traits (Dahlberg 1926, Vandenberg 1962, Hiernaux 1963, Nakata et al. 1974, Susanne 1975, Susanne 1977, Sjøvold 1984, Byard et al. 1984, 1985, Devor et al. 1986, Devor 1987, Sharma and Susanne 1991, Sharma 1998, Sparks and Jantz 2002, Carson 2006). Heritability, considered in the narrow sense, expresses the proportion of total phenotypic variation due to additive genetic variation (Falconer and MacKay 1996). The quantification of the genetic component of craniometric characters is important because they are widely used in phylogenetic studies (Skelton and McHenry 1992, Strait et al. 1997, Skelton and McHenry 1998, Strait and Grine 1999, Collard and Wood 2007) and have been ubiquitously incorporated into population genetic models that analyze the history and structure of human populations (Relethford and Lees 1982, Relethford 1994, Relethford and Harpending 1994, González-José et al. 2001, Relethford 2002, 2004, Roseman 2004). However, it has been argued that this univariate approach to heritability is unable to detect complex patterns of evolution because it does not reflect the inherently multivariate nature of shape (Klingenberg and Leamy 2001, Klingenberg and Monteiro 2005, Myers et al. 2006). Both theoretical and empirical evidences have highlighted that the decomposition and simplification of shape in a suite of univariate measurements may produce inaccurate pictures of the direction of evolution of complex shapes. This is especially relevant for complex morphological structures such as the human skull; even more if we take into account that most craniometric traits defining human skull shape are arbitrary measurements, non-independent between them and non-functionally or developmentally meaningful (Moss and Young 1960, Pucciarelli et al. 1990). In such cases, to complement the information provided by studies using the univariate approach, it is recommended to use a multivariate assessment of shape to address quantitative genetic studies of morphological structures (Klingenberg and Leamy 2001, Klingenberg and Monteiro 2005, Myers et al. 2006).

Geometric morphometrics provides a set of statistical techniques that allow the multivariate analysis of shape (Bookstein 1991, Rohlf and Marcus 1993, Dryden and Mardia 1998, Klingenberg 2002). In combination with the multivariate extension of quantitative genetic methods (Lande 1979), geometric morphometrics can overcome the limitations of the univariate approach. This combined procedure was developed by Klingenberg and Leamy (2001) to explore the patterns of phenotypic and genetic variation of the mouse mandible. Under this methodology, size and shape is captured using landmarks-based morphometrics and the genetic and non genetic components of variance are inspected through the comparison of the phenotypic and genetic covariance matrices of shape (G and P). Since heritability has no direct equivalent in the multivariate context, similarity between G and P, assessed by the magnitude and direction of shape change, is informative about the genetic basis-determination of a particular morphological phenotype.

The human skull is certainly a complex shape. It is a hierarchically integrated unit (Olson and Miller 1958, Lieberman et al. 2000, Bookstein et al. 2003, González-José et al. 2004, Bastir and Rosas 2005) that comprises many osseous structures. Furthermore, it accomplishes different functional requirements, such as surrounding and protecting the brain, the eyes, and supporting the respiratory and the masticatory apparatus. According to Moss and Young (1960), the skull consists of cranial skeletal units whose origin and final shape, size, location, maintenance and growth trajectories are the result of secondary, compensatory and obligatory responses to prior demands exerted by their neighbouring nonskeletal cells, tissues, organs and operational volumes. Depending on their developmental origins, three main regions are usually distinguished within the skull, namely the basicranium, the neurocranium and the face (Sperber 2002). The basicranium derives from the chrondrocranium, which is a cartilaginous precursor of the cranial base; the neurocranium is formed from the desmocranium, from mesodermal and neural crest cells; and finally, the face is developed from the splanchnocranium, which ossifies intramembranously like the cranial vault but only from neural crest precursors (Sperber 2002). These skull regions grow during different ontogenic times and its development is regulated after different genetic and epigenetic factors. The base is the first region to develop, followed by the cranial vault and the face (Sperber 2001). The growth of the neurocranial structures (both the base and the cranial vault) is mainly driven by the growth of the expanding brain and occurs early during the ontogeny, during the prenatal and neonatal periods, while the face develops later, once the brain has finished its growth. The face and the mandible grow during a more extended period of time, reaching its maturity at an early age (Sperber 2001).

Given these complex developmental and functional patterns of the skull, the multivariate approach is strongly advocated in order to investigate the genetic and developmental basis of the human skull phenotype, especially if we recall that integration can modulate, deviate or constrain the evolutionary potential of change (Klingenberg 2004, Hansen and Houle 2004, Merilä and Björklund 2004). Although quantitative traits usually encompass abundant genetic variation, the evolvability of these traits is reduced if genetic variation is constrained by integration of characters (pleiotropic constraints) or by integration among genes (epistatic constraints). These constraints are barriers to evolutionary change (Hansen and Houle 2004) and univariate approaches disregard these complex patterns of covariation between cranial

structures as well as the functional, developmental and ontogenic bases of these regions. Therefore, the estimation of the genetic variation and covariation patterns of such structures is critical to incorporate craniofacial data in models of evolution of quantitative traits.

The main goal of this study is to combine quantitative genetics and geometric morphometrics methods in order to estimate the phenotypic variation observed in the human skull and to separate and describe the genetic from the environmental sources of variation. To address this issue we used a large pedigree-structured series of skulls from the charnel house of Hallstatt (Austria). This cranial collection is exclusive because the names of the individuals are written on the skulls and hence the individuals can be identified at the parish demographical records. Therefore, families were traced back and this genealogical information was used to estimate the genetic variance-covariance matrix (G) from the expected resemblance between relatives (Falconer and MacKay 1996, Lynch and Walsh 1998). Although this is a statistical abstraction obtained without direct observation of the number of loci and alleles, it is expected to contain information about shared functional, developmental and genetic processes between interacting characters (McGuigan 2006). In this study, we explored the phenotypic and genetic patterns of covariation of the human skull and assessed their similarity. To do so, first we inspected the patterns of phenotypic and genetic variation on a global skull shape to account for total variation and morphological integration among cranial structures. Second, we analyzed separately the phenotypic and genetic patterns of variation of the three main developmental regions of the skull (face, neurocranium and basicranium) to account for regionalized variation and integration within structures.

MATERIALS AND METHODS

To perform the quantitative genetic analysis of human skull morphology we measured the skulls of the Hallstatt collection and reconstructed the genealogies of the Hallstatt population. We compiled the complete records of births, marriages and deaths from 1602 to 1900, which included 18,134 individuals. More details about this sample are described at Chapter 3 and at the Appendix of this thesis. At the present work, we analyzed 390 complete skulls, of which 350 individuals fall into the extended, multigenerational genealogies. These skulls date back to the 18th and 19th centuries (40% and 60% respectively) and the sample includes skulls from both sexes (41% females; 59% males). Most of them were adult individuals (91%) and a small proportion of the skulls (12%) were either asymmetric or had slight dysmorphologies possibly related to craniosynostosis.

Skull size and shape was captured by standard geometric morphometric methods. A set of 50 osteological landmarks was recorded on each skull using a 3D Microscribe digitizer (Table 1, Fig. 1). Error measurement was assessed by resampling of 91 skulls that were each measured twice. Analysis of variance showed that repeatability averages over 90%. To account for global and regional patterns of morphological variation, we defined four subconfigurations of landmarks (Table 1) following standard functional and developmental criteria (Marroig and Cheverud 2001, Hallgrímsson et al. 2007). The hemicranial configuration represents a global shape and contains 29 landmarks distributed over the left side of the skull, whereas the others

represent the shape of more localized structures of the skull. The facial and neurocranial subconfigurations contain respectively 23 and 12 symmetrical landmarks and the basicranial subconfiguration includes 12 left landmarks from the cranial base.

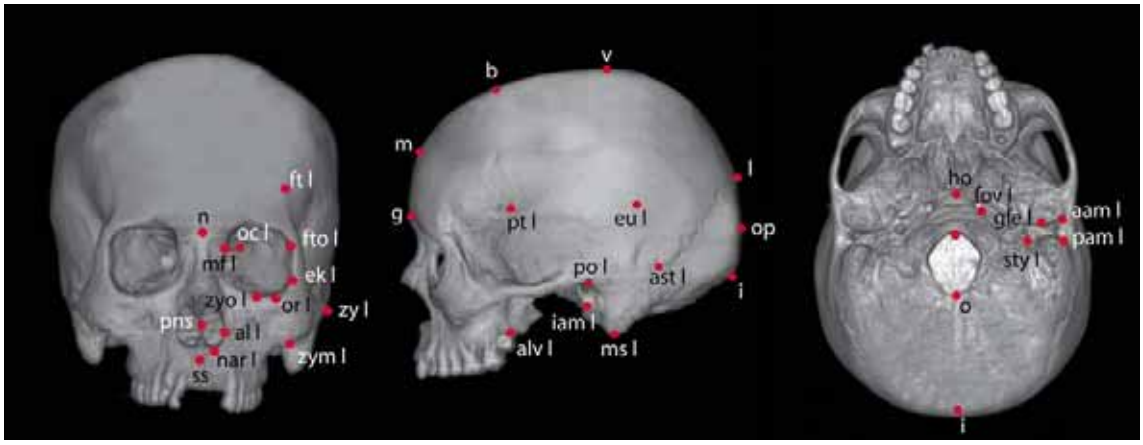

Fig. 1. Midline and left landmarks used in this study. For coding details see Table 1.

Geometric morphometric (Bookstein 1991, Dryden and Mardia 1998) and quantitative genetic methods (Lynch and Walsh 1998) were combined in order to assess skull morphological variation and to estimate the genetic and environmental components underlying the phenotypic variation. To obtain size and shape variation we performed a generalized Procrustes superimposition (Rohlf and Slice 1990). This procedure scales the original landmark configurations to unit centroid size, translates them to a common origin and rotates them until a best-fit criterion is achieved. The resulting fitted configurations lie in Kendall's shape space (Rohlf 1996), which is non-Euclidean, and therefore Procrustes coordinates are projected to a linear tangent space (Dryden and Mardia 1998). Hemicranial, neurocranial, facial and basicranial centroid sizes were used as size measures and they were computed as the squared root of the sum of the squared distances of all landmarks' configuration from their centroid. To perform the geometric morphometric analyses we used a pre-release version of MorphoJ software (C. P. Klingenberg 2007, unpublished).

Size and shape quantitative genetic analysis were performed separately using a restricted maximum likelihood method (REML) implemented by the VCE5 software package (Kovac et al. 2003), which estimates the additive genetic (G), phenotypic (P) and environmental (E) covariance matrices. This method follows a mixed linear model that jointly accounts for fixed and random effects to describe each individual's phenotype. The components of variance are estimated by an iterative procedure that maximizes the likelihood of observing the actual data (Lynch and Walsh 1998). REML analytical methods incorporate multigenerational information from unbalanced datasets. Moreover, they are not limited by assumptions of non-assortative mating, inbreeding or selection (Kruuk 2004).

To obtain the size heritability of each landmark configuration (that is, the heritability of centroid size) we used a univariate model accounting for age, sex and status of deformation as fixed effects. To obtain the shape components of variance we followed the multivariate

approach described by Klingenberg and Leamy (2001). This methodology assesses the phenotypic, environmental and genetic components of biological shapes preserving the original shape configuration. From the Procrustes fitted coordinates, a Principal Components Analysis (PCA) was performed to reduce the dimensionality of the data. PCs accounting for 100% of variation were the phenotypic input data for the quantitative genetic analysis. The multivariate model included shape PCs as dependent variables, centroid size as a covariate, and age, sex and status of deformation as fixed effects. The genetic, phenotypic and environmental covariance matrices were converted back to landmark coordinates with MorphoJ (C. P. Klingenberg 2007, unpublished). To display the dominant features of shape variation and to compare the morphological patterns due to genetic and phenotypic factors, we performed a PCA on each of the G and P covariance matrices.

Table 1. List of digitized landmarks. Codes and definitions used are provided (r right; l left). Each landmark is assigned to one or more skull regions: H, hemicranial configuration; F, facial; N, neurocranial; B, basicranial.

Code	Region	Landmark	Definition
aam l	B	Ant auditory meatus	Most anterior point at the external auditory meatus
al r/l	H, F	Alare	Most lateral point on the margin of the nasal aperture
alv l	H	Alveolar point	Posterior limit of the maxillary alveolar arch at the pterygo-alveolar suture
ast r/l	H, N	Asterion	Point where the lamboidal, parieto- and occipitomastoid sutures meet
b	H, N	Bregma	Point where the coronal and sagittal sutures intersect
ba	H, B	Basion	Midline point on the anterior margin of the foramen magnum
ek r/l	H, F	Ectoconchion	Most lateral and posterior point on the orbital margin
eu r/l	H, N	Euryon	Most lateral point of the braincase perpendicular to the sagittal plane
fov l	B	Foramen ovale	Most posterior point at the foramen ovale
fto r/l	H, F	Frontomalare orbitale	Point where the frontozygomatic suture intersects the orbital margin
ft r/l	F	Frontotemporale	Point where the temporal line gets its most anteromedial position
g	H, N	Glabella	Most anterior midline point on the frontal bone
gle l	B	Glenoid fossa	Most posterior point on the margin of the glenoid fossa
ho	H, B	Hormion	Most posterior midline point on the vomer
i	H, B	Inion	Midline point at the base of the external occipital protuberance
iam l	B	Inf auditory meatus	Most inferior point at the external auditory meatus
l	H, N	Lambda	Midline point of the intersection of the sagittal and lamboidal sutures
m	H, N	Metopion	Midline point where the elevation above the chord from n to b is greatest
mf r/l	H, F	Maxillofrontale	Point where the lacrimal crest meets the frontomaxillary suture
ms l	H, B	Mastoidale	Most inferior point on the mastoid process
n	H, F	Nasion	Midline point where the two nasal bones and the frontal intersect
nar r/l	H, F	Nariale	Most inferior point on the nasal aperture
o	H, B	Opisthion	Midline point at the posterior margin of the foramen magnum
oc l	H	Optic canal	Most superior, medial, and anterior points of the optic canal
op	H, N	Opisthocranion	Most posterior point of the skull in the medial sagittal plane.
or r/l	H, F	Orbitale	Lowest point on the orbital margin
pam l	B	Post auditory meatus	Most posterior point at the external auditory meatus
pns	H, F	Posterior nasal spine	Point at the vomer-palatin junction
po l	H, B	Porion	Uppermost point on the margin of the external auditory meatus
pt r/l	H, N	Pterion	Point where the frontal, parietal, temporal and sphenoides bones meet
ss	H, F	Subspinale	Deepest point seen in the profile below the anterior nasal spine
sty l	B	Stylomastoid foramen	Point at the stylomastoid foramen
v	H, N	Vertex	Midsagittal superior point of the cranium when the skull is in Frankfort plane
zy r/l	H, F	Zygion	Most lateral point on the surface of the zygomatic arch
zym r/l	H, F	Zygomaxillare	Most inferior point on the zygomaticomaxillarysuture
zyo r/l	H, F	Zygoorbitale	Point where the orbital rim intersects the zygomaticomaxillary suture

The distribution of eigenvalues of the G matrices provides information on both the evolutionary potential of the skull and the levels of integration patterns within and among regions (McGuigan 2006). If all the eigenvalues have the same weight, it evidences weak correlations between traits and indicates that shape features are more or less free to evolve. Otherwise, if one eigenvalue is much larger than the remaining ones, it means that covariances are high and thus shape evolution is constrained towards certain directions of shape change. If the rest of eigenvalues are low or even zero then they represent "forbidden" evolutionary trajectories, that is, phenotypes that cannot evolve because they have no additive genetic variation (Kirkpatrick and Lofsvold 1992, Klingenberg 2004). Therefore, the dimensionality of G provides an estimate of the number of independent traits contained in G and points out which regions of phenotypic space are evolutionary accessible and which are not (McGuigan 2006).

RESULTS

Size analysis

The average size heritability of the different configurations of the skull is 0.36 and the results show that there are no substantial differences among them. The hemicranial and the facial configurations show the highest estimations (h^2=0.39, std=0.08; and h^2=0.39, std=0.08 respectively), while the basicranial and the neurocranial configurations show slightly lower estimates (h^2=0.33, std=0.09; and h^2=0.31, std=0.09 respectively).

Shape analysis

The ratio among the genetic and the phenotypic summed eigenvalues provides an estimation of the total amount of genetic variance. This figure indicates that the additive genetic component accounts for about 30% of the total phenotypic variation for each of the configurations. The genetic contribution is higher at the facial (35%), the hemicranial (34%) and the basicranial (33%) configurations, and is lower at the neurocranial (26%) configuration. The eigenvalues of the genetic and the phenotypic covariance matrices show a gradation (Fig. 2-5) that ranges from intermediate values to values close to 0, showing that there is strong integration in the human skull and that evolution across particular multivariate spaces is unlike.

Overall, the variance explained by the eigenvectors of the genetic covariance matrices is higher and more concentrated on the first PCs than the variance explained by the PCs of the correspondent phenotypic covariance matrix, indicating that there are substantial genetic constrains modulating the phenotypic pattern of the human skull. Graphical displays of the morphological variation explained by the first three PCs of the genetic and the phenotypic covariance matrices are presented in Fig. 2-5. The shape changes displayed by the PCs are diverse and affect landmarks from different regions simultaneously, suggesting again the strong canalizing effect of integration (Fig. 2-5). Our discussion is focused on the first PCs since they may track real developmental shifts; lower order PCs may be somewhat arbitrary in terms of shape changes and not as biologically meaningful as the first ones.

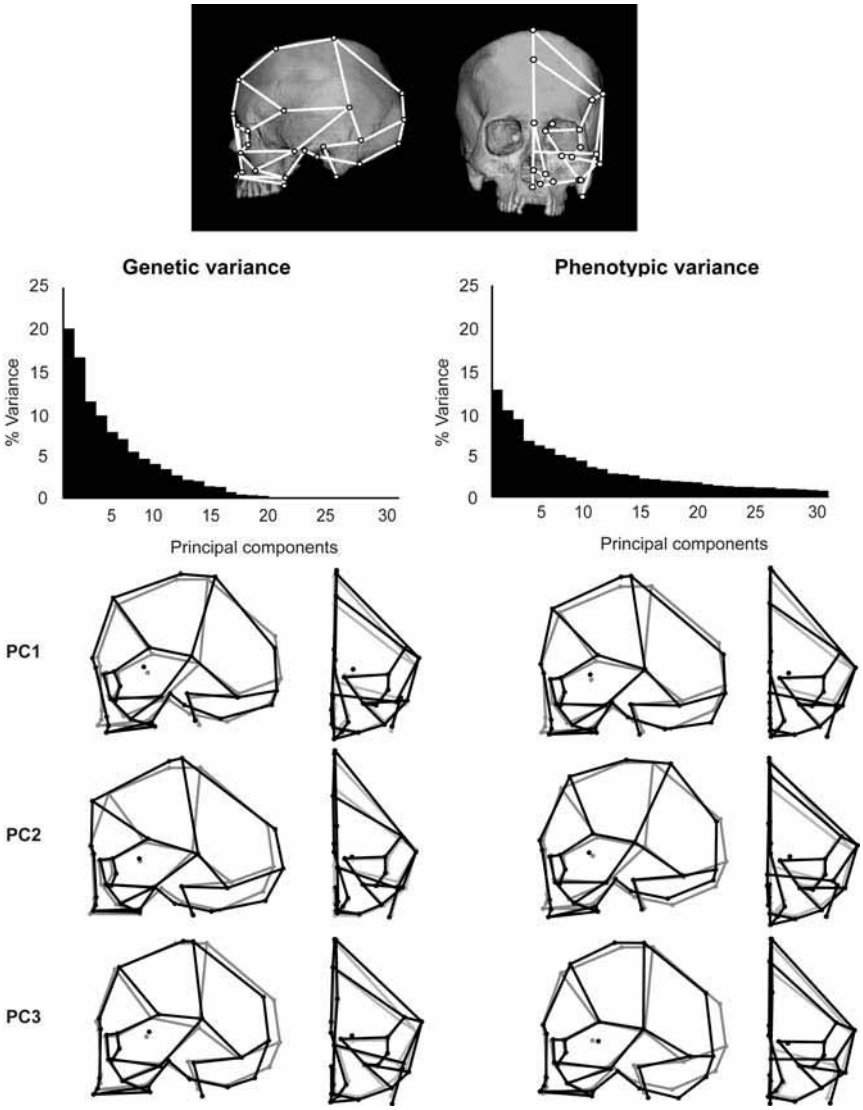

Fig. 2. Hemicranial configuration. The histograms show the variance explained by the PCs of the genetic and the phenotypic covariance matrices. The morphology associated to the three first genetic and phenotypic PCs are displayed as shape changes from the average shape (grey wireframe) to a shape which is at 0.1 Procrustes units far from the mean (black wireframe). Note that the sign of the PCs is arbitrary. For each PC, a lateral and a frontal view of the hemicranial configuration is shown. Due to the high dimensionality of data and to software limitations, only 32 PCs accounting for 90% of the total variation of shape were included in the genetic analysis.

Hemicranial configuration. The morphological patterns described by the genetic and the phenotypic covariance matrices are fairly similar, but there is no one-to-one correspondence between genetic and phenotypic PCs (Fig. 2). The three first PCs explain respectively 48.1% and 31.8% of the total variance and show that shape changes in the face are encompassed with changes in the neurocranium and the basicranium (Fig. 2). This result evidences that there is no complete independence among regions and that skulls behave as an integrated structure. The first genetic and the second phenotypic PCs show that landmarks from face, the neurocranium and the basicranium shift in a integrated way, with similar magnitudes of change but in

different directions: while the cranial vault expands, the face retracts and the cranial base contracts, and vice versa (note that the sign of change is arbitrary). Along with changes in these main developmental regions, we also observe changes in minor functional regions of the face, namely the orbits and the naso-maxillary complex. The second genetic and the first phenotypic PCs mainly concern an antero-posterior stretching-contraction of the cranial vault: the metopion, the bregma and the vertex shift in opposite direction with regard to the lamba, the opisthocranion and the inion. The pterion, the euryon and the asterion follow the direction of change of the landmarks from the posterior cranial vault. Changes at the cranial base are minimal and follow the direction of change of the anterior cranial vault. Both the third genetic and phenotypic PCs show changes that are mostly concentrated at the posterior region of the skull. Midsagittal and lateral landmarks from this region follow opposite directions of change: when the former expands, the latter compress.

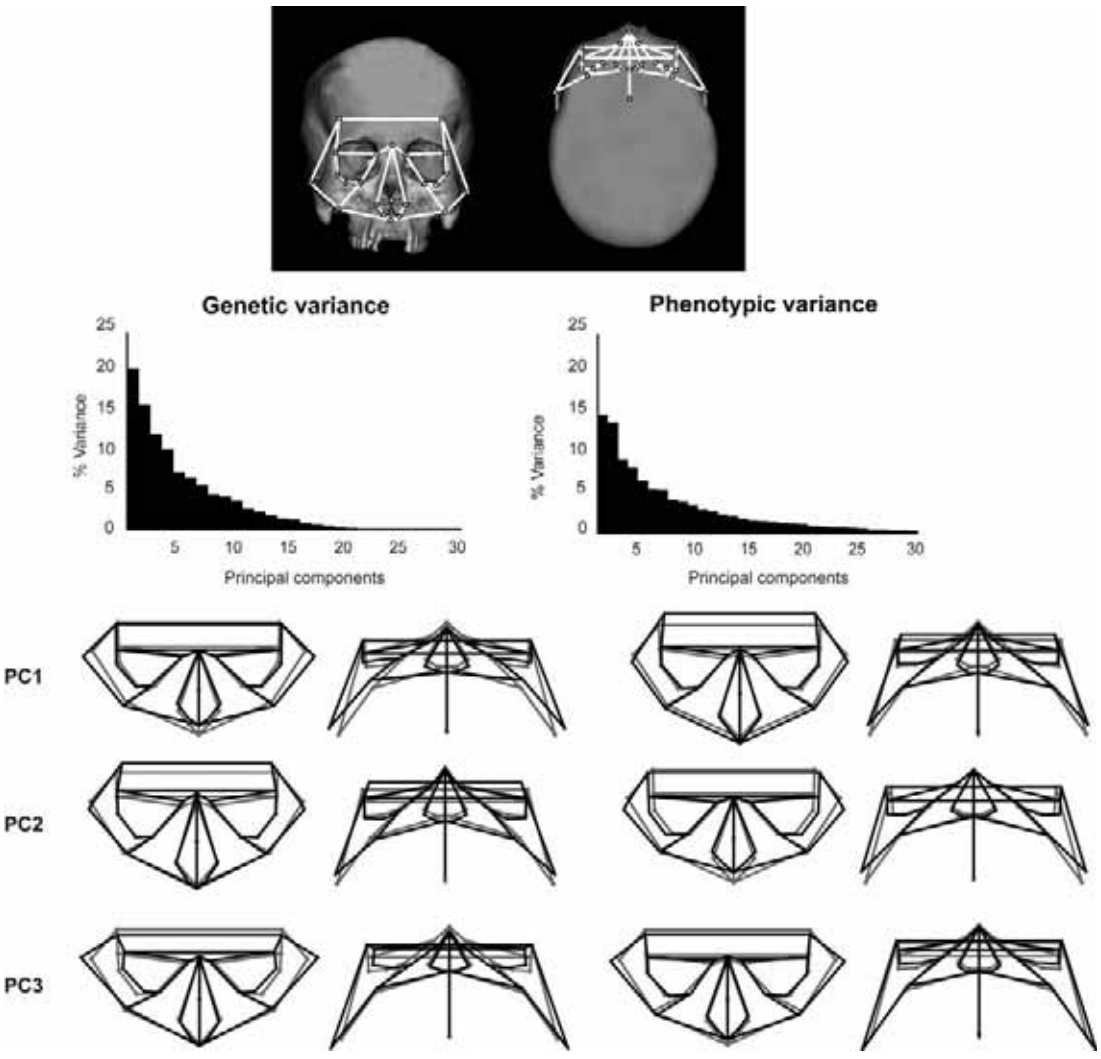

Fig. 3. Facial configuration. For each PC a frontal and a superior view of the facial configuration is shown. The genetic analysis accounted for 100% of the variation of the symmetric component of shape.

Facial configuration. The morphological patterns described by the genetic and the phenotypic covariance matrices are also fairly similar, but again there is no one-to-one correspondence between genetic and phenotypic PCs (Fig. 3). The three first PCs of the genetic and the phenotypic covariance matrices explain respectively 48.1% and 37.7% of the total variance. Total shape changes of the face are linked to shifts in the orbits, the nose and in the zygomatic arches. The first genetic and the second phenotypic PCs show that changes in width are tied to changes in the height of the face. Whereas width increases by an outward movement of the zygions, facial height decreases (by shifts of landmarks at the inferior face), and vice versa. The second genetic and the first phenotypic PCs display major changes at the superior face. The third PCs mainly concern integrated changes between the orbits and the zygomatic region, whereas the nasal complex slightly changes.

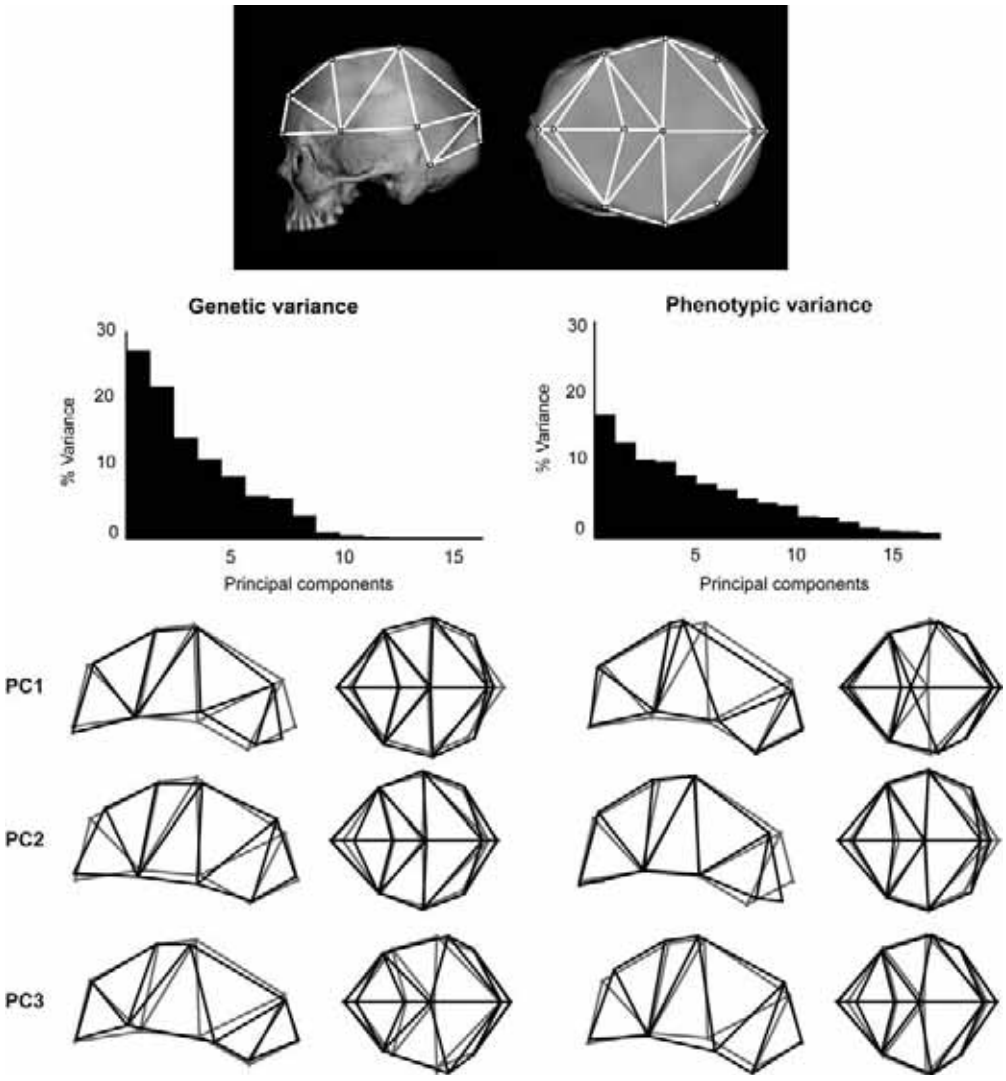

Fig. 4. Neurocranial configuration. For each PC a lateral and a superior view of the neurocranial configuration is shown. The genetic analysis accounted for 100% of the variation of the symmetric component of shape.

Neurocranial configuration. The three first PCs of the genetic and the phenotypic covariance matrices explain respectively 63.5% and 40.8% of the total variance (Fig. 4). The first genetic and the second phenotypic PCs reveal that the main source of variation within the neurocranium is the posterior region of the cranial vault. Midsagittal and lateral landmarks shift in opposite perpendicular directions; that is, a longitudinal compression is followed by an overall lateral widening of the cranial vault and vice versa. The second genetic and the third phenotypic PCs display changes distributed homogeneously throughout the whole vault: landmarks both from the anterior and the posterior region shift in the same direction. Finally, the third genetic and the first phenotypic PCs represent a differential development of the anterior and the posterior regions of the cranial vault; that is, a compression at one region is accompanied by an expansion at the other one.

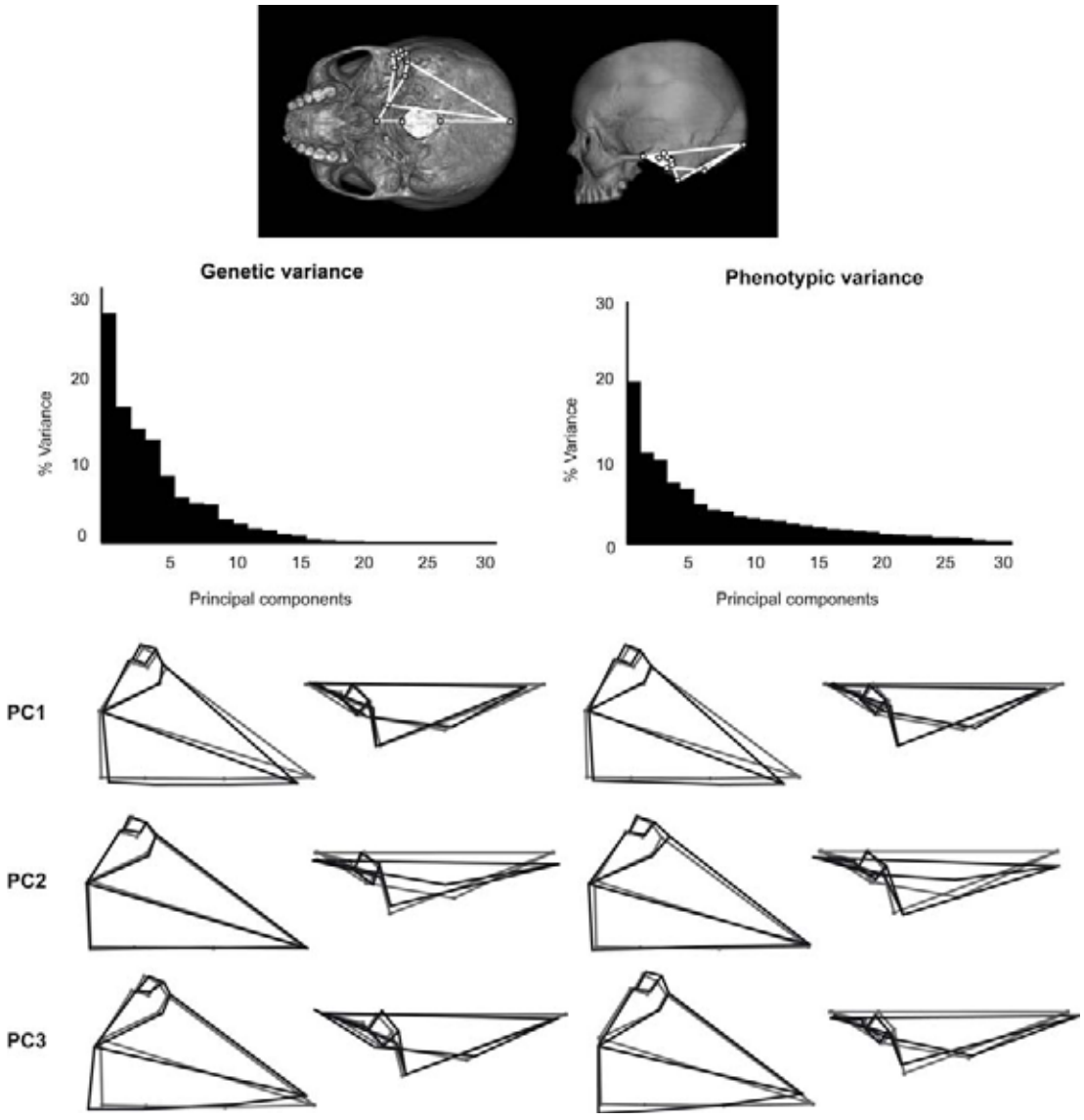

Fig. 5. Basicranial configuration. For each PC an inferior and a lateral view of the basicranial configuration is shown. The genetic analysis accounted for 100% of the total variation of shape. For more details see Fig. 2.

Basicranial configuration. The three first PCs of the genetic and the phenotypic covariance matrices explain respectively 56.1% and 41.4% of the total variance (Fig. 5). In terms of shape changes, the basicranial configuration shows the greatest correspondence between the genetic and the phenotypic covariance matrices. The first genetic and phenotypic PCs display changes in cranial base length: the hormion, the basion and the opisthion move in opposite direction with regard to the inion. The second genetic and phenotypic PCs concern changes in height, with a dorso-ventral compression-expansion of the cranial base. The third PCs reflect a reorganization of the cranial base, including changes in length and width as well as a reorientation of the auditory meatus and the mastoid process.

DISCUSSION

In this study, we have applied a multivariate quantitative genetics approach to skull size and shape in order to investigate the patterns of genotypic variation and covariation underlying human cranial morphology. Our main results show there are considerable amounts of genetic variation in the human skull, distributed fairly homogeneously, but hierarchically structured, among the main developmental regions of the skull. This gives the human skull a substantial potential to evolve and to respond to selection. However, strong patterns of integration suggest that the evolvability of the human skull is also constrained and directed towards certain trajectories of morphological change that would maintain an operational/functional skull shape. This result confirms previous evidence (Chapter 5) emphasizing the important role of developmental mechanisms, which determine the ways through which genetic variation is expressed and how this results in a complex phenotypic shape such as the human skull.

Regarding the genetic substratum of the different regions of the skull, we did not find substantial differences among the genetic component underlying each region. It is commonly assumed that the cranial base is the structure under stronger genetic control and that the face is more prone to be affected by non-genetic effects (Sperber 2001). However, our results indicate that the face, the cranial base and the cranial vault have similar amounts of genetic variation, approximately accounting for 30% of their total variation. This result supports previous evidence suggesting similar levels of heritability within the primate skull (Cheverud and Buikstra 1982, Sjøvold 1984, Cheverud 1996b).

Moreover, our findings indicate that the genetic and the phenotypic patterns of variation underlying the morphology of the human skull are similar but not identical. These results are consistent with those reported by Klingenberg and Leamy (2001), and suggest that the genetic and phenotypic patterns of morphological variation are organized in different ways but expressed through the same processes. In quantitative genetic analysis it is a common practice to use phenotypic data without actual genetic data, assuming that the genetic and the phenotypic covariance matrices are proportional. This is done because usually there is no genealogical information associated with prehistoric skull series and because there is substantial evidence demonstrating that phenotypic variation patterns are a good proxy to study the history and structure of human populations (González-José et al. 2001, 2002, Relethford 2002,

González-José et al. 2003, 2004, Relethford 2004, Ackermann and Cheverud 2004, Martínez-Abadías et al. 2006, González-José et al. 2007). Here we show that despite G and P are not proportional (Chapter 5) they display very similar and consistent patterns of morphological variation.

Evolvability of complex phenotypes should be analyzed considering jointly the patterns of genetic variation and morphological integration. Since univariate quantitative genetic studies are unable to deal with complex covariation patterns among traits, the multivariate approach used here reveals much more powerful and consistent results (Chapter 3). Although overall results are fairly similar and point to the same conclusions (moderate amounts of genetic variation and substantial integration), the multivariate approach provides a more insightful picture of the patterns of evolvability and integration of the human skull. This is pointed out by the relative magnitude and direction of change of the landmarks distributed over the whole structure, which also reveals the relative interdependence among regions. However, and despite its potential, the multivariate approach has seldom been applied, i.e. for the study of the mouse mandible (Klingenberg and Leamy 2001) and the plastron in slider turtles (Myers et al. 2006).

This methodology can also be used as an approach for searching modules in complex phenotypes, as an alternative to modular hypothesis-driven approaches. In the latter, modules are defined a priori according to functional, developmental and environmental criteria and then it is tested if the observed patterns of character correlation actually fit the correlation patterns expected by the assumed model. Conversely, our assessment departs from a different framework, without any previous assumption of shape modularity. Morphological integration can be addressed through geometric morphometrics and quantitative genetics because it provides us a pattern of association between characters based on the genetic variance-covariance matrix. In fact, our results indicate the existence of several modules and reflect the hierarchical nature of them. For example, the entire face shows changes associated to shifts on the vault and the cranial base (Fig. 3), but regional patterns of variation also show more localized and integrated units within the face. The first genetic PC shows that facial breadth covaries with facial height (Fig. 3), the second PC shows that the superior face behaves relatively independent from the inferior face and finally PC3 shows the coordinated changes among the zygomatic arches and the orbits (Fig. 3). However, these latter changes account for lower variance levels, and thus are not as strongly integrated as modules arising in PC1. This combined approach enables to treat complex phenotypes by inspecting its genetic basis and regarding its multivariate nature, allows detecting modules without assuming a priori modular hypotheses, and identifies their relative level and pattern of integration.

This has relevant implications in phylogenetics because integration between characters has confounding effects in cladistic analyses (Skelton and McHenry 1992, Lieberman 1995, Skelton and McHenry 1998, Strait and Grine 1999, Collard and Wood 2000, Strait 2001, Collard and Wood 2007, Lockwood 2007). A fundamental assumption of cladistics is that the characters included in the analysis are independent (Farris 1983, Kluge 1989), which is not certainly the case in complex phenotypes such as the vertebrate skull. This problem might be overcome by treating the integrated characters as a single phylogenetic complex and thus reduce the bias

caused by integration (Strait 2001). A further implication of treating multivariate and integrated structures as such is that homoplasy is less likely in multivariate complex phenotypes than in univariate traits (Polly 2004). Thus, using geometric morphometrics and quantitative genetics should be the natural first step of any cladistic analysis or maximum likelihood analysis based on complex phenotypes.

In this paper, we have attempted to evaluate integration at different levels (genetic and phenotypic). Integrated complexes can be identified at the lowest hierarchical level of integration, the genetic one (Cheverud 1996a) and then it can be checked if they have a correspondence in the phenotypic variance-covariance matrix. If so, it is likely that genetic modules are not substantially modified by epigenetic factors and they should also be detected at higher hierarchical levels, such as the individual and intraspecific ones. To identify complete phylogenetically reliable modules, the approach used here should be further complemented with ontogenetic and interspecific studies in order to account for the whole hierarchical scheme of integration and genetic basis of complex phenotypes (Jernvall 2000).

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5 *Results III*

5.1 *Pervasive genetic integration directs the evolution of human skull shape*

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La integració genètica dirigeix l'evolució de la forma del crani humà

Els cranis dels humans moderns es caracteritzen per tenir una volta cranial gran i arrodonida, una cara retreta i una base flexionada. A més a més, els homínids bípeds presenten el foramen magnum en una posició més avançada que els seus ancestres quadrúpeds. Hi ha autors que consideren que aquestes transformacions són respostes adaptatives a transicions en la locomoció i a canvis cognitius (adquisició d'un cervell més gran), de llenguatge, de dieta, etc. En canvi, hi ha autors que consideren que cadascun dels caràcters que defineixen els humans moderns no s'ha seleccionat per separat, sinó que tots ells formen part d'una resposta integrada a pocs però importants canvis del desenvolupament. En aquest treball es van aplicar mètodes de genètica quantitativa multivariada per simular per separat el procés de selecció de cadascun dels caràcters derivats dels humans.

Per realitzar aquest estudi es va mostrejar la col·lecció de cranis de Hallstatt. En total es van mesurar 390 cranis complets, dels quals 350 queien dintre de les genealogies reconstruïdes. En la mostra s'inclouen individus majoritàriament adults dels dos sexes. Per capturar la forma del crani es van registrar les coordenades tridimensionals de 29 punts craniomètrics repartits per tot el crani. Es van simular cinc escenaris de selecció per testar si la selecció va ser la responsable de produir els caràcters derivats dels humans moderns: la posició avançada del foramen magnum, l'encefalització anterior i posterior, la retracció facial i la flexió de la base del crani.

La primera simulació testava la hipòtesi del bipedisme, mitjançant la selecció d'una morfologia cranial que té el foramen magnum més avançat que la mitjana de la seva població. Els resultats indiquen que a la resposta a la selecció s'ha induït un canvi en el foramen magnum, que està més avançat i que podria ser interpretat com una resposta al bipedisme. Tanmateix, s'observen altres canvis que marquen una major flexió de la base del crani, una cara retreta i un major desenvolupament del neurocrani. Així, s'observa que només seleccionant una posició avançada del foramen magnum obtenim tota la resta de caràcters derivats dels humans moderns. La resposta total a la selecció es va descomposar vectorialment en una resposta directa, que va en la mateixa direcció que el gradient de selecció, i en una resposta correlada, que és perpendicular a la resposta directa. La integració fa que la resposta total es desviï de la direcció del gradient de selecció. Donat que la integració entre regions és molt forta, la resposta total s'assembla molt a la resposta correlada i inclou molts canvis, no només els seleccionats.

La segona simulació testava la flexió de la base del crani. Aquest gradient de selecció es va dissenyar fent que l'hormion, el punt que es situa en la sutura entre la base i la cara, es mogués cap a dalt. Novament, s'observa que a més a més de trobar modificacions a la base del crani i concretament a l'hormion, es produeixen canvis associats al bipedisme, a la retracció facial i a l'encefalització. En aquesta simulació, es detecta que l'efecte de la integració és encara més fort que en l'anterior. La resposta total està molt desviada cap a la resposta correlada perquè la influència de la covariació entre trets és molt intensa.

En la tercera simulació, es va testar la hipòtesi de selecció per la retracció facial, fent que tots els punts de la part anterior de la cara es moguessin cap enrera com un bloc facial. Un cop més, els resultats van mostrar que la resposta total a la selecció inclou canvis morfològics de tot el crani: a més a més de retracció facial, es produeixen canvis relacionats amb el bipedisme, la flexió de la base del crani i l'encefalització. La descomposició vectorial també mostra el fort efecte de la integració.

L'encefalització, que és el quart caràcter derivat dels humans moderns considerat en aquest estudi, es va simular en dos gradients de selecció: un que simulava l'expansió de la zona anterior del neurocrani (tots els punts que delimiten la volta cranial anterior es van moure cap a fora), i un altre que simulava l'expansió de la part posterior (de la mateixa manera, es van expandir els punts del neurocrani posterior). La resposta a la selecció d'ambdues simulacions va incloure tots els caràcters derivats dels humans moderns, indicant la resposta integrada del crani a la selecció.

El resultat global d'aquestes anàlisis de simulació és que no importa la morfologia local que es seleccioni, perquè la integració morfològica obliga a les estructures cranials a respondre globalment i de manera similar. Hem detectat que sota la morfologia craniofacial hi ha una quantitat considerable de variació genètica, però també hem trobat que aquesta variació està fortament subjecta a la integració. Per tant, el potencial evolutiu del crani es veu fortament limitat. A més, s'ha mostrat que hi ha certes morfologies que no poden evolucionar perquè aquestes direccions de canvi morfològic no tenen variació genètica.

En definitiva, tots els resultats destaquen la importància del sistema intern de desenvolupament en marcar els camins de l'evolució. Aquests resultats donen suport a les hipòtesis evo-devo, que consideren que pocs canvis en el desenvolupament poden produir una gran cascada de canvis morfològics. La reacció en cascada podria venir determinada pels forts patrons d'integració genètica que hem detectat en aquest estudi. Aquests resultats suggeririen una nova interpretació de l'evolució del crani humà, perquè a través de la integració morfològica l'evolució de qualsevol dels caràcters derivats dels humans moderns hauria afavorit l'evolució posterior dels altres.

Pervasive genetic integration directs the evolution of human skull shape

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ABSTRACT The evolution of anatomically modern humans was associated to a number of major skull morphological transformations, including a forward movement of the foramen magnum, a cranial vault enlargement, a facial retraction and a cranial base flexion. Whether these derived traits were due to independent selection events or whether they resulted from the inherent morphological integration in the skull has been controversial. To address this issue, we combined quantitative genetics and the methods of geometric morphometrics to analyze genetic variation in skull shape. We use a unique opportunity, the skulls in the charnel-house of Hallstatt (Austria), which provides a large collection of human skulls with associated genealogical data. Our results indicate substantial amounts of genetic variation for some shape features, but also strong constraints corresponding to other shape features that cannot evolve. The genetic architecture of skull shape is therefore subject to strong integration and evidence for genetic evolutionary constraints. Separate simulations of selection for each of the main derived characters of modern human skulls tended to produce similar outcomes with a joint response in all of these traits. These results suggest a reinterpretation of the selective scenario for human evolution because the origin of any one of the derived characters may have facilitated the evolution of the others.

KEYWORDS Human skull, integration, quantitative genetics, geometric morphometrics, evolvability.

INTRODUCTION

The evolution of the modern human skull encompassed the acquisition of several characters: first, there was a forward shift of the foramen magnum associated with the transition to bipedalism, whereas later evolution of modern humans included the development of a globular and expanded cranial vault, a retracted face and strong cranial base flexion (Aiello and Dean 1990, Lieberman et al. 2004). These morphological adjustments have long been considered as adaptive consequences of changes in locomotion, diet, language and cognitive abilities (Wolpoff 1999). Recent genetic analyses suggest that many parts of the human genome have experienced positive selection (Bustamante et al. 2005), for which the possible phenotypic targets include masticatory musculature (Stedman et al. 2004) and brain size (Evans et al. 2005, Mekel-Bobrov et al. 2005). However, there is yet no compelling evidence that the human skull has been shaped by selective forces. An alternative scenario proposes that few basic

developmental changes related to the size and shape of the brain and face may have triggered the whole suite of integrated cranial features of modern humans (Lieberman 1998, Lieberman et al. 2002): an expanded braincase, an orthognatic face and a flexed cranial base with a forward position of the foramen magnum (Aiello and Dean 1990, Lieberman et al. 2004).

We address this issue by combining the methods of geometric morphometrics (Bookstein 1991, Dryden and Mardia 1998) and evolutionary quantitative genetics (Lande 1979, Lynch and Walsh 1998) to examine genetic variation in modern human skull shape and to predict the responses to hypothetical selection for several shape features. Patterns of genetic integration can influence the extent to which selection for specific cranial features will produce responses that are localized or distributed throughout the skull. Strong genetic integration will mold the selection response to conform to its own inherent patterns and therefore may constitute a constraint (Gould 2002), biasing evolution towards certain directions of shape space (Klingenberg and Leamy 2001). In contrast, weak integration provides the flexibility of specific responses to different selection pressures, thus enabling phenotypic change across multiple directions. Genetic integration of cranial shape can be quantified with existing methodology (Klingenberg and Leamy 2001, Klingenberg and Monteiro 2005, Myers et al. 2006) provided there is a sample of skulls with associated genealogical information.

A unique opportunity to conduct this kind of study is the collection of skulls in the charnel house of Hallstatt (Austria). As a local tradition since the 18th century, skeletal remains from the Catholic churchyard were exhumed, skulls were cleaned, and the names of the individuals were written on them, so that parish records make it possible to reconstruct genealogical relationships. The use of this collection allows us to estimate directly the genetic covariance matrix for skull shape and provides a crucial advantage over previous studies of human evolution that have used phenotypic covariance structure as a proxy for genetic data (Ackermann and Cheverud 2004, Roseman 2004, Weaver et al. 2007).

MATERIALS AND METHODS

The sample includes 390 complete skulls, of which 350 individuals fall into extended, multigenerational genealogies. The skulls mainly correspond to adult individuals (91%) from both sexes (41% females; 59% males) born between 1707 and 1885. A small proportion of skulls were either visibly asymmetric (8.2%) or had slight dysmorphologies possibly related to craniosynostosis (3.8%). Strongly dysmorphic skulls were excluded from consideration.

Morphometric analysis

The coordinates of a set of 29 anatomical landmarks distributed over the left side of the entire skull (Fig. 1, Table 1) were recorded with a Microscribe 3D digitizer. Missing values (which accounted for 2.18% of the data) were replaced by multivariate regression or by coordinate reflection when the missing landmark had a symmetric counterpart. The measurement error was quantified for a subset of 91 individuals that were each digitized twice. Analysis of the amounts of shape variation indicated that the component of variation among

individuals exceeded the component of variation between replicate measurements by a factor of 11.5 and is therefore negligible (repeatability is 92%).

Geometric morphometric techniques were used to capture size and shape variation from the coordinate data (Bookstein 1991, Dryden and Mardia 1998, Rohlf 1999). After a generalized Procrustes analysis (Dryden and Mardia 1998), a principal component (PC) analysis was computed in order to reduce the dimensionality of the data, which was necessary due to computational limitations in the genetic analysis. The first 32 PCs accounted for 90% of shape variation and were used in subsequent analyses.

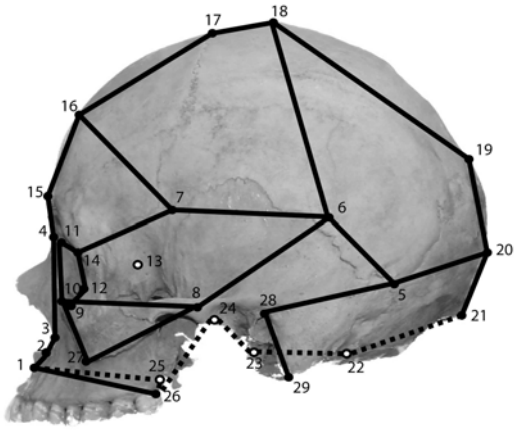

Fig. 1. Landmarks and wireframe used in this study superimposed on a lateral view of a human skull. Note that landmarks with empty circles and dashed lines are not visible from this view (refer to Table 1 for exact location).

Quantitative genetic analysis

Genealogies were compiled from complete church records from 1602 to 1900 and included 350 individuals with preserved skulls and 1089 additional individuals related to them. Restricted maximum likelihood methods (REML) were used to estimate the additive genetic and phenotypic covariance matrices with the software package VCE5 (Kovac et al. 2003). The model included centroid size (Dryden and Mardia 1998) as a covariate, and sex and deformation status (no deformation, asymmetric or dysmorphic) as fixed effects.

Analyses of constraints and response to selection are based on the multivariate breeder's equation $\Delta\mu = \mathbf{G}\mathbf{P}^{-1}s = \mathbf{G}\beta$, where $\Delta\mu$ is the response to selection (change in mean shape), **G** is the additive genetic covariance matrix, **P** is the phenotypic covariance matrix, s is the selection differential and β is the selection gradient (Lande 1979). The importance of genetic constraints was investigated by an analysis of the matrix GP^- (where P^- is the Moore-Penrose generalized inverse of the phenotypic covariance matrix P). This matrix can be interpreted as a multivariate analogue of heritability and indicates the maximal and minimal potential for response to selection of different shape features (Klingenberg and Leamy 2001, Klingenberg and Monteiro 2005). The eigenvectors of this matrix are shape variables that are uncorrelated to each other in terms of their inheritance, and they can therefore be used in univariate quantitative genetic analyses.

Standard errors for the eigenvalues of the **GP**$^-$ matrix were calculated by univariate genetic analyses with VCE5 (Kovac et al. 2003), using the same models as for the analyses of overall shape. New shape scores were derived by multiplying the Procrustes coordinates with each of the eigenvectors of the **GP**$^-$ matrix. For each of the resulting scores, an analysis with VCE5 was run and the standard error of the heritability was used as the standard error of the respective eigenvalues of the **GP**$^-$ matrix.

To test whether selection for specific features of the skull elicits a localized response of just the selected region or an integrated response of the entire skull, we designed five hypothetical selection gradients that represent separately the principal derived features of the modern human skull: bipedalism, base flexion, face retraction, anterior vault enlargement and posterior vault enlargement. To increase our ability to distinguish the effects of different selection regimes, we used highly localized selection gradients affecting only a minimal number of landmarks. This approach simulates what would happen if a particular selection regime were applied to the Hallstatt population; whereas this is not a direct evaluation of events in the human evolutionary lineage, it makes it possible to assess the selection response under the assumption of a conserved genetic and developmental basis for cranial shape.

Table 1. List of igitized landmarks.

Nº	Landmark	Definition
1	Subspinale	Deepest point on the premaxilla between the anterior nasal spine and prosthion
2	Nariale (left)	Most inferior point on the nasal aperture
3	Alare (left)	Most lateral point on the margin of the nasal aperture
4	Nasion	Midline point where the nasal bones and the frontal intersect
5	Asterion (left)	Point where the lamboidal, parietomastoid, and occipitomastoid sutures meet
6	Euryon (left)	Point of greatest breadth of the brain case perpendicular to the midsagittal plane
7	Pterion (left)	Point where the frontal, temporal, parietal, and sphenoid meet; or else the mid point
8	Zygion (left)	Most lateral point of the zygomatic arch
9	Orbitale (left)	Most inferior point on the orbital margin
10	Zygoorbitale (left)	Point of intersection between the orbital rim and the zygomaticomaxillary suture
11	Maxillofrontale (left)	Point where the lacrimal crest of the maxilla meets the frontomaxillary suture
12	Ectoconchion (left)	Most lateral point on the orbital margin
13	Optic canal (left)	Intersphenoidal foramen
14	Frontomalare orbitale (left)	Point where the frontomalar suture crosses the orbital rim
15	Glabella	Most anterior midline point on the frontal bone
16	Metopion	Midline point where the elevation above the chord from nasion to bregma is greatest
17	Bregma	Point where the coronal and sagittal sutures intersect
18	Vertex	Most superior point of the skull in the midsagittal plane
19	Lambda	Point of the intersection of the sagittal and lamboidal sutures
20	Opisthocranion	Most posterior point of the skull in the midsagittal plane
21	Inion	Midline point at the base of the occipital protuberance
22	Opisthion	Midline point at the posterior margin of the foramen magnum
23	Basion	Midline point on the anterior margin of the foramen magnum
24	Hormion	Midline most posterior point on the vomer
25	Post nasal spine	The posterior terminus of the palatal plane
26	Alveolar point (left)	Posterior limit of the maxillary alveolar arch at the pterygo-alveolar suture
27	Zygomaxillare (left)	Most inferior point on the zygomatic synchondrosis
28	Porion (left)	Uppermost point of the external auditory meatus
29	Mastoidale (left)	Most inferior point on the mastoid process

Hypothetical selection scenarios were analyzed as described elsewhere (Klingenberg and Leamy 2001) with minor modifications. We used an auxiliary shape variable proportional to the selection gradient; the use of such a variable makes it possible to analyze direct selection (Lande and Arnold 1983) but avoids the difficulties with visualizing selection gradients on shape (Klingenberg and Monteiro 2005). To ensure that selection gradients were proportional to shape differences, we projected the respective landmark shifts onto the tangent space to shape space (Dryden and Mardia 1998), which can result in smaller shifts of other landmarks. The magnitude of selection gradients was set arbitrarily to ten standard deviations of relative fitness per standard deviation of the respective shape variable. Although this corresponds to an unrealistically high intensity of selection, it makes the subtle changes of shape easily visible (alternatively, these graphs can be interpreted as magnified visualizations of the responses to selection of moderately high intensity). Finally, we decomposed the total response to selection obtained from the multivariate breeder's equation (Klingenberg and Leamy 2001) into a component of direct response in the direction of the selection gradient and a correlated response perpendicular to it.

RESULTS AND DISCUSSION

The eigenvalues of GP^- (Fig. 2), which are the heritabilities for the shape variables that correspond to the respective eigenvectors, showed a gradation from values near one to values very close to zero. The standard errors of these eigenvalues range from 0.0995 to 4.22×10^{-9} (smaller errors tend to be associated with near-zero eigenvalues), which indicates that they were estimated with reasonable precision. The higher eigenvalues correspond to shape features that would respond strongly to selection, whereas the eigenvalues near zero correspond to directions in which the response to selection would be negligible and therefore represent strong or even absolute genetic constraints (Lande 1979). Overall, this analysis indicates that the potential of a response is highly dependent on the direction in which selection acts. Moreover, the spread of these eigenvalues is evidence against the assumption of proportionality of the G and P matrices, which has been made in many studies making evolutionary inferences without actual genetic data (Ackermann and Cheverud 2004, Roseman 2004, Marroig and Cheverud 2004, González-José et al. 2007). Under this assumption, all eigenvalues should be equal up to sampling variation, but in our data they take nearly all the possible range. Overall, this analysis suggests that the potential for response to natural selection strongly depends on the particular shape changes under selection.

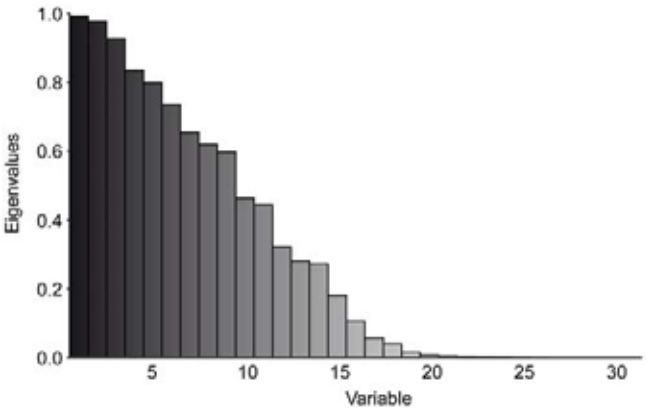

Fig. 2. Eigenvalues of the **GP^-** matrix.

Predicted response to selection

The first selection gradient concerns the forward shift of the foramen magnum associated with the origin of bipedalism (Aiello and Dean 1990): the two midline points of the foramen magnum (opisthion and basion) were moved forward (Fig. 3A). The total response to selection is global and encompasses the complete set of derived features of modern humans: cranial base flexion (more accentuated angle nasion-hormion-basion), facial retraction (posterior and inferior shift of the landmarks on the facial profile), and expansion of the entire cranial vault. This total response consists of a direct response that is localized to the landmarks of the foramen magnum and a correlated response affecting most of the landmarks throughout the skull. The magnitude of the correlated response (0.072) exceeds that of the direct response (0.048), indicating that the direction of response has been deflected substantially from the direction of the selection gradient by an angle of 56° (Table 2).

Table 2. Decomposition of the total response to selection into components of direct and correlated response for the five selection scenarios. The angle between the direct response and the total response is an indication of the deflection by genetic constraints. The numbers indicate the magnitude of the respective responses in units of Procrustes distance (for a strength of selection of 10 standard deviations of relative fitness per standard deviation of the respective shape variable).

	Response to selection			
	Total	**Direct**	**Correlated**	**Angle (°)**
Bipedalism	0.086	0.048	0.072	56.6
Base flexion	0.083	0.022	0.080	74.2
Face retraction	0.104	0.060	0.085	54.5
Anterior enlargement	0.074	0.028	0.068	67.6
Posterior enlargement	0.094	0.071	0.061	40.4

The next selection gradient concerns cranial base flexion (Fig. 3B). Traditionally, it is quantified by the angle nasion-sella-basion (Lieberman et al. 2000). Because our study only included external landmarks, we had to focus on effects of cranial base flexion on external parts of the skull and therefore considered hormion, which is the landmark closest to the 'hinge point' of flexion between the face and the cranial base. Increased flexion will sharpen the angle posterior nasal spine-hormion-basion, so we simulated it by an upward shift of hormion (Fig. 3B). In addition to cranial base flexion, the total response consists of a forward and upward shift of the foramen magnum, retraction of the face, and a general expansion of the braincase (including a widening of the posterior region).

To simulate facial retraction, the landmarks of the nasomaxillary complex were moved backwards into a more posterior position jointly as a facial block (Fig. 3C). Along with the facial retraction we selected for, the total response also included the shift of the foramen magnum associated to bipedalism, cranial base flexion, and an anterior expansion of the braincase.

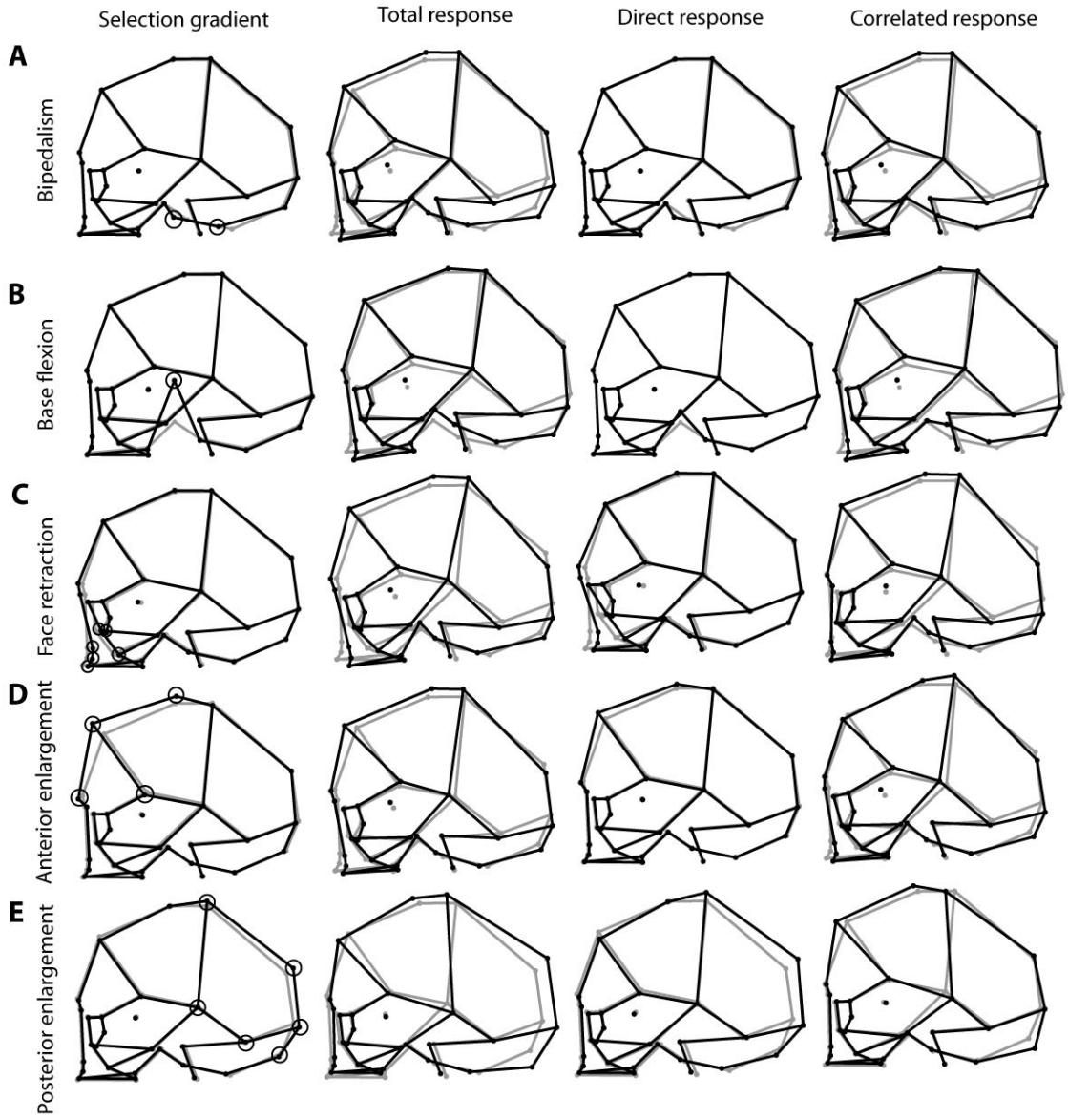

Fig. 3. Hypothetical selection on skull shape. (**A**). Shift of the foramen magnum associated with the acquisition of bipedalism. (**B**) Cranial base flexion. (**C**) Facial retraction. (**D**) Anterior enlargement of the cranial vault. (**E**) Posterior enlargement of the cranial vault. For each selection scenario, the changes from the grey to the black wireframes show the selection gradient (scaled to an arbitrary magnitude of shape change), the total response, the direct response and the correlated response. The grey wireframes show the overall mean shape configuration. Landmarks used to define the selection gradients are marked with circles.

To simulate selection for a larger and more globular cranial vault, we designed two different selection gradients, one for the anterior neurocranial region, and another one for the posterior region. Enlargement and globularity of the anterior neurocranial region was represented by anterior and upward shift of metopion, lateral shift of pterion and smaller shifts of glabella and bregma (Fig. 3D). The total response again includes the whole suite of changes. Enlargement of the posterior neurocranial region was depicted in a similar way by moving

vertex, lambda, opisthocranion, inion, euryon and asterion away from the centre of the skull (Fig. 3E). The total response is primarily an expansion of the entire cranial vault; other changes are difficult to interpret because a forward movement of the foramen magnum and a slight reduction of the face were already included in the selection gradient as a consequence of the projection to tangent space.

All these results are similar in that the direction of the evolutionary response is strongly deflected from the original direction of selection. This was apparent from the differences in shape features between selection gradients and the corresponding responses (Fig. 3) and directly from the angles between the selection gradients and the total responses to selection, which were greater than 45° for all but one of our simulations (Table 2). Moreover, localized selection for each of the derived characters of modern humans consistently yielded a global response that involved the whole set of characters (Fig. 3).

This strong integration suggests an explanation for the longstanding difficulties in finding independent cranial characters in studies of human phylogeny (Skelton and McHenry 1992, Strait et al. 1997, Lieberman et al. 2004). If genetic covariation is so strong that multiple traits consistently respond jointly to selection, as our data suggest, it is doubtful whether they can be regarded as independent phylogenetic characters. Even grouping cranial traits into anatomical or functional complexes (Skelton and McHenry 1992) may not fully overcome this problem because our results indicate that genetic integration is pervasive throughout the entire skull, suggesting that even the trait complexes are interdependent. Integration may also enhance the likelihood that independent evolutionary changes in different lineages produce similar shape changes, which would help to account for the homoplasy that has made it difficult to infer phylogenies from craniodental characters (Skelton and McHenry 1992, Strait et al. 1997).

Our analyses indicate that the developmental integration in the skull, as it is manifest in the structure of the genetic and phenotypic covariance matrices, has a major effect on the outcome of selection and thus suggest a reinterpretation of the adaptive context the evolution of the human skull. Genetic information suggests that many parts of the genome have experienced recent selection (Bustamante et al. 2005), for which the possible phenotypic targets include masticatory musculature (Stedman et al. 2004) and brain size (Evans et al. 2005, Mekel-Bobrov et al. 2005). Our results suggest that specific genetic changes or selection pressures such as these may drive continuing evolution in the shape of the entire skull. Likewise, it is conceivable that the derived characters of modern humans may not have arisen independently by adaptive evolution in response to a separate selection pressure each, but that the origin of one trait may have facilitated the evolution of the whole suite of characters.

If patterns of genetic covariation in the Hallstatt population can be taken as representative of more general conditions in human evolutionary history, our results have implications for palaeoanthropology. For instance, it is possible that selection associated with the origin of bipedalism might have facilitated the subsequent evolution of the globular shape, facial retraction and cranial base flexion of modern human skulls (Fig. 3A), and that the evolution of those three traits was mutually enhanced by the genetic integration among them. This hypothesis emphasizes the role of the developmental and genetic system in determining the

potential for evolutionary response. It therefore differs from previous hypotheses such as the postural hypothesis (Demes 1985), which have focused on functional changes and thus changes in the selective regime imposed by bipedalism. This perspective unites quantitative genetics and comparative developmental approaches (Lieberman et al. 2004) to provide a more comprehensive understanding of human evolution.

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6 *Results IV*

6.1 *Detecting natural selection in modern human skulls*

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Detectant l'acció de la selecció natural en cranis humans moderns

En evolució humana, es considera que la selecció natural ha sigut una de les principals forces determinants del canvi evolutiu i de l'adaptació de les espècies que formen el nostre llinatge. Tanmateix, hi ha poques evidències directes de l'acció de la selecció en els humans. Les evidències disponibles provenen majoritàriament dels estudis moleculars, que han detectat traces de selecció positiva en gens involucrats amb el desenvolupament d'un cervell més gran o amb l'adquisició del llenguatge. També hi ha evidències de selecció positiva en caràcters dentals en primats i en variables demogràfiques d'èxit reproductiu en humans. No obstant, no n'hi ha cap sobre l'acció de la selecció en el crani humà, tot i que s'assumeix implícitament que la morfologia cranial humana ha evolucionat com a resposta adaptativa a pressions de selecció. L'objectiu d'aquest estudi és aplicar mètodes geneticoquantitatius per obtenir estimes directes de selecció en humans, combinant dades morfològiques amb dades d'èxit reproductiu i de vida.

Aquest tipus d'anàlisi s'ha aplicat anteriorment per detectar l'acció de la selecció en trets morfològics, d'història de vida i del comportament. Els principals resultats obtinguts en poblacions salvatges d'animals i de plantes indiquen que la selecció direccional pot ser molt forta, mentre que la selecció estabilitzadora sol ser més dèbil i menys prevalent. Per explorar els efectes de la selecció, és important realitzar una aproximació multivariada a la forma que es vol estudiar perquè la selecció actua sobre fenotips complexos i no sobre caràcters individuals. A més, els fenotips complexos estan estructurats per forts patrons d'integració que produeixen respostes correlades a la selecció i desvien

les trajectòries evolutives de la seva direcció de canvi original. Combinant mètodes de morfometria geomètrica i de genètica quantitativa multivariada, es poden estimar els efectes directes i indirectes de la selecció.

En aquest treball hem aplicat aquesta metodologia per estimar si la selecció natural ha afavorit algun tipus particular de morfologia cranial en una població d'humans moderns (Hallstatt, Àustria). Els patrons generats s'han comparat amb els patrons de canvis seculars observats en la mateixa població en un període de 200 anys. Per això, hem analitzat una mostra de 377 cranis complets d'individus adults d'ambdós sexes, per als quals teníem informació genealògica i demogràfica associada. Per a cada individu, es van obtenir quatre mesures d'història de vida: fecunditat (nombre total de fills), èxit reproductiu (nombre de fills que han sobreviscut fins als 15 anys), lambda individual (λ, que és una mesura que té en compte tant els períodes reproductius com el nombre de fills que sobreviuen als 15 anys) i longevitat (anys complets viscuts). Per detectar la selecció en el crani, totes les anàlisis s'han realitzat a partir de regressions multivariades d'aquestes mesures demogràfiques sobre mesures de forma i de mida del crani. Per estimar els patrons de canvi secular es van realitzar regressions multivariades de la forma i la mida cranial sobre una variable temporal, l'any de naixement dels individus.

Els resultats indiquen una forta acció de la selecció direccional en la forma del crani i una acció més moderada de la selecció estabilitzadora en la mida del crani. Tanmateix, s'ha trobat que la resposta evolutiva esperada a aquests règims selectius no es corresponen amb els patrons evolutius observats a la població. Això indicaria que altres forces evolutives han contribuït en l'evolució de la morfologia cranial a la població de Hallstatt durant els segles XVIII i XIX. Aquestes forces poden ser la deriva gènica, el flux gènic i el mestissatge, o bé l'acció de la selecció natural en altres trets no mesurats però que estiguin indirectament relacionats amb la forma del crani.

Per les seves característiques, és poc probable que la deriva gènica hagi estat un dels factors evolutius més influents en l'evolució del crani d'aquesta població. De fet, s'ha detectat que tot i que Hallstatt era una població geogràficament aïllada, els seus nivells de consanguinitat eren especialment baixos en comparació amb altres poblacions europees de l'època. Això indica que es van produir importants moviments poblacionals i que la població de Hallstatt presentava una quantitat considerable de variació genètica. El flux gènic, per sí sol, tampoc hauria estat un factor suficient per provocar aquesta disrupció de patrons perquè s'ha comprovat que la majoria d'immigrants que arribaven a Hallstatt procedien de poblacions veïnes. Finalment, es considera

que altres factors importants responsables d'aquests patrons poden haver estat la correlació genètica entre caràcters i la covariació amb factors ambientals sobre la morfologia cranial.

En aquest treball hem aportat evidències directes de l'acció de la selecció natural tant en la forma com en la mida del crani humà, però hem detectat també que els efectes d'aquestes forces selectives han quedat difosos sota els efectes d'altres forces evolutives. Així es destaca la complexitat del crani humà i es posa de manifest que aquest respon a les forces evolutives a través de complexes xarxes genètiques i d'interaccions epigenètiques.

Detecting natural selection in modern human skulls

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ABSTRACT In human evolution, selection is implicitly assumed to be one of the main forces driving evolutionary change and adaptation, but direct evidence of this is rarely available, especially for morphological traits such as skull size and shape. The main goal of this study is to assess how life-history and fitness measures relate to skull morphological variation, which is the most direct evidence of natural selection. To do this, we used a unique large collection of modern human skulls with genealogical associated data from Hallstatt (Austria). We combined morphological and demographical data and applied multivariate quantitative genetic methods to estimate selection on a three dimensional reconstruction of the skull morphology. Then, we compared the obtained selected pattern with the secular changes observed in this population over a period of almost 200 years. Our results show that selection significantly acted on the evolutionary changes observed in the skull morphology of the Hallstatt's population during the 18th and 19th centuries. Indeed, we detect relatively strong directional selection on skull shape and weak stabilizing selection on skull size. However, we find that the expected responses to these selection regimes do not correspond to the actual evolutionary patterns of skull morphology. Therefore, these results emphasize the major role of selective forces both in human skull size and shape, but suggests that microevolutionary factors other than natural selection are also contributing to the evolution of the skull and are obscuring the effects of natural selection.

KEYWORDS Human skull, natural selection, fitness, quantitative genetics, geometric morphometrics.

INTRODUCTION

The role of natural selection in shaping the evolution of phenotypes is a major goal in evolutionary biology. Selection is assumed to be one of the main evolutionary forces driving human evolution (Wolpoff 1999), although direct and consistent estimates of the action of selection on cranial morphology is rarely available.

Most evidence of selection on humans comes from molecular and genomic studies (Bustamante et al. 2005). For instance, it has been reported that genes involved in reducing jaw-muscle size (Stedman et al. 2004) and enhancing language acquisition (Enard et al. 2002) as well as brain development (Evans et al. 2005, Mekel-Bobrov et al. 2005) have been a recent target of selection in the human lineage. A study of life-history traits in a human preindustrial population has also suggested that female fitness measures may have responded to optimizing selection, although there is indication that strong genetic constraints may have reduced their

evolutionary potential (Pettay et al. 2005). In primates, there is also molecular, morphological and developmental evidence for positive selection on dental traits. It has been reported that a gene involved in tooth development is strongly selected in primates (Pereira et al. 2006), and that molar size is significantly correlated with lifetime fitness in howler monkeys (DeGusta et al. 2003). These results are consistent with studies of life-history traits and dental growth that have correlated the evolution of developmental changes with hominid speciation (Ramírez-Rozzi and Bermúdez de Castro 2004).

Concerning the evolution of craniofacial form in humans, some studies have suggested that rather than adaptation by natural selection, genetic drift may have played a major role (Dean et al. 1998, Ackermann and Cheverud 2004, Roseman 2004, Roseman and Weaver 2004, Harvati and Weaver 2006b, Weaver et al. 2007). According to these, the emergence of modern cranial features and thus the divergence between Neanderthals and humans are by-products of population isolation and random genetic drift (Dean et al. 1998, Harvati and Weaver 2006b, Weaver et al. 2007). Likewise, several studies have suggested that geographical distance would explain the patterns of morphological variation within modern human populations (Roseman and Weaver 2004, Harvati and Weaver 2006a), with the exception of some nasal adaptations to extreme cold environments (Roseman 2004, Harvati and Weaver 2006a).

These retrospective analyses of selection on humans, however, were performed without previous knowledge of the genetic architecture of skull morphology, assuming that phenotypic variation reflects the underlying genetic variation. As the evolutionary response to selection does depend on inheritance (Lande and Arnold 1983), an entirely quantitative genetic approach to estimate selection on human's skull morphology was thus lacking. The main goal of this study is to obtain direct estimates of selection in humans. To our knowledge, this is the first attempt to demonstrate how fitness relates to the phenotypic and genetic variation of skull morphology, which is the most consistent evidence of natural selection.

Selection on quantitative traits

There is a large literature on the quantitative genetic basis of morphological, life-history and behavioural traits (Endler 1986, Kingsolver et al. 2001). Overall, these studies evidenced that in natural populations directional selection can be quite strong (Endler 1986), whereas stabilizing selection is generally weaker and less prevalent (Kingsolver et al. 2001).

Selection is likely to act on entire phenotypes rather than on individual traits in isolation (Lande and Arnold 1983) and adaptation is an inherently multivariate process (Blows 2007). Therefore, it is important to focus on multiple rather than on single traits when exploring selection on quantitative traits (Endler 1986, Kingsolver et al. 2001, Hoekstra et al. 2001). Strong covariation between traits is pervasive (Lande and Arnold 1983) and therefore correlated selection should be considered as one of the main forces driving the evolution of biological complexes, such as morphological ones. Correlated responses primarily arise as a consequence of pleiotropy (Falconer and MacKay 1996) and their effect on selection is to deviate the evolutionary trajectories from the originally selected direction (Blows and Hoffmann 2005).

Multivariate regression analyses allow the estimation of both the direct and indirect effects of selection on correlated quantitative traits (Lande and Arnold 1983). However, few studies to date have attempted to estimate selection on more than two traits (Blows 2007). Several reviews have suggested that published results about natural selection on quantitative traits could be misleading, since they might be biased upwards because of insufficient statistical power to detect selection (Hersch and Phillips 2004) or downwards because they ignored the complex correlation patterns between traits (Blows 2007). Moreover, shape is usually described as a suite of linear measurements and most studies of selection on shape do not account for the three-dimensional nature of complex shapes. This issue can be overcome by combining the multivariate methods of quantitative genetics and geometric morphometrics (Klingenberg and Monteiro 2005). However, only one study investigating natural selection on flower shape (Gómez et al. 2006) has applied this approach and has tested evolutionary hypotheses of shape in a true multivariate fashion.

These methods provide a means to detect the action of selection and to distinguish between the different selective forces that may have caused an evolutionary change. According to quantitative genetics, directional selection should induce a permanent change in the distribution of a heritable trait (Freeman and Herron 2004), shifting the mean trait value towards one end of the distribution. Morphologies under strong and consistent directional selection are expected to evolve towards a selected direction of change, the morphology representing the individuals with higher fitness. Phenotypically, these genetic changes may be detected as changes in the population mean between generations. If evolution is due to directional selection, the evolutionary patterns should correspond with the morphological changes observed in a population over time. In other words, directional selection and secular trends should share the same direction of morphological change. Under stabilizing selection, individuals with intermediate characteristics are favoured by decreasing genetic variation and stabilizing on a mean trait value (Freeman and Herron 2004). Morphologies under the effect of stabilizing selection should not change over time since it is expected that they have already reached their optimal phenotype. Finally, under disruptive selection individuals with extreme values at both ends of the distribution are selected for. In some cases, this type of selection may be responsible for speciation (i.e. if individuals from each extreme become geographically isolated).

Here, we combined morphological and demographical data in order to estimate the actual selection on skull morphology in a human population from Hallstatt (Austria). Furthermore, we compared the putative selected morphologies with the secular changes observed in this population over a period of almost 200 years. To do this, we use a unique large collection of skulls with genealogical associated data and apply the multivariate quantitative genetic methods (Lande and Arnold 1983, Phillips and Arnold 1989, Blows and Brooks 2003) to assess selection on a three dimensional reconstruction of the skull morphology. This will allow us to identify the targets of selection on both skull size and shape, as well as to detect and quantify the intensity of the selective forces may have been driving skull evolution in this population.

MATERIALS AND METHODS

To test the action of natural selection on human skull morphology, we measured the skulls of the Hallstatt collection and reconstructed the genealogies of the corresponding population. Both procedures are fully described in previous works (Chapters 3, 4, 5 and Appendix) Here, we focus on the estimation of the fitness measures and the specific morphometric analyses developed to assess the effect of selection on skull size and shape on those individuals showing the highest fitness.

Fitness measures

The genealogies were reconstructed from the complete parish records of births, marriages and deaths from the period 1602-1900, which included 18,134 individuals. To estimate fitness measures, we only included those individuals with complete individual life histories, who married at least once and who survived to adulthood and reproduction (N=2,549).

We estimated several life-history and fitness measures, such as fertility (considered as the total number of offspring), lifetime reproductive success (LRS) (estimated as the number of children raised to adulthood), longevity (considered as the age at death) and individual lambda (λ). This was computed as the dominant eigenvalue (λ) of the population projection matrix (Leslie matrix) derived from the times of births of offspring and death of each individual (McGraw and Caswell 1996). The projection matrices were computed with a time resolution of one year, and the λ values therefore indicate the intrinsic rate of increase per year, whereas other studies used different intervals, e.g. 5 years (Käär and Jokela 1998). To obtain a biologically more meaningful measure of fitness, we calculated the rate of increase per generation by raising λ to the 29th power (29 years is the generation time in this population, computed as the average age at birth of the first child). In the estimation of both λ and LRS measures, only offspring surviving to the age of 15 years were considered. For each of these fitness measures, relative fitness was computed by dividing the individual fitness values by the respective mean value.

Morphometric analyses

We analyzed a sample of 377 complete adult skulls from both sexes (155 females, 222 males). A set of 29 landmarks representing a global skull shape was registered with a Microscribe digitizer. Repeated measures on 91 skulls showed that repeatability is higher than 90%. The Hallstatt's skull collection covers a temporal span of nearly 200 years, although most of the skulls derive from the 19th century. The number of individuals with complete fitness and morphological information is 331 for the estimation of individual lambda (λ), 352 for fertility and LRS and 376 for longevity.

Shape information was captured from the coordinate data by a generalized Procrustes fit (Dryden and Mardia 1998). Size information was extracted computing centroid size, which is the squared root of the summed distances between the centroid and each landmark coordinate (Dryden and Mardia 1998). All morphometric analyses were performed with MorphoJ software

(C.P. Klingenberg 2007, unpublished). The analyses of selection on shape were conducted by multiple regression of relative fitness on shape measures (Lande and Arnold 1983, Phillips and Arnold 1989, Gómez et al. 2006). To reduce dimensionality of the data, we included only the first 15 principal components (PCs) of shape in the regression analysis, accounting for 71% of the total shape variation. The analyses of selection on size were conducted by multiple regression of relative fitness on centroid size (Lande and Arnold 1983, Phillips and Arnold 1989, Gómez et al. 2006). To avoid potential biases due to sexual dimorphism, sex was always included as a covariate in the regression analyses.

The regression model for shape included the scores of 15 PCs and their squares and pairwise cross-products. The selection gradient (linear selection) was computed from a multiple regression of relative fitness on the PC scores. Nonlinear selection was estimated from quadratic regression of relative fitness on the PC scores and their squares and cross-products. The matrix of nonlinear selection (gamma) was assembled from the coefficients of the second-order terms (Lande and Arnold 1983, Phillips and Arnold 1989). Statistical significance of selection was assessed by permutation tests, using as the test statistic the amount of fitness explained by the regression. Separate permutation tests were run for total selection (using the full regression model) as well as for the linear (only PC scores) and nonlinear components of selection (difference between full and linear models).

The strength of selection was computed as a standardized selection coefficient. For linear selection, this was done by scaling the magnitude of the selection gradient by the phenotypic standard deviation of a shape variable corresponding to the direction of the selection gradient. The strength of nonlinear selection was computed by scaling the eigenvalues of the gamma matrix (Phillips and Arnold 1989) by the phenotypic standard deviation of a shape variable defined by the corresponding eigenvector, and is equivalent to a standardized selection coefficient for that shape variable (Blows and Brooks 2003). This value is useful for population comparisons because it measures the force of selection in units of phenotypic standard deviation (Arnold and Wade 1984).

To assess if the selected morphologies (that is, those associated with the highest fitness scores) actually fitted the evolutionary trends of the Hallstatt sample (that is, the observed changes in phenotype over time), we estimated the predicted response of selection to the selected morphologies and then we compared them to secular trends. The predicted response to selection was computed using the multivariate breeders' equation (Lande 1979), as extended for geometric morphometrics (Klingenberg and Leamy 2001).

To perform these calculations we used the genetic and the phenotypic variance-covariance matrices obtained in previous works with the same population (Chapter 5) and a detailed description of the methodology can be found there. Secular trends were assessed as the regression of individual's skull shape and size against their year of birth: a pooled within-group regression was performed considering sex as the grouping variable, and the statistical significance was assessed by a permutation test with 10,000 randomization rounds.

RESULTS

Selection on shape

Actual selection. Significant directional selection on skull shape was consistently found by each of the multivariate regressions of relative fitness on shape (Table 1). This suggests that individuals with higher fitness show particular skull morphologies representing one end of the shape distribution. However, no significant stabilizing or disruptive selection on skull shape was detected, since any of the permutation tests for nonlinear selection was statistically significant (Table 1).

Table 1. Selection on skull shape. Analysis based on multivariate regressions of shape variables (15 PCs) on several fitness measures. The null hypothesis expects no selection at all. Permutation tests were performed to detect total selection, linear selection (directional selection) and non linear selection (stabilizing or disruptive selection). Significant p-values are bolded and indicate that there is selection in skull shape. The strength of selection is given for both linear and nonlinear selection gradients. For nonlinear selection, the maximum and the minimum eigenvalues of the gamma matrix along with their associated standard errors are provided.

	Total	**Linear Selection**		**Non Linear Selection**				
	p	*p*	*Strength*	*p*	*Max eigenvalue ± SE*	*Strength*	*Min eigenvalue ± SE*	*Strength*
Lambda	**0.2651**	**0.0456**	0.2843	0.4947	1706.81±486.31	0.2266	-2288.43±626.62	-0.2707
Fertility	**0.0301**	**0.0018**	0.3522	0.2342	1941.28±520.28	0.2476	-1901.78±610.22	-0.2163
LRS	**0.3791**	**0.0082**	0.3398	0.6336	1932.68±479.84	0.2692	-1831.09±581.26	-0.2390
Longevity	**0.0008**	**0.0000**	0.1692	0.3821	636.16±163.04	0.0757	-450.07±162.48	-0.0649

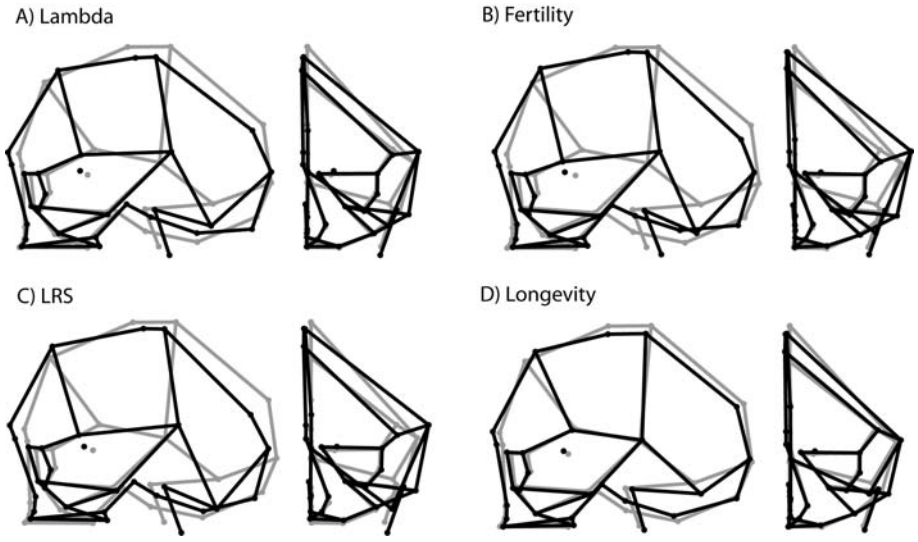

Fig. 1. Selection on skull shape. Multivariate regressions of shape on measures of reproductive fitness such as (A) individual lambda, (B) fertility, (C) lifetime reproductive success (LRS), as well as on one measure of lifetime success such as (D) longevity. Significant linear selection gradients (Table 1) are visualized as changes in landmarks positions from the mean shape configuration (grey wireframe) to the skull shape of selected individuals showing the highest fitness scores (black wireframe). Both lateral and frontal views of the hemicranial configuration are provided.

The morphological changes associated to the linear selection gradients obtained after each fitness measure are displayed in Fig. 1. These include major changes of the cranial vault and the cranial base, as well as minor changes of the face. The favoured shape is associated with a forward shift of the anterior cranial vault (which expands anteriorly and mediolaterally), a downward movement of the posterior cranial vault, a reduction of the cranial base and a slight retraction of the face. This general pattern is particularly pronounced in the regressions of reproductive fitness on skull shape (Fig. 1A-C), whereas it is weaker for the regression on longevity (Fig. 1D). This is no surprising since longevity may display more complex patterns of evolution and might be subject to more environmental influences. The strength of directional selection on skull shape is quite high. The average strength is 0.29, which is considerably higher than the median value for standardized selection gradients of 0.16 reported in a wide range studies of natural selection in quantitative traits (Kingsolver et al. 2001).

Response to selection. For each linear selection gradient, we estimated the total response to selection using the multivariate breeder's equation (Fig. 2). These analyses showed that the total response to selection involved similar changes as those depicted by linear selection gradients, including a reduction of the posterior cranial vault as well as of the cranial base, an expansion of the anterior cranial vault and a slight retraction of the face. The selection gradients mainly involved a dorso-ventral compression of the skull, which is weak in the total predicted response to selection because indirect selection through correlated characters tended to maintain the globularity of the cranial vault (Table 2).

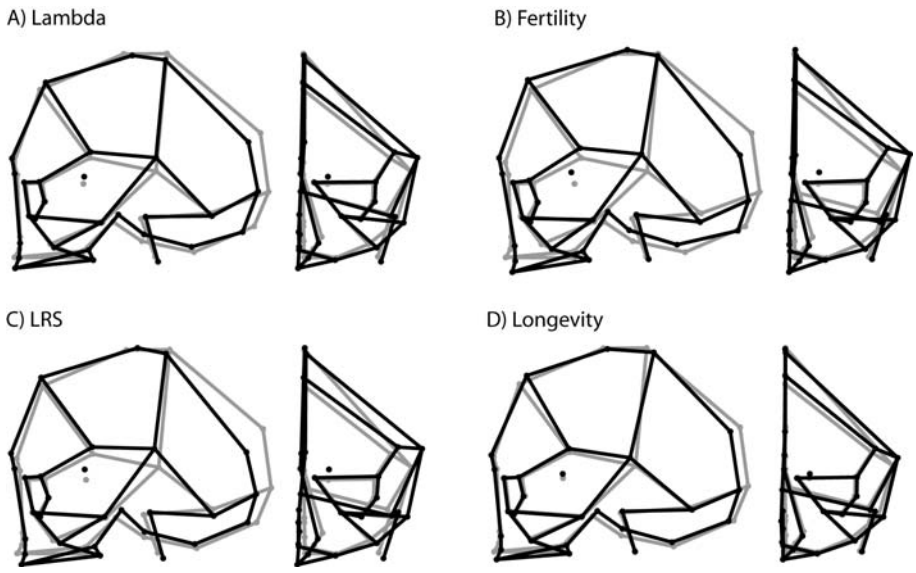

Fig. 2.. Predicted response to selection. Each selected shape, that is, the significant linear selection gradients obtained from the regressions of skull shape and fitness, was used as a selection gradient in the multivariate breeder's equation to estimate the total response to selection. The responses are scaled by a factor of 50 and are displayed as shape changes from the grey (mean shape configuration) to the black wireframe.

Table 2. Decomposition of the total response to selection into components of direct and correlated response for the four fitness measures. The angle between the direct response and the total response is an indication of the deflection by genetic constraints. The numbers indicate the magnitude of the respective responses in units of Procrustes distance.

	Response to selection			
	Total	**Direct**	**Correlated**	**Angle (°)**
Lambda	0.00157	0.00088	0.00129	55.7
Fertility	0.00227	0.00121	0.00192	57.8
LRS	0.00223	0.00133	0.00179	53.4
Longevity	0.00119	0.00068	0.00098	55.2

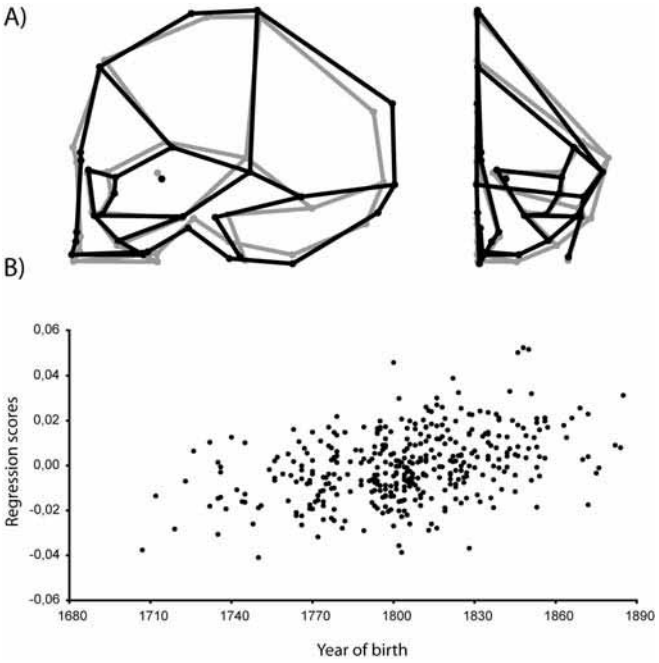

Fig 3. Secular trends on skull shape: multivariate regression of shape on time. (A) Patterns of skull shape are visualized as landmark displacements from the consensus morphology (grey wireframe) to the morphology associated to higher regression scores. (B) Shape regression scores plotted against year of birth.

Secular trends. The regression analysis of shape on time was statistically significant (p<0.0), and showed that approximately 1% of the morphological variation can be predicted from the year of birth. This indicates that the overall shape of the skull has actually changed through the temporal span considered in this study (from 1707 to 1885). The secular trend consists of an expansion of the posterior cranial vault, a deflection of the cranial base and, to a lesser extent, a projection of the face (Fig.3). In comparison to the morphological patterns displayed by the expected responses to selection, this shape change is in an opposite direction to the one predicted by the previous selection analyses (Figs. 1 and 2).

Selection on size

Actual selection. To detect selection on skull size, we separately computed regressions of fitness measures against centroid size. In contrast to the results obtained with shape, we detected significant total and nonlinear selection for skull size (Table 3). Negative non linear selection differentials indicated that stabilizing selection is operating on skull size. Therefore, skull size is not expected to change over time since stabilizing selection would favour individuals with an averaged-sized skull. Only when the fitness estimate was longevity, we also found significant directional selection on skull size (Table 3). The strength of stabilizing selection on size is 0.09 and is thus not as strong as the strength of directional selection on shape.

Secular trends. The regression of centroid size on time (year of birth) yielded statistical significant results (p=0.003). The percentage of predicted size variation was low (2.3%), but the regression analysis shows that there is a weak but significant tendency to reduce skull size over

time (regression coefficient=-0.07). Again, this result does not confirm the expectation of no change on skull size predicted by the selection analyses.

Table 3. Selection on skull size. Analysis based on multivariate regression of centroid size on several fitness measures.

	Total	**Linear**		**Non Linear**		
	p	*p*	*Strength*	*p*	*Gamma ± SE*	*Strength*
Lambda	**0.0126**	0.2836	0.0611	**0.0068**	-0.000573±0.000210	-0.1154
Fertility	**0.0363**	0.3659	0.0537	**0.0169**	-0.000525±0.000216	-0.1058
LRS	**0.0318**	0.3470	0.0601	**0.0142**	-0.000548±0.000224	-0.1103
Longevity	**0.0000**	0.0000	0.0855	**0.0015**	-0.000239±0.000074	-0.0457

DISCUSSION

Here we report that selection significantly operated on the skull evolutionary changes observed in Hallstatt during the 18th and the 19th centuries. Indeed, we detect strong directional selection on skull shape (Table 1) and weak stabilizing selection on skull size (Table 3). However, we find that the expected responses to these selection regimes do not correspond to the actual evolutionary patterns of skull morphology (Fig. 3). This suggests that microevolutionary factors other than natural selection are also contributing to the evolution of skull morphology in the Hallstatt's population.

A similar result was found in a quantitative genetic analysis of antler size in red deer (Kruuk et al. 2002), in which directional selection for increased size was detected but the actual evolutionary shift was a decline in antler size over the analyzed period. Further analyses revealed that estimates of selection were inflated by an environmental covariance between antler size and reproductive success, which is strongly dependent on the nutritional state. This suggested that no evolution occurred because antler size is indirectly correlated with fitness, leading to an overestimate of the expected response to selection (Kruuk et al. 2002).

As shown here, unraveling the evolutionary patterns of complex structures, such as the human skull, is complicated because several microevolutionary forces might be acting simultaneously and the structure might respond to each of them following different directions of shape change. In order to depict a more complete picture of the evolution of complex structures, it is therefore useful to estimate both the actual selection patterns and the secular trends as complementary approaches. Secular trends describe the phenotypic changes occurred in successive generations and shows how a morphology has evolved over a period of time. These changes may be due to a wide range of factors and are a poor indicator of the actual forces of selection (Lande and Arnold 1983). However, the analyses of selection by its own only account for selective forces and disregard other microevolutionary forces that may play a role in the structure's evolution.

In the Hallstatt population, other factors such as genetic drift, gene flow, or natural selection acting on traits indirectly related to cranial traits may have also contributed to the evolution of the skull morphology. Hereby we discuss each of these factors. Genetic drift causes

random evolution of traits regardless of their fitness and manifests strongly in small populations (Falconer and MacKay 1996). In such cases, the influence of stochastic variation is higher and alleles can be fixed or removed from the gene pool just by chance. Moreover, in small populations genetic variation is also reduced by inbreeding, which tends to increase homozygosity within individuals over time (Falconer and MacKay 1996). Geographically, Hallstatt is a semi-isolated village located at the far end of a lake and surrounded by mountains and glaciers. During the 18th and the 19th centuries, the village could only be reached by boat or by footpaths across the mountains and the population was rather small: the number of inhabitants fluctuated between 1,500 and 2,000 (Kurz 2002). However, it is unlikely that genetic drift was the main factor responsible for the reported evolutionary patterns since low levels of endogamy and consanguinity have been found in this population for the analyzed period (Esparza, pers. comm.). Consanguinity levels were particularly low after 1850, when the first road and railway were constructed and the population was opened to new comers. The average level of consanguinity in Hallstatt is 10 times lower than other European populations from the same period (Boëtsch et al. 2002). This suggests that Hallstatt underwent important populational movements throughout its history and that this population has considerable levels of genetic diversity. Moreover, genetic drift has no preferred direction of change and we did find in both analyses of selection and secular trends a significant direction of change in skull shape and size (Tables 1 and 3).

Substantial amounts of gene flow and admixture is therefore a more plausible explanation for the observed evolutionary patterns. Historically, the salt mine industry attracted many people to Hallstatt, who established there to work on the mines. The population definitely opened to migration exchange at the end of the 19th century, after the Industrial Revolution, with increased mobility and improved means of transport (Urstöger 1984). Within skull genealogies, around 20% of the population was from immigrant origin (Sjøvold 1995), but it has been reported that most immigrants came from the nearest villages to Hallstatt, such as Goisern, Ischl, Ausee and Gosau (Sjøvold 1986). Despite substantial gene flow among these populations, admixture would not be a sufficient explanation because the genetic background of these populations is expected to be fairly homogeneous. It is unlikely that populations from the surroundings areas were subject to opposing selection pressures.

Finally, the lack of correspondence between the responses to selection and secular trends could also be produced by constraints imposed by genetic correlation among traits or because of environmental covariance. The response to selection would then have been masked by opposing changes in environmental conditions. In Hallstatt, a mining village surrounded by high mountains, environmental conditions may have also played a role in this process. Factors not measured here such as the nutritional state or adaptations to the lack of sun or to iron-deficiency might be regarded as potential explanatory causes.

Here, we have provided direct evidence of the evolutionary forces that have contributed to the evolution of skull morphology of a human population. Our results emphasize the major role of selective forces both in skull size and shape. Stabilizing selection on skull size is weak but significant, whereas directional selection on skull shape is quite strong. In fact, directional selection on skull shape is almost twice as much higher as the overall median strength value

reported by Kingsolver et al. (2001), whereas stabilizing selection on skull size is at the same level as reported in other studies (Kingsolver et al. 2001). This supports previous findings suggesting that directional selection is stronger and may be more common than stabilizing selection (Merilä et al. 2001a, Merilä et al. 2001b, Kruuk et al. 2002). Thus, selective forces shouldn't be disregarded in studies of human evolution (Roseman and Weaver 2004, Weaver et al. 2007). Nevertheless, the effects of these selective regimes are obscured by the effects of other microevolutionary forces. Whether one force or another is predominant can differ between populations and between periods of time, depending on the historical and biological backgrounds of each population. The human skull is a complex structure, which is under a wide range of evolutionary forces. This study highlights that the skull responds to these evolutionary forces through complex and widespread networks of genetic and epigenetic interactions (Lieberman et al. 2004, Hallgrímsson et al. 2007).

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7 *Results & Discussion*

Results & Discussion

Substantial amounts of genetic variation underlying both size and shape and pervasive genetic integration are the two main aspects that characterize the genetic architecture of the human skull (Chapters 3 to 6).

The main developmental regions of the human skull (namely the face, the neurocranium and the basicranium) have similar amounts of genetic variation. This result was consistently obtained from two independent approaches, the univariate (Chapter 3) and the multivariate approach (Chapter 4). This result confirms previous evidence indicating that within the primate skull, facial, neurocranial and basicranial dimensions show similar levels of heritability (Cheverud and Buikstra 1982, Sjøvold 1984, Cheverud 1996b). It has been suggested that the face is more prone to plastic changes because its growth is more extended into the postnatal period (Kohn 1991, Strand Vidarsdóttir et al. 2002, Bastir and Rosas 2004b). The results herein indicate that the contribution of environmental variation is not significantly greater in facial than in neurocranial or basicranial dimensions (Chapters 3 and 4), at least when the whole regions are considered. A closer look to the phenotypic variation patterns reveals that some specific dimensions have non significant heritability estimations and thus are completely subject to environmental variation. However, these dimensions are not restricted to the face, but are also found at the neurocranium and the basicranium (Chapter 3). Therefore, more complex patterns need to be advocated to explain the plasticity of the human skull.

The multivariate approach to craniofacial variation (Chapter 4) reveals more complex patters of variation than the univariate approach (Chapter 3). It shows that both at the genetic and the phenotypic levels, variation is not concentrated within specific developmental or functional regions (Chapter 4). Variation is more widespread throughout the skull and involves several regions. Although this methodology has been rarely applied, it has been proved

both theoretically and experimentally that it depicts a more insightful picture of the patterns of evolvability and integration of complex phenotypes (Klingenberg and Leamy 2001, Myers et al. 2006). Moreover, it provides an experimental approach, not grounded in previous assumptions of modularity, to search integration patterns and modules (Chapter 4).

Despite the high levels of contribution of the genetic variation to the cranial phenotype, the evolvability of the skull is restricted by morphological integration (Chapters 3 to 6). For instance, the evolvability of a trait depends more on its variability (the capacity of traits to vary) than on the standing level of variation (Wagner and Altenberg 1996). Skull variability is strongly dependent on morphological integration. Each of the analyses performed in this thesis have pointed out, in a way or another, that integration patterns are strong (Chapters 3 and 4) and that the ability to respond to selection is constrained by correlated variation (Chapters 5 and 6).

The results obtained in this thesis support previous evidence indicating that the skull is a highly and hierarchically integrated structure, with complex covariation patterns both within and among regions (Cheverud 1982, 1995, Lieberman et al. 2002, Bookstein et al. 2003, Ackerman and Cheverud 2004a, Bastir and Rosas 2004b, González-José et al. 2004, Hallgrímsson et al. 2007). The evidence provided by these studies was obtained from the analysis of phenotypic covariation patterns. The analyses herein (Chapters 3, 4 and 5) shows that at the genetic level, covariation patterns are even more complex and more structured. Moreover, they indicate that the genetic and phenotypic covariance matrices are similar, but neither identical (Chapter 3) nor proportional (Chapter 5). In studies applying population genetic models to investigate the history and structure of human populations (Steadman 2001, González-José et al. 2003, Ackermann and Cheverud 2004b, Roseman 2004, Roseman and Weaver 2004, González-José et al. 2005a, Stojanowski 2005, Harvati and Weaver 2006a, Martínez-Abadías et al. 2006, Stojanowski and Schillaci 2006, González-José et al. 2007) it is a usual practice to assume that the phenotypic covariance matrix is proportional to the genetic covariance matrix (Cheverud 1988) because genetic data is rarely available. Results from Chapter 5 show that this assumption is not straightforward, at least in the Hallstatt sample. The genetic covariance matrix is not full rank, indicating that there are genetic constraints (shape features that can not evolve because they do not have sufficient genetic variation) and shows that genetic variation is more concentrated than phenotypic variation (Chapters 4 and 5).

Due to pervasive genetic integration, there are no clear boundaries among regions and integration patterns do no reflect true functional or developmental modules (Chapters 3 and 4). According to Klingenberg (2004) 'modules are units that are made internally coherent by manifold interactions of their parts, but are relatively autonomous from other such units with which they are connected by fewer or weaker interactions'. The results obtained in the present thesis indicate that the face, the neurocranium and the basicranium are highly integrated. In some aspects of genetic and phenotypic variation, the face shows a slight relative independence and some modularity within minor functional regions (Chapters 3 and 4), but the general pattern is dominated by integration. Integration is in fact detected at different scales. When the whole skull is considered, the face, the neurocranium and the basicranium tend to behave as a unit, showing the clearest correspondence between genetic and phenotypic covariance patterns (Chapter 4). At a smaller scale, integration is observed among certain functional regions (e.g. the orbits and zygomatic arches), but not in others (e.g. anterior and posterior parts of the neurocranium).

A key region responsible for these general integration patterns is the cranial base (Chapters 3 and 4), because it acts as a hinge structure between the face and the neurocranium (Lieberman et al. 2000a, 2000b). As previously found in the mice cranium (Hallgrímsson et al. 2007), the patterns of integration in the human skull are dominated by strong genetic covariation among the breadth measures of the basicranium, the neurocranium and the face (Chapter 3). Conversely, traditional hypothesis of integration suggesting a distinction among brachycephalic and dolicocephalic skulls (Enlow and Hans 1996) are not supported by the phenotypic and genetic correlation patterns found in the Hallstatt's sample (Chapter 3). Under this hypothesis, maximum cranial breadth should be positively correlated with facial breadth and negatively correlated with facial height and neurocranial length and height. However, the analyses herein only found a significant correlation between neurocranial and facial breadth (Chapter 3). This supports previous findings (Lieberman et al. 2000a, Bastir and Rosas 2004b) and suggests that this kind of terminology, which is still extensively used (Goodman 1995, Goodman 1997, Gonzalez et al. 2003, Gravlee et al. 2003, Fiedel 2004), does not reflect the genetic architecture of the human skull.

Another consequence of morphological integration is that it strongly influences the evolutionary response of the skull to selection (Chapters 5 and 6). Results from the simulation analysis of the evolution of the main derived characters of the skull of modern humans (Chapter 5) show that independently

of which morphology is selected, the skull always responds in a global way. This results from pervasive genetic integration, which produces jointly the whole suite of derived characters. Therefore, the forward shift of the foramen magnum, the development of a globular and expanded cranial vault, a retracted face and a strong cranial base flexion did not evolve as a response to independent selective scenarios. It is more likely that the evolution of each of the derived characters enhanced the evolution of the others, suggesting a re-interpretation of the selective context of human evolution (Chapter 5).

This hypothesis emphasizes the role of the developmental and genetic system in determining skull evolvability, as opposed to other hypothesis that focus on functional changes and external pressures (Weidenreich 1924, Dart 1925, Schultz 1942, DuBrul 1950, Schultz 1955, DuBrul and Laskin 1961, Demes 1985). For instance, the morphological changes associated with the evolution of bipedalism may have enhanced the evolution of a more globular and expanded neurocranial shape. Afterwards, this feature could have been favoured by selection for bigger and more complex brains, as molecular evidence suggests (Evans et al. 2005, Mekel-Bobrov et al. 2005). It has been suggested that humankind skills, such as intelligence, language and social organization arose due to the ability of the brain to expand within an osseous hard resistant case (Wolpoff 1999). Jointly with these traits, neurocranial shape may have evolved in correlation to facial retraction and cranial base flexion. Therefore, although time and integration may have blurred the signals of strong selection in the human skull, this is not evidence against the action of natural selection on the human skull.

The evolution of morphological characters that differentiate species from each other is achieved through alterations in the inherited pattern of growth and development (Thompson et al. 2003). Evo-devo hypotheses about tinkering, the idea that small changes of existing systems can lead to big changes (Lieberman et al. 2004, Hallgrímsson et al. 2006, 2007) are supported by this study (Chapter 5). According to these, few changes in key genes triggered modern human craniofacial form. Genetic changes influencing the timing of gene expression during growth and development, as well as genetic or epigenetic changes altering key developmental pathways, may produce substantial changes in the phenotype (Thompson et al. 2003). The results of the present thesis point out the major role that development may have played in the evolution of the human skull. It is likely that genetic integration regulated the cascade of morphological effects driven by small developmental changes (Chapter 5). These developmental changes remain unknown but

future research should seek for the genetic and the developmental basis of these observed patterns of craniofacial variation in modern humans.

Lieberman et al. (2004) hypothesized that the following developmental pathways could be involved in the shift to the cranial morphology of modern humans. Regarding the face, the authors pointed out that any change reducing overall rates of facial growth would be a plausible explanation for the smaller and less projected faces of modern humans. For instance, they suggest that modifications of the regulatory system of growth hormones could have played a role (as for example, the growth hormone-insulin-like growth factor-I, GH-IGFI axis; and the thyroid, TH axis). Regarding the cranial base, they suggested that the most likely explanation for the more enlarged and flexed anterior cranial fossa in modern humans are secondary epigenetic interactions between the cranial base and the neighboring soft and skeletal tissues (especially the brain and the face). Thus, genes whose expression regulates the relative size of the temporal and frontal lobes (e.g. *C21orf5*) are promising gene candidates. Moreover, the genes that regulate the formation of the anterior cranial base precursors (such as *Br* and *shh*) may also play an important role. The prediction is that an alteration of the expression of these genes inducing larger mesenchymal condensations would produce a larger anterior cranial base region in modern humans (Lieberman et al. 2004).

According to this hypothesis, few changes in key genes triggered modern human craniofacial form (Lieberman et al. 2004): 'possibly one in the brain that caused a longer, more flexed cranial base, another that caused overall diminution of facial size, and a third that involved increased globularity of the neurocranium'. The conclusion is that evolutionary changes occur through shifts early in development that make use of pre-existing developmental pathways to generate novel but highly integrated morphologies (McBratney and Lieberman 2003). This is supported by several studies reporting that facial morphological differences between modern humans and hominid fossils are established early in ontogeny, before two years old (Ponce de Leon and Zollikofer 2001, Ackermann and Krovitz 2002).

On the other hand, epigenetic interactions may have also played an important role in human evolution. Interactions through the cranial base are especially relevant because this region integrates the face and the neurocranium (Lieberman et al. 2000a, 2000b). The cranial base responds to brain growth and translates these forces both to the face and the neurocranium. The interactions between the face and the cranial base are established at the anterior chondrocranial skeletal elements (sphenoides and alisphenoides) through the

osteogenic growth produced at the synchondrosis. Therefore, the study of the genetic and developmental factors that regulate this process may shed light to the evolution of the human skull.

The approach developed by Lieberman et al. (20004) has provided a new framework for methodological assessment of craniofacial morphology, combining the techniques of geometric morphometric shape analysis and developmental evolutionary biology for studies of human evolution. The works by Lieberman et al. (2000a, 2000b, 2002, 2004) jointly with those of Hallgrímsson et al. (2004, 2005, 2006, 2007) provide relevant results, but even more interesting, raise new questions and challenging hypothesis about the mechanisms by which modern human craniofacial form was achieved.

Taking into account the profound effects of morphological integration (Chapter 5), it is difficult to consider that the main derived characters of modern humans are single independent characters. Therefore, they may not be suitable for phylogenetic and cladistic analyses because the fundamental assumption of independence of cladistic analysis is not fulfilled. Strait (2001) points out that 'the solution to this problem is to treat integrated features as a single phylogenetic complex and to weight the complex as if it were an independent character'. Many studies attempted to overcome this problem by grouping functional characters (Skelton and McHenry 1992, Strait et al. 1997, Skelton and McHenry 1998a, 1998b, Strait and Grine 1999). Nevertheless, no consistent results were found. This indicates that morphological integration has a great confounding effect for phylogenetic and cladistic studies. Lieberman et al. (2004) stated that 'it is important to identify morphological traits that are mostly the result of genetic expression (and thus that could be useful for taxonomic classification and phylogenetic analysis because they are heritable) and to differentiate them from those morphological traits whose expression is entirely dependant on epigenetic/environmental factors'. This is important, but as discussed above, morphological integration should also be assessed with great precision.

This is relevant because it has been shown that skeletal morphology can produce completely misleading evolutionary trees (Collard and Wood 2000): cranial morphology suggests that gorillas and chimpanzees were monophyletic; whereas most molecular data points that the monophyletic group is formed by chimpanzees and humans (Patterson et al. 1993, Rokas and Carroll 2006). This fact does not imply that morphometric characters have no phylogenetic imprint at all, but emphasizes the functional and developmental complexity of the skull morphology. Recently, a paper demonstrated that allometry is another

important confounding effect for phylogenetic studies (Gilbert and Rossie 2007). When these factors are adequately assessed, morphological data provide as valid results as molecular data (Gilbert and Rossie 2007). Hence, it is crucial to isolate discrete osseous morphologies that are highly heritable, relatively independent from adjacent skeletal units, and truly homologies.

Finally, the quantitative genetic analysis allowed to detect that selection significantly operated on the skull evolutionary changes observed in Hallstatt during the 18th and the 19th centuries (Chapter 6). Strong directional selection was detected on skull shape and weak stabilizing selection on skull size. The strength of directional selection was surprisingly high, twice as much as the overall median strength value reported by other studies in animal populations (Kingsolver et al. 2001). However, the results also showed that there is no correspondence between the expected responses to these selection regimes and the secular trends occurred in Hallstatt during this period (Chapter 6). This suggests that microevolutionary factors other than natural selection are also contributing to the evolution of skull morphology.

These results suggest that natural selection shouldn't be disregarded as an important factor driving human skull evolution (Chapter 6). Some studies have suggested that genetic drift was the main evolutionary force responsible for the divergence between Neanderthals and humans as well as for the diversification of modern humans (Dean et al. 1998, Ackermann and Cheverud 2004b, Roseman and Weaver 2004, Harvati and Weaver 2006b, Weaver et al. 2007), with the exception of some nasal adaptations to extreme cold environments (Roseman 2004, Harvati and Weaver 2006b). These retrospective analyses of selection on humans were performed without previous knowledge of the genetic architecture of skull morphology. However, genetic variation provides the crucial raw material for long-term evolution (Thompson et al. 2003) and the evolutionary response to selection does depend on inheritance (Lande and Arnold 1983).

In the analyses herein (Chapter 6) an entirely quantitative genetic approach to estimate selection on human's skull morphology was performed. Direct estimates of the components of selection in humans were obtained by combining demographical and morphological data. The results of these analyses indicated that the evolution of such a complex phenotype should be considered under a multifactorial framework (Chapter 6). The final outcome of evolution is likely the result of several microevolutionary forces acting simultaneously and producing different effects on the cranial phenotype. In

order to reach a more comprehensive understanding of this process, all of these factors should be taken into account.

What is outstanding from all of these results (Chapters 3 to 6) is that morphological integration is a key factor in evolution, which regulates the variability of the human skull. Morphological integration is weak enough to allow populational diversification of cranial forms, but strong enough to preserve an operational structure of the human skull.

8 Conclusions

Conclusions

The main conclusions arisen from this thesis are the following:

1. There is a substantial amount of genetic variation underlying both size and shape of the human skull. The three main developmental regions of the skull (namely the face, the neurocranium and the basicranium) show similar amounts of genetic variation.
2. These high amounts of genetic variation would confer the human skull a high ability to evolve. Nevertheless, this is restricted by complex patterns of covariation among cranial regions.
3. The human skull is a highly integrated structure, both at the genetic and the phenotypic level. Genetic integration is pervasive and hierarchically structured.
4. Craniofacial variation is not regionalized, but widespread throughout the skull since there's no single, simple shape change associated to neither genetic nor phenotypic variation.
5. Integration is detected at different scales. When the whole skull is considered, the face, the neurocranium and the basicranium tend to behave as a unit, showing the clearest correspondence between genetic and phenotypic covariance patterns. However, the face shows some degree of independence. At a smaller scale, integration is observed among certain functional regions (e.g. the orbits and the zygomatic arches), but not in others (e.g. the anterior and posterior parts of the neurocranium).
6. The cranial base is a key region that integrates the face and the neurocranium and settles down the overall cranial shape, constraining facial and neurocranial dimensions.

7. Covariation between cranial widths is a dominant integrative feature that has been conserved across the evolution of the mammalian skull.

8. Traditional hypothesis of integration suggesting a distinction among brachycephalic and dolicocephalic skulls do not reflect the genetic architecture of the human skull.

9. The genetic and the phenotypic covariation matrices are similar but not identical or proportional. Genetic covariation matrices show more complex and structured patterns of morphological integration than the phenotypic covariation matrices. This should be taken into account in studies using the phenotypic covariation matrix as a proxy of the genetic covariation matrix.

10. Genetic covariation matrices also evidence for genetic constraints, which reduce the evolutionary potential of the human skull. These correspond to shape features that can not evolve because they do not have sufficient genetic variation.

11. The evolvability of the human skull is constrained and directed towards certain trajectories of morphological change that would maintain an operational and functional skull shape.

12. The combination of geometric morphometrics and multivariate quantitative genetics is a robust and powerful approach to explore the evolutionary patterns of complex phenotypes. Unlike univariate approaches, multivariate approaches can detect more complex evolutionary patterns because they do not disregard covariation among traits and thus reveal the anatomical, developmental and functional complexity of the human skull.

13. This methodology is an alternative approach for searching modules in complex phenotypes. As opposed to modular hypothesis-driven approaches, no previous assumption of shape modularity needs to be formulated.

14. Development has played a major role in the evolution of the human skull. It is likely that genetic integration may have regulated the cascade of morphological effects driven by small developmental changes, as evo-devo hypotheses suggest.

15. Taking into the strong effects of morphological integration, it is difficult to consider that the main derived characters of modern

humans are single independent characters. This result has thus profound implications for phylogenetic and cladistic analyses.

16. The origin of any one of the derived characters of modern humans may have facilitated the evolution of the others, which suggests a re-interpretation of the selective scenarios for human evolution.

17. The morphological changes associated with the evolution of bipedalism may have enhanced the evolution of a more globular and expanded neurocranial shape, which could be favoured afterwards by selection for bigger and more complex brains, as molecular evidence suggests. Jointly, these traits may have evolved in correlation to facial retraction and cranial base flexion. Therefore, although time and integration may have blurred the signals of strong selection in the human skull, this is not evidence against the action of natural selection on the human skull.

18. In fact, natural selection has significantly acted on human skull evolution. Over the last 200 years, strong directional selection on skull shape and weak stabilizing selection on skull size has been detected at Hallstatt's population.

19. Other microevolutionary forces contributed to the evolution of skull morphology but in opposite directions to those selected, causing a non correspondence between secular trends and the response to selection patterns. Forces such as genetic drift and gene flow would not be sufficient causes. Environmental factors may have also participated in this process.

20. The skull is under the effect of multiple evolutionary forces, acting at the same time and towards similar or different directions of shape change. The skull responds to these pressures through complex and widespread networks of genetic and epigenetic interactions.

21. The multivariate approach undertaken in this study clearly reflects the complex morphological nature of the human skull. The Hallstatt material is a unique collection of skulls with associated genealogical data. The information that can be derived from the study of this material can shed light to the understanding of the evolutionary process of the human skull.

9 *Resum de la Tesi Doctoral*

Introducció

EL PROJECTE HALLSTATT

Aquesta tesi és el resultat final d'un projecte que va ser finançat l'any 2004 per la Wenner-Gren Foundation for Anthropological Research (Beca de recerca individual nº 7149). El projecte es titula *Quantitative genetics of craniofacial traits: a functional approach to heritability* i el seu objectiu final és integrar eines morfomètriques i biodemogràgiques amb mètodes de genètica quantitativa per estimar la variació genètica i la capacitat evolutiva de la morfologia craniofacial humana.

Les anàlisis que aquí es presenten estan basades en l'estudi d'una col·lecció de cranis humans procedents de Hallstatt, un petit poble situat als Alps austríacs. Aquest material esquelètic s'ha anat acumulant a Hallstatt des de l'any 1775 i inclou més de 700 cranis decorats. Aquesta mostra és única en el món perquè constitueix la col·lecció de cranis més gran coneguda amb informació genealògica associada. Això fa que l'estudi d'aquesta mostra sigui d'especial interès pels estudis de biologia evolutiva, perquè representa una oportunitat única per aplicar mètodes de genètica quantitativa a l'estudi del crani humà.

La col·lecció es va originar al segle XVIII gràcies a una tradició local que ha perdurat fins molt recent: per honrar els seus avantpassats, els familiars reclamaven que les seves restes esquelètiques fossin desenterrades passats uns 10 anys de la defunció (Burgstaller 1961). L'enterramorts era qui s'encarregava de netejar i decorar el crani amb motius florals i religiosos i d'escriure el nom del difunt al front del crani (Sauser 1956). Aquests cranis van quedar dipositats a la cripta de l'església catòlica de Hallstatt. A partir de la informació demogràfica dels arxius parroquials (1602-1900), es van poden reconstruir les genealogies de les famílies de Hallstatt i gràcies al nom que portaven escrit al front, es van poder identificar els cranis dintre de les genealogies. Aquest costum no es circumscrivia només a Hallstatt, sinó que es practicava en moltes regions austríaques i alemanyes que envolten els Alps. Tanmateix, a Hallstatt és

a l'únic lloc on la tradició ha perdurat tants anys i on s'ha conservat la col·lecció de cranis original.

Principalment, aquest projecte ha consistit en la recol·lecció i l'anàlisi de dos tipus de dades: morfomètriques i biodemogràfiques. Per una banda, les dades craniomètriques van ser registrades mitjançant tècniques de morfometria geomètrica amb l'objectiu de quantificar la morfologia cranial. Per una altra banda, les dades demogràfiques van aportar la informació necessària per reconstruir les genealogies de la població de Hallstatt. Finalment, es van combinar aquestes dues bases de dades per aplicar mètodes de genètica quantitativa i estimar les fonts de variació genètica i ambiental que determinen el fenotip craniofacial humà. Així, s'ha pogut estimar l'heretabilitat de mesures craniomètriques, el component de variació genètica de la forma i de la mida de les estructures craniofacials i s'han pogut testar hipòtesis sobre la selecció natural i l'evolució del crani al llarg del llinatge humà.

Història i demografia de Hallstatt

Hallstatt es troba a la província de Salzkammergut, als Alps orientals (47°34'N 13°39'E), a uns 70 km SE de Salzburg. El poble està envoltat de glaceres i es troba a la riba d'un gran llac, el Hallstättersee, format pel riu Traun. La història del poble ha estat tan íntimament lligada amb l'extracció de la sal que fins i tot el nom del poble està relacionat amb aquest mineral. Durant milers d'anys, les mines de sal han estat la principal font de recursos d'aquesta regió. La història de la població de Hallstatt s'inicià en temps neolítics (12.000 a.C) i els primers indicis de l'activitat minera daten del 5.000 a.C., establint-se com les mines de sal més antigues del món.

El Catolicisme es va començar a estendre pel Salzkammergut als voltants de l'any 300 d.C., i la primera evidència d'una església catòlica a Hallstatt data del segle XII. L'església actual, St. Mary Kirche, així com la cripta i la capella de St. Michael, van ser construïdes a finals del segle XV. Fins al segle XVI, la població de Hallstatt era catòlica, però després de la Reforma, el Luteranisme es va introduir a Àustria i va anar captant adeptes en la població. A Hallstatt, fins l'any 1850, tota la població estava registrada a la parròquia catòlica però llavors les esglésies es van separar completament i cada individu era enregistrat en els arxius de la seva parròquia corresponent. A l'any 1845, Hallstatt tenia 1930 habitants, dels quals 58.3% eren catòlics i 41.7% eren protestants (Kurz 2002).

Entre els segles XVIII i XIX, el nombre d'habitants de Hallstatt va fluctuar entre 1500 i 2000 habitants, però a partir del segle XX va començar a decaure (Kurz 2002). El nivell mínim d'habitants es va assolir al cens de l'any 2001, en el que menys de mil habitants estaven enregistrats a Hallstatt. A diferència d'altres pobles de la regió, la població de Hallstatt s'ha mantingut bastant estable al llarg d'aquests tres últims segles. En el patró demogràfic de Hallstatt es poden distingir les tres fases típiques de la transició demogràfica europea (Kurz 2002).

Tot i que fins a finals del segle XIX Hallstatt era una població geogràficament aïllada, els nivells de consanguinitat s'han mantingut especialment baixos al llarg de la seva història (Esparza, com. pers.). El primer mitjà de transport introduït a Hallstatt va ser un vaixell de vapor en l'any 1862 i la primera carretera no va ser construïda fins l'any 1875 (Urstöger 1984). Fins llavors, només es podia accedir al poble a través de passos muntanyencs i mitjançant barques que circulaven pel llac. Tot i així, les mines de sal van atraure a molta gent de la regió que anava a Hallstatt a treballar. Aquests moviments poblacionals explicarien els baixos nivells de consanguinitat trobats en aquesta població. Actualment, encara es continua extraient sal però la principal activitat econòmica es sustenta en el turisme, donat que Hallstatt ha estat declarada patrimoni cultural i paisatgístic de la humanitat (UNESCO 1996).

Estudis previs: la col·lecció de cranis de Hallstatt

Els primers estudis sobre la col·lecció de cranis de Hallstatt es van realitzar a finals del segle XIX (Zuckerkandl 1883, 1898) i les primeres revisions sistemàtiques de la col·lecció daten de mitjans del segle XX (Sauser 1956, Olbrich 1962). Durant els anys 80, el Dr. Sjøvold, de la Stockholms Universitet, va realitzar tota una sèrie d'estudis sobre l'heretabilitat dels caràcters mètrics i no-mètrics de la morfologia craniofacial (Sjøvold 1984, 1986, 1987, 1990, 1995). A part d'aquests estudis, la col·lecció de cranis de Hallstatt no es va tornar a analitzar fins l'any 2006. Aleshores, es van recalcular les heretabilitats dels caràcters craniomètrics però utilitzant tècniques estadístiques més sofisticades (Carson 2006a, 2006b). Els estudis d'aquests dos autors mostren diferències entre ells, però coincideixen en mostrar que els caràcters craniomètrics presenten heretabilitats moderades.

EL CRANI HUMÀ

El crani humà és una estructura òssia molt complexa, formada per uns 45 elements esquelètics i que creix sota el control de factors tant genètics com epigenètics (Sperber 2001). El crani dels mamífers s'organitza en tres regions principals: la cara, el neurocrani o volta cranial, i el basicrani o base del crani (Sperber 2001). Aquestes regions tenen orígens embrionaris diferents i compleixen múltiples funcions. La base i la volta del crani recobreixen i protegeixen el cervell, mentre que la cara protegeix els ulls i dóna suport als aparells masticatori i respiratori (Sperber 2001). A nivell del desenvolupament dels ossos, es poden fer dues grans distincions: els ossos que es formen a partir d'un procés d'ossificació endocondral, com per exemple els ossos de la cara o de la volta cranial i que són els que formen el dermatocrani; i els ossos que es formen a través d'un procés d'ossificació cartilaginosa, com per exemple els ossos de la base del crani, que formen el condrocrani. A diferència dels ossos del dermatocrani, que es formen per ossificació directa, els ossos del condrocrani es formen a partir d'un precursor cartilaginós que s'ossifica secundàriament en el desenvolupament (Carlson 1999). Per tant, els ossos del condrocrani depenen de la formació i del creixement d'aquest cartílag i es poden veure afectats per l'acció diferencial de factors genètics i epigenètics que influenciïn les vies del desenvolupament que regulen aquest procés.

En el moment del naixement, el ossos que formen el crani humà encara estan en procés de creixement: els centres d'ossificació (fontanel.les) continuen produint teixit ossi fins que cada ós assoleix la seva forma i mida final. A mesura que creixen, els ossos es van fusionant a través de les sutures cranials i acaben formant una estructura única. Les tres regions que conformen el crani (la cara, el neurocrani i el basicrani) es desenvolupen en moments diferents sota l'acció de diferents factors. La base del crani és la primera regió que es desenvolupa, seguida de la volta del crani. La cara és, finalment, la última regió que es desenvolupa (Sperber 2001). El creixement de les estructures neurocranials (tant la base com la volta del crani) es produeix durant els primers estadis de l'ontogènia (prenatal i neonatal) i ve determinat, en gran mesura, pel creixement del cervell. La cara creix durant un període més extens i arriba a la seva forma definitiva ben entrat el període postnatal. Les estructures cranials relacionades amb el desenvolupament dels òrgans sensorials estan pràcticament formades en el moment de néixer. El predomini del neurocrani respecte la cara és màxim durant el període prenatal (8:1), però es redueix progressivament després del naixement: 6:1 als dos anys d'edat, 4:1 als cinc anys d'edat. En arribar a l'edat adulta, s'assoleixen les proporcions 2:1 definitives (Sperber 2001).

L'estructura dels ossos, però, també es modifica al llarg de la vida. Tot i que el crani creix com una unitat compacta, cada ós presenta les seves pròpies taxes de creixement. El creixement global del crani és el resultat d'un creixement diferencial, és a dir, és el resultat d'una combinació coordinada de múltiples trajectòries de creixement regionals (Enlow 2000). Segons aquest principi, els ossos canvien la seva forma i augmenten la seva mida a través de patrons osteogènics locals. Aquests patrons vénen donats per dos processos: la remodelació i l'absorció (Sperber 2001). La remodelació és una combinació de deposició osteoblàstica i de reabsorció osteoclàstica, produïda en resposta a les matrius funcionals periosteals i que provoca canvis de forma en els ossos (Enlow 2000). La transposició, en canvi, consisteix en desplaçaments dels ossos causats per forces exercides pels teixits tous que envolten el crani. Tots dos processos, poden ocórrer o bé en la mateixa direcció o bé en direccions contràries (Enlow 2000).

La deposició de nou teixit ossi es produeix tant a la superfície de l'ós, fent que augmenti el gruix dels ossos, com a les sutures, que estan formades per teixit connectiu (Sperber 2001). Les diferents sutures cranials es fusionen en diferents moments de la vida de l'individu i, per tant, els patrons de fusió sutural poden ser utilitzats com a marcador diagnòstic de l'edat. Les sutures romanen obertes fins els 20-25 anys d'edat, però a partir d'aquest moment comencen a tancar-se. Aquest procés s'acaba cap als 40 anys i llavors les sutures van desapareixent. El tancament prematur d'una sutura (o craniosinostosi) inhibeix el creixement en la direcció esperada però per compensar la manca de creixement estimula un creixement anormal en altres direccions (Sperber 2001). Així, el crani continua creixent però causa malformacions.

La integració entre les estructures cranials i el seu creixement controlat són necessaris per un desenvolupament normal del crani. La morfogènesi inicial és directament dependent de l'expressió de gens *homeobox* (Siebert & Swindler 2002) que codifiquen per factors de transcripció que regulen l'expressió gènica durant els primers estadis del desenvolupament. Aquest procés és d'especial rellevància per determinar els patrons de diferenciació cel·lular que donaran lloc als diferents components craniofacials. Durant els estadis posteriors, factors tant genètics com ambientals influeixen el desenvolupament del complex craniofacial (Sperber 2001). El genotip estableix les regles arquitectòniques necessàries per construir el crani, però com que el creixement ossi és un procés lent i gradual, i la remodelació es produeix al llarg de la vida, l'expressió fenotípica final també està modulada per factors nutricionals, bioquímics i físics. També són importants els factors funcionals, a

través dels quals el desenvolupament de teixit tous associats com els músculs i els òrgans nerviosos poden influenciar en el creixement dels ossos (Moss & Young 1960). No obstant, encara es desconeix com els canvis en la morfologia cranial es correlacionen amb canvis determinats genètics (Siebert & Swindler 2002).

TENDÈNCIES EVOLUTIVES EN HOMÍNIDS

El registre fòssil és una de les fonts d'informació més importants que tenim sobre l'evolució del llinatge dels homínids. De totes les restes fòssils, el crani és la estructura que millor es conserva i que més s'ha utilitzat per reconstruir arbres filogenètics (Strait & Grine 1999). L'evolució de la forma del crani ha tingut una importància cabdal en l'evolució del llinatge humà. Entre les espècies d'homínids existeixen nombroses diferències morfològiques a nivell del crani, però aquestes diferències no són dicotòmiques, sinó que el que observem és una gradació, un *continuum*, i les fronteres entre espècies no són clares (Collard & Wood 2000).

Tot i així, s'han descrit quatre caràcters derivats dels humans moderns (Aiello & Dean 1990): una posició més avançada del foramen magnum (que és una cavitat que es troba a la base del crani i a través de la qual es connecta la espina dorsal amb el cervell), una volta cranial més gran i més arrodonida, una forta flexió de la base del crani i una marcada retracció facial. La posició anterior del foramen magnum s'ha contemplat com una adaptació dels homínids al bipedisme (Ahern 2005). El segon caràcter derivat dels humans moderns, i potser un dels més importants en el procés d'hominització, és l'increment de l'encefalització. Aquesta és la tendència evolutiva dels homínids per desenvolupar voltes cranials més grans i més arrodonides. Possiblement, l'encefalització es va originar en resposta a un increment del volum del cervell (Lieberman et al. 2004, Holloway et al. 2004). Els dos últims caràcters, la retracció facial i la flexió de la base del crani, haurien evolucionat de manera associada junt amb l'encefalització (Lieberman et al. 2002, 2004). Anatòmicament, aquestes característiques estan ben definides, però malauradament es desconeixen les bases genètiques, evolutives i del desenvolupament que han determinat aquestes tendències morfològiques.

S'han proposat dos tipus d'hipòtesis per explicar l'evolució del crani humà. Tradicionalment, les hipòtesis adaptatives han considerat que la forma del crani humà és el resultat de respostes adaptatives a la selecció de diversos

factors, com ara la locomoció bípeda, els canvis de dieta i el desenvolupament d'un cervell més gran que facilités l'adquisició del llenguatge i d'habilitats cognitives més complexes (Wolpoff 1999). Per una altra banda, hipòtesis més recents basades en la biologia evolutiva del desenvolupament consideren que la morfologia cranial dels humans és el resultat d'uns pocs canvis en els patrons que regulen el desenvolupament del crani humà i que es produeixen durant els primers estadis de l'ontogènia (Lieberman et al. 2004). Així, un petit canvi en el desenvolupament podria desembocar en una gran cascada de canvis morfològics que serien dependents els uns dels altres (Hallgrímsson et al. 2006, 2007).

Modularitat i Integració Morfològica

Malgrat la seva gran complexitat anatòmica, la característica que dificulta veritablement l'estudi del crani humà és l'existència de forts patrons d'integració morfològica entre regions (Lieberman et al. 2000b, Hallgrímsson et al. 2004, Lieberman et al. 2004, Bastir & Rosas 2005, Hallgrímsson et al. 2006). És a dir, les tres regions del crani no són independents entre sí, sinó que estan integrades entre elles de forma més o menys intensa a través de patrons de covariació (Klingenberg 2005). Això fa que quan una zona es modifica també faci modificar a les altres, donant una resposta de canvi morfològic unitària i global. A més a més, és molt important tenir en compte els patrons d'integració morfològica perquè poden limitar l'evolució en determinats sentits de canvi morfològic (Klingenberg 2005). El crani no és una estructura que pugui evolucionar lliurement, sinó que està constreta per la integració amb l'objectiu de mantenir una morfologia que arquitectònicament sigui viable, funcional i operativa (Ackerman & Cheverud 2004a). La variabilitat fenotípica craniofacial s'expressa, per tant, en un rang limitat que ve determinat per la integració morfològica (Klingenberg 2005). A més a més, la integració morfològica assumeix que els trets craniomètrics relacionats tant a nivell funcional com del desenvolupament s'hereten conjuntament (Cheverud 1995). Així, com la selecció afavoriria trets associats, es considera que el crani evoluciona com una unitat coordinada (Cheverud 1995).

Hi ha autors que han proposat l'existència de sis mòduls funcionals en el crani dels primats (Cheverud 1982, 1988, 1995, 1996b, Marroig & Cheverud 2001, Ackerman & Cheverud 2004a, Marroig & Cheverud 2004): dintre de les estructures neurocranials, distingeixen la volta cranial, la base del crani i l'òrbita; mentre que dintre de la cara, distingeixen la regió oral, la regió nasal i la regió zigomàtica. Els resultats d'aquestes investigacions han donat suport a la

hipòtesi de les matrius funcionals (Moss & Young 1960), evidenciant que la covariació entre unitats que compleixen una mateixa funció és més forta que entre divisions òssies no funcionals. Segons Ackermann & Cheverud (2004a), dintre de les estructures facials i neurocranials hi ha una forta integració morfològica, i aquests autors sustenten que la gran diversitat de formes present en l'ordre dels primats és el resultat de canvis en les magnituds relatives de covariació entre aquestes estructures, tot i que s'ha mantingut el patró general d'integració.

Per una altra banda, s'ha evidenciat que la cara és una estructura integrada però relativament independent del neurocrani i del basicrani (Lieberman et al. 2000a, 2000b, Zollikofer & Ponce de León 2004, Bastir & Rosas 2005). Tanmateix, s'ha mostrat que la base del crani també presenta una relativa independència (Bastir & Rosas 2006) tot i ser un fort element integrador i una peça clau en l'evolució de la forma del crani dels primats (Lieberman et al. 2000a, 2000b, 2002, 2004). Des del punt de vista filogenètic, s'ha suggerit que els trets facials no serien tan informatius com els trets neurocranials (Collard & Wood 2000, Collard & O'Higgins 2001) perquè es considera que la cara, en desenvolupar-se més tardanament i durant el període postnatal, està més influenciada per l'ambient i és més susceptible als canvis plàstics (Strand Vidarsdóttir et al. 2002, Bastir & Rosas 2004b).

Finalment, també és important destacar que la integració no es limita als components ossis del crani, sinó que també s'han detectat patrons de covariació entre el crani i el cervell (Richtsmeier et al. 2006).

MORFOMETRIA GEOMÈTRICA

La morfometria geomètrica és la metodologia que s'ha utilitzat en aquesta tesi per quantificar la forma cranial. La morfometria geomètrica s'ha definit com la fusió entre la biologia i la geometria. Concretament, és una aproximació molt útil per la caracterització quantitativa, l'anàlisi i la comparació estadística entre formes biològiques (Bookstein 1991, Marcus et al. 1996, Dryden & Mardia 1998, Lele & Richtsmeier 2001). La morfometria geomètrica és un mètode basat en l'enregistrament de coordenades cartesianes que va ser desenvolupada per analitzar la forma (i els canvis morfològics) en un espai bi- o tridimensional. La morfometria geomètrica inclou un ampli corpus de tècniques gràfiques i estadístiques i representa un nou paradigma per l'anàlisi de la forma (Bookstein 1991, Marcus et al. 1996, Dryden & Mardia 1998, Lele &

Richtsmeier 2001). Les tècniques de morfometria geomètrica han tingut un gran èxit en molts camps de la biologia i han constituït una veritable revolució pels estudis morfomètrics (Bookstein 1991, Rohlf & Marcus 1993, Klingenberg 2002, Adams et al. 2004). Les claus d'aquest èxit són una major precisió per capturar la forma biològica, una major robustesa dels mètodes estadístics, una major capacitat de visualització de la forma (2 i 3 dimensions) i una gran disponibilitat de programes estadístics gratuïts per realitzar aquestes anàlisis.

En morfometria geomètrica, es considera que la forma (*form* en anglès) és la combinació de mida (*size*) i de forma (*shape*). D'aquí en endavant, quan es parla de forma es refereix al terme anglès de *shape*. A diferència de la morfometria clàssica, la forma no s'obté mitjançant mesures linears o angulars, sinó que es captura mitjançant el registre de coordenades cartesianes (x, y si es treballa en 2 dimensions; x, y, z si es treballa en 3 dimensions) d'un conjunt de punts que s'ubiquen sobre els objectes que es volen analitzar. Per poder comparar diferents formes, és necessari que els punts registrats siguin homòlegs, o bé a nivell anatòmic i funcional, o bé a nivell purament geomètric.

Els principis de la morfometria geomètrica estan basats en la capacitat de transformar la geometria dels espècimens en punts d'un espai abstracte anomenat morfoespai i vice versa (Kendall et al. 1999). Les propietats matemàtiques i estadístiques dels morfoespais són complexes però estan descrites en detall (Bookstein 1991, Marcus et al. 1996, Dryden & Mardia 1998, Lele & Richtsmeier 2001, Zelditch et al. 2004). En definitiva, el morfoespai es podria considerar com un gràfic on cada punt representa la morfologia d'un sol individu (o el que és el mateix, la configuració de punts que representa la forma d'un individu). A més a més, qualsevol forma possible correspondria a un punt del morfoespai. Els morfoespais solen ser multidimensionals i no Euclidians, de manera que per realitzar les anàlisis estadístiques prèviament es projecta el morfoespai a un pla linear tangent (Slice 2001).

Existeixen diferents metodologies per realitzar anàlisis de morfometria geomètrica, però una de les més utilitzades i la que s'ha fet servir en aquesta tesi és el mètode Procrustes (Rohlf & Slice 1990, Bookstein 1991). La sobreimposició Procrustes aconsegueix capturar la informació de mida i de forma dels objectes que s'analitzen. Per una banda, la mida s'estima mitjançant una mesura anomenada "mida centroide", que tècnicament es defineix com l'arrel quadrada del sumatori de distàncies al quadrat des del punts registrats fins al "centroide", que seria el centre de gravetat de la configuració de punts (Dryden & Mardia 1998). I per una altra banda, la forma s'estima estandarditzant les configuracions de punts per la mida, la posició i l'orientació

(Dryden & Mardia 1998). Així, un cop eliminats aquests efectes amb la sobreimposició Procrustes, totes les diferències que s'observen representen únicament diferències de forma (Bookstein 1991, Zelditch et al. 2004). Les coordenades que resulten d'aquesta anàlisi, les coordenades Procrustes, es poden utilitzar per estimar una configuració de forma mitjana (Dryden & Mardia 1998), així com per realitzar tot tipus d'anàlisis estadístiques multivariants.

GENÈTICA QUANTITATIVA

En els últims anys, s'ha demostrat àmpliament la validesa de l'ús dels caràcters craniomètrics en estudis de genètica de poblacions (Relethford 1994, 2002, González-José et al. 2004) i de la història de les poblacions humanes (González-José et al. 2003, Stojanowski 2004, Brace et al. 2005, Stojanowski 2005, Martínez-Abadías et al. 2006, Stojanowski & Schillaci 2006, González-José et al. 2007). Tot i així, es desconeix el grau d'influència genètica i no-genètica en l'expressió fenotípica dels caràcters craniomètrics. La genètica quantitativa permet l'estudi d'aquest tipus de caràcters complexos, que són aquells que mostren variació contínua i són mesurats en una escala mètrica. L'objectiu dels estudis de genètica quantitativa és estimar les components de variació genètica i ambiental dels fenotips (Falconer & MacKay 1996, Lynch & Walsh 1998).

Aquesta qüestió és d'una importància cabdal pels estudis de biologia evolutiva perquè qualsevol tret biològic només pot respondre a la selecció si té suficient variació genètica heretable. En el cas de la morfologia cranial dels humans, només entendrem la seva evolució si tenim informació genètica que ens permeti discernir entre els factors genètics i ambientals que determinen l'expressió fenotípica de la variació morfològica del crani. A més, per identificar marcadors filogenètics fiables, és important identificar quins caràcters cranials són més el resultat de l'expressió genètica i quins són més influenciables pels factors ambientals (Strait 2001, Lieberman et al. 2004, Collard & Wood 2007).

Mitjançant els mètodes de genètica quantitativa, podem descomposar la variació fenotípica com la suma de dos factors: la variació genètica i la variació ambiental (Falconer & MacKay 1996, Lynch & Walsh 1998). La matriu de variació genètica s'obté a partir de la semblança esperada entre parents. Aquesta informació prové de les genealogies, que ens indiquen el grau de

parentiu entre individus. Tota la variació que s'explica per parentiu es considera variació genètica, mentre que la resta de variació es considera variació residual o ambiental. Existeixen diversos mètodes per realitzar aquesta descomposició (Falconer & MacKay 1996, Lynch & Walsh 1998); en aquesta tesi s'han utilitzat les tècniques estadístiques més sofisticades, basades en el mètode de màxima versemblança (REML). Aquest mètode consisteix en un procés iteratiu que va descomposant la variació fenotípica en genètica i ambiental i al final es queda amb aquella descomposició que és més versemblant, és a dir, que la seva probabilitat de ser certa sigui més alta (Falconer & MacKay 1996, Lynch & Walsh 1998).

L'heretabilitat de qualsevol tret pot ser estimada utilitzant els mètodes de genètica quantitativa, però el principal problema sobre l'estudi del crani humà és la tria de caràcters. Tradicionalment, s'han utilitzat un conjunt de mesures linears o angulars per definir la forma del crani (Martin & Saller 1957, Howells 1973). El problema és que aquestes mesures són arbitràries i no reflecteixen els patrons funcionals o del desenvolupament cranial (Pucciarelli et al. 1990). A més a més, si s'estudia cada caràcter per separat, no es tindran en compte els patrons d'integració morfològica. Les formes biològiques són inherentment multivariades (Klingenberg & Monteiro 2005) i han de ser estudiades, per tant, amb tècniques que permetin realitzar un anàlisi multivariat de la forma, com les tècniques de morfometria geomètrica. Klingenberg & Leamy (2001) van desenvolupar una metodologia que permet combinar les tècniques de morfometria geomètrica i de genètica quantitativa per investigar l'arquitectura genètica de formes biològiques complexes.

Amb tota la informació obtinguda amb els mètodes genètico-quantitatius, també es pot estimar la resposta a la selecció, aplicant l'equació de Lande (Lande 1979), que és la versió multivariada de l'equació de resposta a la selecció (Falconer & MacKay 1996, Lynch & Walsh 1998). Aquesta equació indica que si es coneix la intensitat de la selecció, es pot estimar la resposta a la selecció a partir de les matrius de covariació genètica i fenotípica. La intensitat de la selecció ve representada pel diferencial de selecció, que és la diferència entre la morfologia mitjana de la població parental abans i després de la selecció. L'aproximació mutivariada a la resposta a la selecció permet estimar la capacitat evolutiva de les estructures que s'analitzen, perquè té en compte tant el substrat de variació genètica, com els patrons de covariació genètica, que limiten el potencial evolutiu.

Pel que fa a la morfologia craniofacial humana, s'han fet molts esforços per estimar l'acció de la selecció natural (Marroig et al. 2004, Ackermann &

Cheverud 2004b, Roseman 2004, Marroig & Cheverud 2004, Roseman & Weaver 2004, Harvati & Weaver 2006a, Weaver et al. 2007). Aquests estudis han aportat evidència indirecta que mostra que gran part dels canvis evolutius que han resultat en la forma craniofacial dels humans moderns són el resultat de la deriva gènica i no de la selecció adaptativa. Tanmateix, tots aquests estudis han basat les seves anàlisis en la matriu de covariació fenotípica, sense tenir un coneixement previ de la matriu de covariació genètica. En aquesta tesi s'adrecen directament totes aquestes qüestions.

Objectius

Els principals objectius d'aquesta tesi són:

- Combinar mètodes de genètica quantitativa i de morfometria geomètrica per analitzar els components de variació del fenotip cranial humà.
- Quantificar els patrons de variació-covariació genètica, fenotípica i ambiental de la morfologia craniofacial humana, a través de dos tipus de caràcters craniomètrics:
    - Caràcters univariats: Mesures clàssiques, com distàncies lineals entre punts craniomètrics.
    - Caràcters multivariats: Reconstruccions tridimensionals de la forma de les principals regions cranials (cara, neurocrani i basicrani)
- Analitzar els patrons d'integració morfològica del crani humà, tant a nivell fenotípic com genètic.
- Estimar la capacitat evolutiva del crani humà.
- Simular l'evolució dels caràcters derivats de la morfologia craniofacial dels humans moderns.
- Detectar l'acció de la selecció natural en el crani humà, combinant dades demogràfiques d'èxit reproductiu amb dades morfològiques.

Aquests objectius han estat assolits mitjançant l'anàlisi de la col·lecció de cranis amb informació genealògica associada de Hallstatt (Àustria). Els resultats obtinguts s'han discutit en quatre manuscrits que seran tramitats per la seva

publicació. El corresponent resum en català de cada manuscrit es troba a l'inici de cadascun dels capítol de resultats (Capítols 3 a 6).

Resultats & Discussió

Els dos principals aspectes que caracteritzen l'arquitectura genètica del crani humà són, per una banda, els importants nivells de variació genètica que determinen tant la mida com la forma del crani humà. I, per una altra banda, els patrons dominants d'integració genètica (Capítols 3 a 6).

Les principals regions del crani humà (la cara, el neurocrani i el basicrani) mostren nivells similars de variació genètica. Aquest mateix resultat es va obtenir a partir de dues aproximacions independents: tant a partir de l'aproximació univariada (Capítol 3) com de la multivariada (Capítol 4). Aquest resultat confirma evidències prèvies que indiquen que en el crani dels primats, les dimensions facials, del neurocrani i de la base del crani tenen nivells d'heretabilitat similars (Cheverud & Buikstra 1982, Sjøvold 1984, Cheverud 1996b). S'havia suggerit que la cara era una estructura que estava més subjecta als canvis plàstics perquè el seu creixement s'estén més en el període postnatal (Kohn 1991, Strand Vidarsdóttir et al. 2002, Bastir & Rosas 2004b). Els resultats aportats per aquesta tesi indiquen que la contribució de la variació ambiental no és significativament més alta en les dimensions facials que en les dimensions del neurocrani o de la base del crani (Capítols 3 i 4), al menys quan es consideren globalment aquestes regions. Si s'exploren els patrons de variació fenotípica amb més deteniment, s'observa que hi ha certes dimensions que tenen heretabilitats no significatives i que per tant, estan totalment subjectes a la variació ambiental. Tanmateix, aquestes dimensions no es concentren a la regió facial, sinó que també es troben al neurocrani i a la base del crani (Capítol 3). Així, per explicar la plasticitat del crani humà és necessari invocar patrons de variació més complexes.

L'aproximació multivariada a la variació craniofacial (Capítol 4) revela patrons de variació més complexes que l'aproximació univariada (Capítol 3). L'aproximació multivariada mostra com, tant a nivell genètic com fenotípic, la variació no es concentra en regions funcionals o del desenvolupament específiques (Capítol 4), sinó que la variació està dispersa al llarg de totes les regions cranials. Tot i que aquesta metodologia ha estat molt poc aplicada, s'ha comprovat tant teòricament com experimentalment que és un mètode que

aporta una visió més completa dels patrons d'integració i de la capacitat evolutiva de fenotips complexes com el crani humà (Klingenberg & Leamy 2001, Myers et al. 2006). A més a més, aquesta metodologia es pot fer servir com un mètode experimental per explorar els patrons d'integració morfològica i per identificar estructures modulars (Capítol 4). A diferència d'altres mètodes, aquest no parteix d'assumpcions prèvies sobre la modularitat de l'estructura que es vol analitzar.

Malgrat els alts nivells de variació genètica, la capacitat evolutiva del crani està limitada per la integració morfològica (Capítols 3 a 6). La capacitat evolutiva d'un tret depèn més de la seva variabilitat (la capacitat dels trets per variar) que dels mateixos nivells de variació (Wagner & Altenberg 1996). La variabilitat del crani és fortament dependent de la integració morfològica. Totes les anàlisis realitzades en aquest treball indiquen, d'una manera o d'una altra, que els patrons d' integració morfològica en el crani humà són forts (Capítols 3 i 4) i que la capacitat de respondre a la selecció està constreta per la variació correlada (Capítols 5 i 6).

Els resultats obtinguts en aquesta tesi recolzen evidències prèvies que indiquen que el crani és una estructura fortament integrada. Aquesta integració s'estructura jeràrquicament a través de patrons de covariació complexos dintre i entre regions (Cheverud 1982, 1995, Lieberman et al. 2002, Bookstein et al. 2003, Ackerman & Cheverud, 2004, Bastir & Rosas 2004a, González-José et al. 2004, Hallgrímsson et al. 2007). La evidència aportada per aquests estudis es va obtenir a partir de l'anàlisi dels patrons de covariació fenotípica. Les anàlisis realitzades en aquest treball (Capítols 3, 4 i 5) mostren que a nivell genètic, els patrons de covariació són encara més complexos i encara més estructurats que els patrons de covariació fenotípica. A més a més, indiquen que les matrius de covariació genètica i fenotípica són molt semblants però no idèntiques (Capítol 3) ni proporcionals (Capítol 5). En els estudis que apliquen models de genètica poblacional per investigar sobre la història i l'estructura de les poblacions humanes (Steadman 2001, González-José et al. 2003, Ackermann & Cheverud 2004b, Roseman 2004, Roseman & Weaver 2004, González-José et al. 2005, Stojanowski 2005, Harvati & Weaver 2006a, Martínez-Abadías et al. 2006, Stojanowski & Schillaci 2006, González-José et al. 2007), és una pràctica usual assumir que la matriu de covariació fenotípica és proporcional a la matriu de covariació genètica (Cheverud 1988) perquè normalment no es disposa d'informació genètica associada. Els resultats del Capítol 5 mostren que aquesta assumpció no és tan directa, al menys en la mostra de Hallstatt. La matriu de covariació genètica no és de rang complet, fet que indica l'existència de límits genètics (morfologies que no poden evolucionar perquè no tenen

suficient variació genètica associada) i que mostra que la variació genètica està més concentrada que la variació fenotípica (Capítols 4 i 5).

Degut als forts patrons d'integració morfològica, no hi ha fronteres clares entre les regions cranials i els patrons d'integració no reflecteixen veritables mòduls funcionals ni del desenvolupament (Capítols 3 i 4). Segons Klingenberg (2004), 'els mòduls són unitats internament coherents establertes a través de la interacció de les seves parts, però que són relativament autònomes de la resta d'unitats, a les quals també estan connectades però a través de menys interaccions i més febles'. Els resultats obtinguts en aquesta tesi indiquen que la cara, el neurocrani i la base del crani estan fortament integrats. En alguns aspectes particulars de la variació genètica i fenotípica, la cara mostra una relativa independència i un cert grau de modularitat en regions funcionals menors (Capítols 3 i 4), tot i que el patró general està dominat per la integració. La integració es detecta a diferents escales. Quan es considera tot el crani, la cara, el neurocrani i el basicrani tendeixen a comportar-se com una unitat, mostrant la correspondència més clara entre els patrons de covariació genètica i fenotípica (Capítol 4). A una escala més petita, la integració s'observa entre certes regions funcionals (com per exemple entre les òrbites i els arcs zigomàtics), però no entre d'altres (com per exemple entre les parts anterior i posterior del neurocrani).

Una regió clau responsable d'aquests patrons generals d' integració morfològica és la base del crani (Capítols 3 i 4), perquè actua com a frontissa entre la cara i el neurocrani (Lieberman et al. 2000a, 2000b). Com ja es va trobar en el crani de ratolins (Hallgrímsson et al. 2007), els patrons d'integració del crani humà estan dominats per una forta covariació genètica entre les amplades màximes de la base del crani, el neurocrani i la cara (Capítol 3). Contràriament, les hipòtesis tradicionals d'integració que suggereixen una distinció entre cranis braquicèfals i dolicocèfals (Enlow & Hans 1996) no estan recolzats pels patrons de correlació genètica i fenotípica trobats a la mostra de Hallstatt. Sota aquestes hipòtesis, l'amplada màxima del neurocrani hauria d'estar positivament correlada amb l'amplada facial i negativament correlada amb l'alçada facial, la longitud neurocranial i l'alçada neurocranial. No obstant, les anàlisis realitzades en aquesta tesi només van trobar una correlació significativa entre les amplades del neurocrani i de la cara (Capítol 3). Aquest resultat recolza descobriments anteriors (Lieberman et al. 2000a, Bastir & Rosas 2004b) i suggereix que aquest tipus de terminologia, l'ús del qual encara està molt estès (Goodman 1995, 1997, Gravlee et al. 2003, Gonzalez et al. 2003, Fiedel 2004), no reflecteix l'arquitectura genètica del crani humà.

Una altra conseqüència de la integració morfològica és que influeix molt intensament en la resposta evolutiva del crani a la selecció (Capítols 5 i 6). Els resultats obtinguts a partir de l'anàlisi de simulació de l'evolució dels principals caràcters derivats del crani dels humans moderns (Capítol 5) mostren que independentment de la morfologia que es seleccioni, el crani sempre respon d'una forma global. Això resulta dels patrons dominants d'integració genètica, que fa que es produeixin conjuntament tot el conjunt de caràcters derivats. Així, la posició avançada del foramen magnum, el desenvolupament d'una volta cranial més gran i més arrodonida, la retracció facial i la flexió de la base del crani no van evolucionar com a resposta a escenaris selectius independents. És més probable que l'evolució de cadascun dels caràcters derivats promogués l'evolució dels altres. Això suggereix una reinterpretació del context selectiu de l'evolució humana (Capítol 5).

Aquesta hipòtesis emfatitza el paper del desenvolupament i del sistema genètic en determinar la capacitat evolutiva del crani, a diferència d'altres hipòtesis que es centren més en canvis funcionals i pressions evolutives externes (Weidenreich 1924, Dart 1925, Schultz 1942, DuBrul 1950, Schultz 1955, DuBrul & Laskin 1961, Demes 1985). De fet, és plausible considerar que els canvis morfològics associats amb l'evolució del bipedisme puguin haver afavorit l'evolució d'una forma neurocranial més globular i més expandida. Posteriorment, aquesta morfologia podria haver estat afavorida per la selecció per un cervell més gran i més complex, tal i com evidencien els estudis moleculars (Evans et al. 2005, Mekel-Bobrov et al. 2005). S'ha suggerit que les habilitats humanes, com la intel·ligència, el llenguatge i l'organització social complexa van sorgir gràcies a la capacitat del cervell per expandir-se en una "caixa" òssia resistent (Wolpoff 1999). Juntament amb aquests trets, la forma del neurocrani hauria evolucionat de forma correlada amb la retracció facial i la flexió de la base del crani. Així, tot i que el temps i la integració morfològica semblen haver esborrat les senyals directes de la selecció, aquest fet no és cap evidència en contra de l'acció de la selecció en el crani humà.

L'evolució dels caràcters morfològics que diferencien unes espècies de les altres s'aconsegueix a través d'alteracions heretades dels patrons de creixement i del desenvolupament (Thompson et al. 2003). Els resultats d'aquest estudi (Capítol 5) recolzen les hipòtesis evo-devo, que parteixen de la idea que petits canvis en els sistemes existents poden portar a grans canvis (Lieberman et al. 2004, Hallgrímsson et al. 2006, 2007). Segons aquestes hipòtesis, pocs canvis en punts clau del sistema genètics podrien haver desencadenat la morfologia cranial dels humans moderns. Canvis genètics que influenciïn els patrons temporals d'expressió gènica durant el creixement i el desenvolupament, així

com canvis genètics i epigenètics que alterin rutes clau del desenvolupament, poden produir canvis substancials del fenotip (Thompson et al. 2003). Els resultats d'aquesta tesi destaquen la importància del paper que pot haver jugat el desenvolupament en l'evolució del crani humà. És plausible que la integració genètica hagi regulat la cascada d'efectes morfològics desencadenada per aquests petits canvis del sistema del desenvolupament (Capítol 5). Aquests canvis del sistema del desenvolupament encara són desconeguts, per això futures investigacions haurien d'estar encaminades a explorar les bases genètiques i del desenvolupament d'aquests patrons de variació craniofacial observats en els humans moderns.

Lieberman et al. (2004) van suggerir que les següents rutes del desenvolupament podrien estar involucrades en el canvi que va portar a l'evolució de la morfologia craniofacial moderna. Pel que fa a la cara, aquests autors van indicar que qualsevol cavi que reduís les taxes de creixement facial podrien donar una explicació plausible a les característiques facials dels humans moderns (una cara més petita i menys projectada cap endavant). Per exemple, aquests canvis podrien estar associats a modificacions del sistema que regula la producció de les hormones del creixement (com els factors de creixement tipus insulina de l'eix GH-IGFI i els de l'eix tiroïdal TH). Pel que fa a la base del crani, van suggerir que l'existència de fosses cranials anteriors més grans i més flexionades en humans moderns es deu a interaccions epigenètiques entre la base del crani i els teixits tous i esquelètics amb els que està en contacte (el cervell i les estructures facials). Així, els gens que regulen la mida relativa dels lòbuls frontals i parietals del cervell (com per exemple el *C21orf5*) podrien ser bons gens candidats. De la mateixa manera, els gens que regulen la formació dels precursors de les parts anteriors de la base del crani (com el *Br* o el *shh*) també podrien jugar un paper important. La predicció és que una alteració en els patrons d'expressió d'aquests gens que induís una major condensació de cèl·lules mesenquimàtiques podria haver produït un major desenvolupament de les fosses cranials anteriors en els humans moderns.

Segons aquestes hipòtesis, pocs canvis en gens clau van ser els responsables de l'evolució del crani humà (Lieberman et al. 2004): 'possiblement un en el cervell que va causar una base del crani més llarga i més flexionada, un altre que va provocar una disminució general de la mida de la cara i un tercer que va portar a un augment de la globularitat del neurocrani'. En definitiva, la conclusió és que els canvis evolutius ocorren a través de pocs canvis que es produeixen durant les primeres etapes de l'ontogènia a partir de les xarxes del desenvolupament ja existents i que generen morfologies noves però fortament integrades (McBratney & Lieberman 2003). Aquesta hipòtesi

està recolzada per diversos estudis que suggereixen que les diferències morfològiques facials entre els humans moderns i els homínids fòssils s'estableixen aviat en l'ontogènia, abans dels dos anys d'edat (Ponce de Leon & Zollikofer 2001, Ackermann & Krovitz 2002).

Per una altra banda, les interaccions epigenètiques també poden haver jugat un paper important en l'evolució humana. Les interaccions entre la base del crani són especialment rellevants perquè aquesta regió integra la cara i el neurocrani (Lieberman et al. 2000a, 2000b). La base del crani respon al creixement del cervell i transmet aquestes forces tant a la cara com al neurocrani. Les interaccions entre la cara i la base del crani s'estableixen a través dels elements esquelètics del condrocrani anterior (alisfenoides i esfenoides) a partir del creixement osteogènic que es produeix a les sincondrosis. Així, l'estudi dels factors genètics i del desenvolupament que regulen aquest procés poden aportar informació rellevant per l'estudi de l'evolució del crani humà.

L'aproximació desenvolupada per Lieberman et al. (2004) ha aportat un nou context metodològic per analitzar la morfologia craniofacial, combinant les tècniques de morfometria geomètrica i de la biologia evolutiva del desenvolupament per als estudis d'evolució humana. Els treballs de Lieberman et al. (2000a, 2000b, 2002, 2004), juntament amb els de Hallgrímsson et al. (2004, 2205, 2006, 2007) aporten resultants rellevants, però potser encara més interessant, formulen noves qüestions i hipòtesis sobre els mecanismes a través dels quals es va desenvolupar la morfologia craniofacial humana.

Si es tenen en compte els profunds efectes de la integració morfològica (Capítol 5), es difícil considerar que els principals caràcters derivats dels humans moderns siguin caràcters independents. Si això és així, aquests caràcters no serien adequats per les anàlisis filogenètiques i cladístiques perquè no complirien l'assumpció bàsica d'independència de les anàlisis cladístiques. Strait (2001) va indicar que 'la solució a aquest problema consisteix en tractar els caràcters integrats com complexes filogenètics independents i en donar-los-hi el mateix pes que qualsevol caràcters independent'. Molts estudis han intentat salvar aquest obstacle agrupant caràcters funcionals (Skelton & McHenry 1992, Strait et al. 1997, Skelton & McHenry 1998a, 1998b, Strait & Grine 1999). Tanmateix, els resultats obtinguts no són coincidents. Lieberman et al. (2004) va afirmar que 'és important poder identificar trets morfològics que siguin principalment el resultat de l'expressió genètica (i que, per tant, puguin ser útils per les classificacions taxonòmiques i per les anàlisis filogenètiques perquè són heretables) i diferenciar-los d'aquells trets l'expressió

dels quals depèn en gran mesura de factors epigenètics o ambientals'. Aquesta qüestió és important, però com s'ha discutit anteriorment, la integració morfològica també hauria de ser analitzada amb gran precisió.

Això és rellevant perquè s'ha mostrat que la morfologia esquelètica pot representar arbres filogenètics completament erronis (Collard & Wood 2000): la morfologia cranial suggereix que els goril·les i els ximpanzés son monofilètics, mentre que la majoria de les dades moleculars indiquen que el grup monofilètic està format per ximpanzés i humans (Patterson et al. 1993, Rokas & Carroll 2006). Aquest fet no implica que els caràcters morfomètrics no tinguin cap senyal genètica, però fica de manifest la complexitat funcional i del desenvolupament de la morfologia cranial. Recentment, un treball va demostrar que l'al.lometria és un altre factor que esbiaixa les anàlisis fiologenètiques (Gilbert & Rossie 2007). Quan aquests factors es tracten adequadament, les dades morfològiques aporten resultats tan vàlids com les dades moleculars (Gilbert & Rossie 2007). Així, és d'una importància cabdal aconseguir identificar morfologies òssies discretes que siguin altament heretables, relativament independents d'unitats esquelètiques adjacents i que siguin veritables homologies.

Finalment, les anàlisis geneticoquantitatives realitzades van permetre detectar que la selecció havia actuat significativament en els canvis evolutius observats a Hallstatt durant els segles XVIII i XIX (Capítol 6). Es va detectar una forta acció de la selecció direccional en la forma del crani i una feble acció de la selecció estabilitzadora en la mida del crani. La força de la selecció direccional detectada va ser sorprenentment intensa, fins a dos cops més alta que la força mitjana obtinguda en altres estudis realitzats amb poblacions animals (Kingsolver et al. 2001). Tanmateix, els resultats també mostren que no hi ha una correspondència entre la resposta a la selecció esperada i els patrons de canvis seculars observats a Hallstatt durant aquest període (Capítol 6). Això indica que altres factors microevolutius també han contribuït a l'evolució de la morfologia cranial.

Aquests resultats suggereixen que la selecció natural no s'hauria de deixar de banda i s'hauria de continuar considerant com un factor important per l'evolució del crani humà (Capítol 6). Alguns estudis han suggerit que la deriva gènica va ser la principal força evolutiva responsable de la divergència dels Neandertals i els humans moderns, així com de la diversificació dels humans moderns (Dean et al. 1998, Ackermann & Cheverud 2004b, Roseman & Weaver 2004, Harvati & Weaver 2006a, Weaver et al. 2007), amb excepció d'algunes morfologies nasals que s'haurien desenvolupat com adaptació a

climes extremadament freds (Roseman 2004, Harvati & Weaver 2006b). Aquestes anàlisis retrospectives sobre la selecció en humans es van realitzar sense tenir un coneixement previ de l'arquitectura genètica del crani humà. No obstant, la variació genètica és la matèria primera sobre la qual actua l'evolució a llarg termini (Thompson et al. 2003) i la resposta evolutiva a la selecció depèn de l'heretabilitat (Lande & Arnold 1983).

En les anàlisis d'aquesta tesi es va realitzar una aproximació completament geneticoquantitativa per estimar la selecció en la morfologia cranial. Combinant dades demogràfiques i morfològiques, es van obtenir mesures directes dels components de la selecció en humans. Els resultats d'aquestes anàlisis indiquen que l'evolució d'un fenotip tan complex hauria de ser contemplat sota una perspectiva multifactorial (Capítol 6). El resultat final de l'evolució és probablement el producte de múltiples forces microevolutives que actuen simultàniament i produeixen diferents efectes sobre el fenotip cranial. Per comprendre amb profunditat aquest procés, tots aquests factors haurien de ser considerats.

El més destacat de tots aquests resultats (Capítols 3 a 6) és que la integració morfològica és un factor clau en l'evolució que regula la variabilitat del crani humà. La integració morfològica és lo suficientment feble com per permetre la diversificació poblacional de la forma cranial, però és també lo suficientment forta com per preservar una estructura operacional del crani humà.

Conclusions

Les principals conclusions emergents d'aquest treball de tesi doctoral s'enumeren a continuació:

1. Hi ha una quantitat considerable de variació genètica que determina tant la mida com la forma del crani humà. Les tres principals regions del crani (la cara, el neurocrani i el basicrani) presenten nivells similars de variació genètica.

2. Aquestes grans quantitats de variació genètica conferirien al crani una gran capacitat per evolucionar. Tanmateix, aquesta capacitat està limitada per patrons complexes de covariació entre regions.

3. El crani humà és una estructura fortament integrada, tant a nivell genètic com fenotípic. La integració genètica és dominant i està estructurada jeràrquicament.

4. La variació craniofacial no està regionalitzada, sinó que s'estén per tot el crani de manera que no s'observa cap tret cranial individual associat a una determinada variació genètica o fenotípica.

5. La integració es detecta a diferents nivells. Quan es considera tot el crani, la cara, el neurocrani i el basicrani tendeixen a comportar-se com una unitat, mostrant la correspondència més clara entre els patrons de covariació genètica i fenotípica. No obstant, la cara mostra una relativa independència. A una escala menor, la integració s'observa entre determinades regions funcionals (com per exemple entre les òrbites i els arcs zigomàtics), però no en altres (com les regions anterior i posterior del neurocrani).

6. La base del crani és una regió clau a través de la qual s'integren la cara i el neurocrani. El crani estableix els fonaments de la forma cranial, establint límits entre les dimensions facials i del neurocrani.

7. El patró integrador dominant ve determinat per la covariació entre les amplades màximes del crani. Aquest patró s'ha conservat al llarg de l'evolució dels cranis dels mamífers.

8. Les hipòtesis tradicionals d'integració morfològica que suggereixen una distinció entre cranis braquicèfals i dolicocèfals no reflecteixen l'arquitectura genètica del crani humà. Els patrons de correlació genètica i fenotípica entre les dimensions facials i neurocranials no segueixen el patró esperat de variació.

9. Les matrius de covariació genètica i fenotípica són similars però no idèntiques ni proporcionals. Les matrius de covariació genètica mostren patrons d'integració morfològica més complexes i estructurats que les matrius de covariació fenotípica. Aquest resultat s'hauria de tenir en compte en aquells estudis que utilitzen la matriu de covariació fenotípica en substitució a la matriu de covariació genètica.

10. Les matrius de covariació genètica també indiquen l'existència de límits genètics al canvi morfològic, que redueixen el potencial evolutiu del crani humà. Aquests límits corresponen a característiques morfològiques que no poden evolucionar perquè no tenen suficient variació genètica heretable.

11. La capacitat evolutiva del crani humà està restringida i dirigida cap a determinades trajectòries de canvi morfològic que mantindrien una forma cranial operativa i funcional.

12. La combinació dels mètodes de la morfometria geomètrica i de la genètica quantitativa multivariada constitueix una aproximació robusta i potent per explorar els patrons evolutius de fenotips complexos. A diferència de les aproximacions univariades, les aproximacions multivariades poden detectar patrons evolutius més complexes perquè tenen compte la covariació entre trets i per tant revelen la complexitat anatòmica, funcional i del desenvolupament del crani humà.

13. Aquesta metodologia és una aproximació alternativa per buscar mòduls en fenotips complexes. A diferència d'altres aproximacions basades en hipòtesis, no és necessari formular cap assumpció prèvia de modularitat.

14. El desenvolupament ha jugat un paper molt important en l'evolució del crani humà. És probable que a través de la integració genètica s'hagi regulat la cascada d'efectes morfològics desencadenada per uns pocs canvis en el programa del desenvolupament, com suggereixen les hipòtesis evo-devo.

15. Si es consideren els forts efectes de la integració morfològica, és difícil acceptar que els principals caràcters derivats dels humans moderns siguin caràcters independents. Aquest resultats té doncs implicacions profundes per les anàlisis filogenètiques i cladístiques.

16. L'origen de qualsevol dels caràcters derivats dels humans moderns pot haver facilitat d'evolució dels altres, fet que suggereix una reinterpretació dels escenaris selectius de l'evolució humana.

17. Els canvis morfològics associats a l'evolució del bipedisme podrien haver estimulat l'evolució d'una volta cranial més gran i més arrodonida, que posteriorment podria haver estat afavorida per la selecció per un cervell més gran i més complex, com indiquen les evidències moleculars. Juntament, aquests trets haurien evolucionat en correlació amb la retracció facial i la flexió de la base del crani. Així, tot i que el temps i la integració semblen haver esborrat els efectes de la selecció en el crani humà, això no pot ser interpretat com absència d'acció de la selecció natural.

18. De fet, la selecció natural ha actuat en l'evolució del crani humà. A la població de Hallstatt s'ha detectat que durant els últims 200 anys una

forta selecció direccional ha actuat sobre la forma del crani i una dèbil selecció estabilitzadora ha influenciat la mida cranial.

19. Altres forces microevolutives van contribuir a l'evolució de la morfologia cranial però en direccions oposades a les seleccionades; per això els patrons seculars no es corresponen als patrons de resposta a la selecció. Forces com la deriva gènica o el flux gènic no serien causes suficients d'aquest patró. Els factors ambientals també poden haver participat en aquest procés.

20. El crani està sota l'acció de nombroses forces evolutives, que actuen en al mateix temps i dirigint el canvi morfològic o bé cap a la mateixa direcció o bé en direccions diferents i fins i tot oposades. El crani respon a aquestes pressions a través de complexes xarxes d'interacció genètica i epigenètica.

21. L'aproximació multivariada que s'ha dut a terme en aquest estudi reflecteix la gran complexitat morfològica del crani humà. La mostra de cranis de Hallstatt (Àustria) constitueix un col·lecció única perquè disposa d'informació genealògica associada i a partir del seu estudi es podrà aprofundir en el coneixement dels processos evolutius del crani humà.

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11 Appendix

Appendix

In this section are provided all the details about sample composition and genealogy reconstruction: the number and characteristics of the analyzed skulls, the way in which the decorated skulls were identified, the procedure for skull measurement, as well as the process of familiar reconstruction. Hence, two datasets are presented: the craniometric and the demographic.

SAMPLE: SKULL DATABASE

Data collection was carried out in Austria between November and December 2004 in collaboration with Dr. Sjøvold. During that stay, Sjøvold's pedigrees were revised and extended and only the skulls falling into his pedigrees were measured. The skulls were identified, taken out from the charnel house and measured at an equipped room of the parish house, which is some meters away from the charnel house. The measuring could not be carried out at the charnel house because there was neither electrical supply, nor heating in there. After measuring, the skulls were returned to their place.

The Hallstatt skull collection consists of more than one thousand skulls, from which around 700 are decorated. The collection represents a time span of more than two hundred years, since the oldest skull dates back from 1775 and the last one was included in 1986. From the whole decorated skull collection, almost 60% of the skulls were identified. The majority of the identified skulls derive from the 19th century: 51.8% of the individuals were born between 1800 and 1849 century and 64.6% died between 1850 and 1900.

SKULL IDENTIFICATION

Skull identifications were made by Sjøvold, and herein it is described how this was accomplished. Identifications were based on the decoration and writings of the skulls as well as on the church records of births, marriages and deaths,

which extend back to 1602. The first step was to identify the skulls by the name painted on his forehead, and afterwards they were looked up in the parish records. Obviously, the name was the main information for identification, but the kind and pattern of decoration also provided valuable clues. Each gravedigger had a personal artistic style decorating the skulls, and this was helpful in order to establish an approximate date of decease of "uncertain" individuals. In several cases, it even allowed the differentiation between several persons sharing the same name. When an individual was unequivocally found at the parish, his family was traced back as many generations as possible.

Although skull identifications were carried out with extreme care, they totally rely on several factors that are almost out of the research control, such as the previous identification made by the gravedigger and the accuracy of the name inscription or the information provided by the parish records. All of these should be regarded as potential sources of error. Some of these errors have been noticed and corrected, but it should be admitted that a low proportion of undetectable erroneous information might be present in the dataset presented in this thesis.

The work of the gravedigger was of crucial importance for this study because it was compelling to trust his identifications. However, in an attempt to further validate them, the individual's sex assignment was confirmed by performing a discriminant function analysis. It is acknowledged that this is just a partial appraisal, since it only detects those cases in which a female and a male have been confused and neglects those cases where two individuals of the same sex may have been exchanged. However, other kinds of errors are almost impossible to detect. Results showed that 8 skulls presented a posterior probability higher than 0.85 of being the opposite sex. These individuals were considered as misidentifications and were thus removed from the dataset.

Despite this fact, it must be noted that this error is expected to be low for several reasons: first, because it is logic to expect that gravediggers paid the most attention when exhuming the ancestors of their neighbors (or even of their relatives); and second, because if any mistake was made, the most probable is that members of the same family buried within the same grave were confused. Considered together, the above observations significantly reduce the expected error and/or its influence in the analyses performed, which in any case would underestimate the additive genetic component of the morphological phenotype.

When reanalysing the skeletal material, thanks to Sjøvold's photographic records dating back to the 80's, the current status of the skulls was compared

to its appearance during Sjøvold's first surveys. Stunningly, it was found that the names of several individuals had been changed because the skulls had been redecorated during the last few years. These skulls were included in the sample but using the old-worn but correct name.

Regarding the accuracy of the parish records, just some misprints or few obvious errors could be detected, as such that provided information about a given family that was biologically unfeasible (e.g. when a mother had two children in less than several months, or when the mother was too young to give birth to a baby, etc). In any case, information concerning motherhood will always be more assured than that of fatherhood.

In spite all of these pitfalls (which are acknowledged but considered rather small), it should be remarked that this dataset is one of the richest ones in the anthropological field, and that it is very rare to find ancient complete demographical information associated to a large and well conserved skull collection. Thus, once all the information has been checked out as thoroughly as possible, this dataset should be analysed in deep detail because it can shed light into many biological and evolutionary aspects of the human skull.

SKULL MEASUREMENT

Skulls were measured using a 3D digitizer, a Microscribe G2X (Immersion, Inc), and herein is described the way in which proceeded the landmark recording. The measurement protocol included 65 anatomical landmarks that were digitized in two consecutive recordings. The skulls had to be oriented in two different positions because there was no way to access all the facial, neurocranial and basicranial points from a single orientation. The crania were fixed at the table with plasticine and each recording was made as follows. At the first recording, the skulls were placed lying on the posterior neurocranial region (e.g. the nape) in order to digitize landmarks mostly representing facial structures, though a few neurocranial points were also included. At the second recording, the skulls were placed laying on its right side and recorded points represented mainly the neurocranial and basicranial regions.

In order to automatically match the two recordings, three landmarks (namely the nasion, bregma and hormion) were used as custom reference frame in the MUS software (Microscribe Utility Software). This way, the digitized landmark coordinates were always oriented within the same frame despite the orientation of the skull: nasion was defined as the origin of coordinates (x:0, y:0, z:0), hormion was constrained to point the x direction (x, y:0, z:0), and bregma to point the y direction (x, y, z:0). In order to guarantee the accuracy of this procedure, nasion was measured in both orientations, so

that it was verified that its coordinates were always close to 0 (though a deviation of less than 1 mm was accepted). These three landmarks were selected due to several reasons: first, because they are anatomical landmarks that can be easily located; second, because they were accessible in both orientations; and third, because this procedure also oriented landmark coordinates along the sagittal plane (therefore theoretical sagittal landmarks had a z coordinate equal to 0; and symmetrical right and left landmarks only differed in the sign of the z coordinate, one being negative and the other one positive).

The landmark coordinates were registered in an Excel spreadsheet, along with information for every individual about its name, sex, age and any other kind of relevant skull characteristics (as for example skull and dental pathologies, missing or deformed parts, maturity of dentition, etc.).

SAMPLE COMPOSITION

Originally, 406 individuals were measured. The exact number of individuals used in the specific analyses is detailed in the Materials and Methods section of each results chapter (Chapters 3 to 6). The bulk of skulls were measured in Hallstatt (at the *Beinhaus* of the Hallstatt charnel house and at the *Hallstatt Museumsverein*), but also at the *Anatomisches Institut* in Innsbruck, the *Naturhistorisches Museum* and the *Österreichisches Museum für Volkskunde* in Vienna.

The sample includes individuals from both sexes. The majority of them are adults, though there are also a number of children and juveniles. Exact numbers are also reported at the Materials and Methods section of each results chapter (Chapters 3 to 6). Besides the age reported at the demographical records, age was also assessed by skeletal and dental criteria: adulthood was determined by a fully closed spheno-occipital suture, as well as by definite dentition and molar eruption.

Skull conservation is fair good, although high levels of alveolar resorption are frequent. Moreover, as much as 11.1% of the individuals showed craniosynostosis, a premature cranial suture closure which results in a characteristic occipital flattening and prominent forehead. Strongly deformed individuals were not considered.

DATA PREPARATION AND MISSING DATA

To ascertain the validity of the analyses, we accounted for outlier and missing data. Outlier points for each landmark coordinate were detected by means of Box and Whisker plots (assuming a value of 1.5 as outlier coefficient). Overall

mean percentage of outliers was less than 1%. These points were deleted and considered as missing data.

Missing data treatment started with the quantification of percentages of missing cases per landmark and per individual. Those landmarks showing more than 10% of missing cases and those individuals presenting more than 20% of missing landmarks were deleted from the whole database. Six landmarks were thus removed from the database: ectomolare right and left (61.52%), inner prosthion (60.29%), palate (56.37%), prosthion (56.13%), and inner petrous (20.30%). Most of these landmarks correspond to the oral region because missing values were mainly due to alveolar resorption. Furthermore, 16 fragmentary individuals were also removed from the database. Finally, the overall percentage of missing data was of 2.18%.

Missing values were replaced by two different methods. If the missing landmark had a symmetric counterpart (as for example the nariales, the asterions, or the orbitales), it was directly replaced by coordinate reflection: this was done by copying the x,y,z coordinates of the symmetric landmark and changing the sign of the z coordinate. If not, missing data were replaced by multiple regression. This method analyzes the relationship between a dependent variable and several independent or predictor variables by performing least-squares multiple linear regression. This method is preferable to other methods using the mean or mode distribution because the multiple regression takes many factors into account to predict the values of the missing data and thus also reflect individual size or sex differences.

FAMILY RECONSTRUCTION

The genealogies used in the present thesis were reconstructed by Dr. Mireia Esparza from the Universitat de Barcelona, an expert in biodemographical research working in the research team led by Dr. Miquel Hernández. Dr. Esparza joined the Hallstatt project in 2005 in order to analyze the demography of Hallstatt's population and the heritability of life-history traits and fitness measures in humans (such as reproductive span, longevity, fecundity, age at first and last birth, mean interbirth interval, offspring survival, and lifetime reproductive success). This project is independent but complementary to the research project presented in this thesis.

The first step for family reconstruction was to collect all the available demographic information from Hallstatt population from the 17th to the 19th century. The original parish records are preserved at Hallstatt's Catholic Church: the records from 1602 to 1852 consist of book records with consecutive entries handwritten in German Gothic lettering; afterwards church

records were registered in alphabetical order in Latin writing. Before 1852, all the population were registered at the Catholic Church, but after that year the Protestants were registered at their own parish books. The parish also has an alphabetical typed copy for the records covering the first period (1602-1852), which was reproduced from the original books at the sixties. Original registers contained more exhaustive information than typed registers, which include basic information like name, surname, date of birth, marriage or death, name of the parents (though not always) and age. Original registers also provide information about grandparents, occupation, familiar house, cause of death, etc.

All the demographic registers were gathered thanks to Sjøvold's collaboration. In the first place, typed records were photocopied, which included four baptisms books, two of marriages, three of deaths and one of immigrants. On the second place, original German Gothic handwritten records were also consulted in order to complete the information and to fill up some gaps found at the typed registers. And finally, original registers from 1852 to 1900 both from the Catholic and the Protestant parishes were photographed in order to extend the analyzed period.

The second step for family reconstruction was to assemble all this information in a single database. Note that this database includes all the population from Hallstatt from 1602 to 1900, not only population concerning families from which crania are available. Birth, marriages and death records were transcribed into computer using separate Excel spreadsheets, and thus three databases were created: a birth database (BDB), a marriage database (MDB) and a death database (DDB). From these, two interrelated databases were constructed: an individual database (IDB) and a family database (FDB). The individual database was created through the combination of the birth and death databases (IDB=BDB+DDB); whereas the family database was created from the marriage database (FDB>MDB). A specific software tool for family database reconstruction was created using Ruby on Rails (V. Jalencas 2007, unpublished), although the whole database was personally revised case by case. The main problem of family reconstruction is that there are no univocal relationships between registers, so that individuals are identified by a number of coincidences rather than by exact matching between databases.

The individual database (IDB) was set up as follows. First, male individuals were created by seeking the correlation between surname and name at BDB and DDB. An identification number (ID) was given to each individual. In order to verify this initial identification, it was checked if the date of birth was consistent with the age at death, a piece of information that was usually

available at the death registers. If the age at death was not recorded, it was checked out that there were no incompatibilities between the dates of birth and death (for instance, it was verified that the date of death was posterior to the date of birth, or that the difference in years between birth and death was considerable enough if the death register reported that the individual was married or widower, etc.). Whenever it was possible, it was also checked if the name of the parents were coincident at the two databases (BDB and DDB). If the individual was just registered either at the birth or at the death records (because the individual had emigrated from or immigrated to Hallstatt), the individual was created based on any of these recordings. If individuals were identified according to the death register and the age at death was available, an approximate date of birth was estimated.

This kind of identification was only done with male individuals because females changed their surname when they got married, adopting the surname of their husband. Therefore, it was necessary to look at the marriages database (MDB) before female individuals could be identified. Families (FDB) were created from the marriage registers (MDB) and an identification number was given to each family (which is independent from the individual's ID). The reconstruction started with those marriages where the male was bachelor (or when it was not specifically reported that he was widower). These families were linked to the male individuals already created at the IDB, checking that the name, surname and dates of birth and death consistently matched. Once this was finished, the same protocol was repeated for widower males by searching among the married males, and having always checked that the first wife died before the widower husband married again.

Finally, females were assigned to families (FDB). At the same time as families were created, females were searched at the birth (BDB) and death (DDB) databases and incorporated to the individual database (IDB), following exactly the same procedure as with males. Married female individuals have two surnames (the surname of their husband, as well as their maiden surname). Those females that did not marry were created and given an ID number afterwards.

Once all male and female individuals were created at the IDB database and all families were founded from their corresponding marriages (FDB), offspring was assigned to each family. This was done by looking for coincidences among the parents' name and surname between BDB and FDB. This step was repeated looking for the correspondences between DDB and FDB, because it was noticed that if children had died on the same day they had been born, they were only registered at one of the two databases, either BDB

or DDB. When all of this was achieved, it was confirmed that everything was consistent, and that the "story" of every family was biologically feasible.

Finally, as it was detected that within the same family different variants of the same surname occurred, surnames were unified choosing the most frequent variant among all the families. If several variants were almost equally common, they were accepted as different if they still exist at present Hallstatt population. This was done by searching the different variants at the Austrian telephone directory (http://www.herold.at/). When variants were not found, they were substituted and unified using the current variant. According to this, all members of the same family shared the same surname's variant.

Following this procedure, the demographical database was completed and included 18,134 individuals.