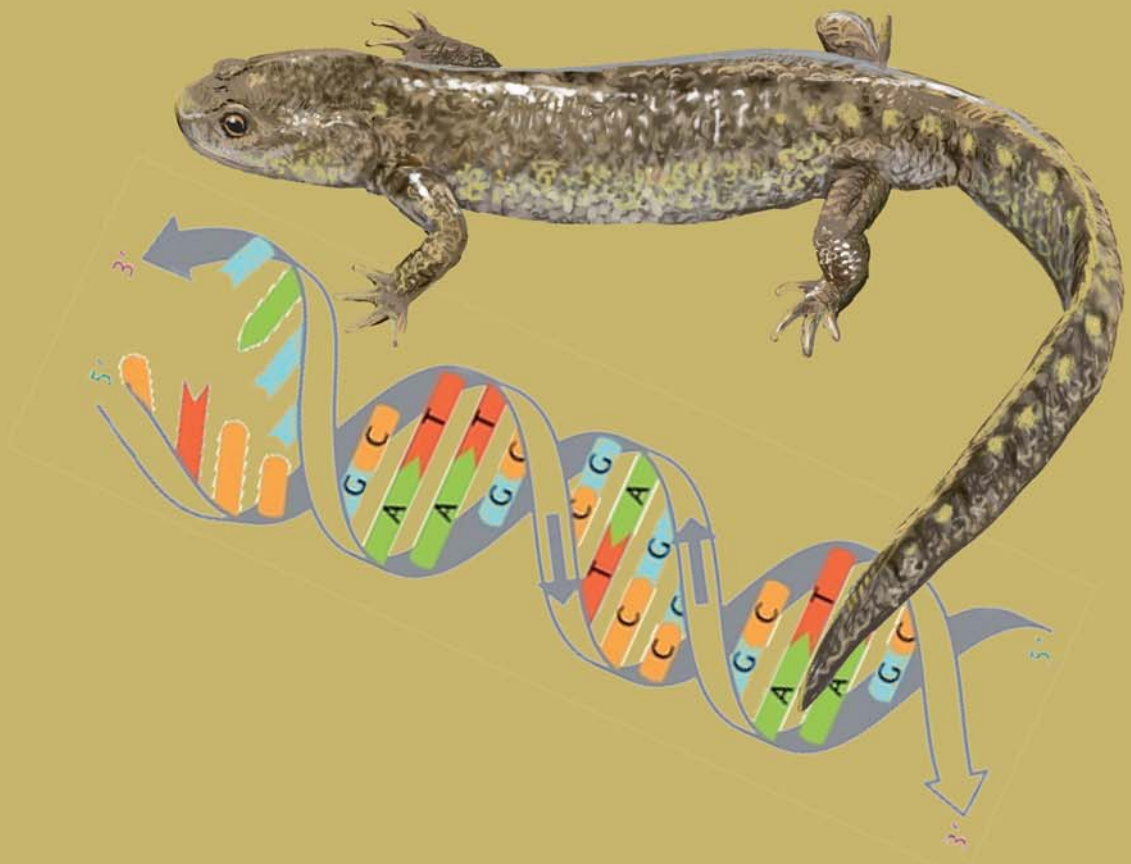
Tesi doctoral

Conservation genetics of the critically endangered Montseny brook newt (*Calotriton arnoldi*)



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Advantage or burden – habitat fragmentation in a critically endangered amphibian species (*Calotriton arnoldi*) and its implications for species conservation

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Running title: Consequences of habitat fragmentation

Abstract

Endemic species with restricted geographic ranges suffer potentially the highest risk of extinction and pose a special challenge for conservation. If these species are further fragmented into genetically isolated subpopulations, the risk of extinction might be even higher. Habitat fragmentation is generally considered to have negative effects on species survival, although there is also sporadic evidence for neutral or even positive effects. Nevertheless, these effects have been dismissed in conservation biology. Endemic to a small mountain range, the Montseny brook newt (*Calotriton arnoldi*) is one of Europe's amphibian species with the smallest distribution range (8 km^2) and is considered critically endangered according to the IUCN. Here, we analyzed its population structure using 24 microsatellite loci and found that habitat fragmentation due to a natural barrier has resulted in strong genetic division of populations into two sectors, with no detectable migration between sites. Although effective population size estimates suggested critically low values for all populations, we found very low levels of inbreeding or relatedness among individuals within populations. In contrast, genetic diversity in *C. arnoldi* is in the range and therefore comparable to populations of amphibian species with much larger distribution ranges. Our extensive study shows that natural habitat fragmentation had no negative effect on an evolutionary time scale and it seems that species may evolve reproductive strategies (e.g. mating preferences) to cope with rather small population sizes. Nevertheless, habitat fragmentation must be considered distinct from habitat loss or degradation. The effect of both should be regarded independently when planning the conservation strategies for an endangered species.

Introduction

Among threatened species, those that are endemic to a restricted spatial area should *per se* experience a higher risk of extinction. In general, the fragmentation of a species range into smaller subunits by external factors such as anthropogenic activities (Blank *et al.* 2013; Martínez-Cruz *et al.* 2007; Storfer *et al.* 2013) or climatic events (Garner *et al.* 2004; Veith *et al.* 2003; Zancolli *et al.* 2014) is known to pose a major threat, as gene flow among small or isolated sub-populations may be very restricted (Ewers & Didham 2006; Kim *et al.* 1998; Sunny *et al.* 2014). Long-term survival of these populations may be compromised due to stochastic and drift events (Sunny *et al.* 2014).

Therefore, one of the main goals in conservation biology is to identify the genetic structure and diversity of species at the population level and the current patterns of gene flow between populations (Apodaca *et al.* 2012 and references therein). The amount of gene flow among populations of a given species highly depends on its dispersal propensity (i.e. the probability of dispersal between habitat patches) and realized dispersal rates (Slatkin 1994). Organisms with lower dispersal rates are more susceptible to isolation than those with higher dispersal rates. Thus, dispersal may counteract the loss of gene flow among populations and, therefore, has been shown to be an important factor for the long-term survival of species (Allentoft & O'Brien 2010). Strong genetic differentiation among populations is a sign of interrupted gene flow and most likely a lack of dispersal of individuals between them. In conservation biology, mainly non-natural external factors such as human-induced changes are considered to have caused habitat fragmentation, hindering individuals to disperse (Templeton *et al*. 1990). However, both species-intrinsic and naturally driven processes might also lead to strong genetic differentiation of sub-populations which, in consequence, could have the same effect as non-naturally induced causes. Such processes may include, for example, differential habitat adaptation of populations (e.g. Manenti & Ficetola 2013; Steinfartz *et al.* 2007; Urban 2010; Vences & Wake 2007). In general, the division of a species into populations via habitat fragmentation has been considered to pose negative effects on species survival and persistence of populations. However, it has been suggested that in some instances habitat fragmentation can have neutral or even positive effects (Fahrig 2003; Templeton *et al.* 1990).

Amphibians are generally considered to have limited dispersal abilities, causing genetic differentiation across small geographic scales (Monsen & Blouin 2004 and references therein), although more recent studies indicate that in some cases dispersal propensities have been underestimated (e.g. Smith & Green 2005). The notable sensitivity of amphibians to environmental changes and habitat fragmentation are other factors that may reinforce patterns of sharp genetic discontinuities over short distances (Savage *et al.* 2010; Storfer *et al.* 2013; Velo-Antón *et al.* 2013). Therefore, data on gene flow among amphibian populations can also help in determining if a metapopulation structure exists (Marsh & Trenham 2001; Smith & Green 2005; Storfer 2003; Storfer *et al.* 2013). Whether populations of a given species act as a metapopulation has a direct influence on management programs and the appropriate conservation strategies that should be taken, such as determining the number of

breeding lines and translocation actions (Neuwald & Templeton 2013; Sunny *et al.* 2014).

The Montseny brook newt (*Calotriton arnoldi*) is only known from the Montseny Natural Park in the NE Iberian Peninsula and represents one of the most spatially restricted European amphibian species (Sillero *et al.* 2014). Here, its disconnected populations are found within a restricted altitudinal range in seven geographically close brooks. Although the historic range of this species is unknown, currently it occupies a total area of 8 km^2 (Amat & Carranza 2009). Moreover, its habitat is fragmented into two main sectors (eastern and western) on both sides of the Tordera River valley and separated by unsuitable terrestrial habitat. The current census population size of this species has been estimated to be less than 1500 adult individuals (Amat & Carranza 2005), whereas estimates for its effective population size are missing. Hence, *C. arnoldi* is one of the most endangered vertebrates in Europe and it is classified as critically endangered by the International Union for Conservation of Nature (IUCN) (Carranza & Martínez-Solano 2009). Recent human activities such as the extraction of large amounts of water for commercial purposes, deforestation and the building of tracks and roads are disturbing the habitat and are affecting the species negatively (Amat 2005; Amat & Carranza 2006; Amat *et al*. 2014; Barber *et al.* 2014). A previous study based on mitochondrial (Cyt *b*) and nuclear (RAG-1) sequence analysis already suggested a high degree of genetic isolation between the eastern and western sectors, which is further supported by morphological differentiation (Valbuena-Ureña *et al.* 2013 – Chapter IV). Thus, the observed fragmentation of this species into highly genetically isolated populations is probably the result of an ancient naturally driven intrinsic fragmentation process rather than caused by the recent human disturbances mentioned above. However, a detailed analysis based on population structure, gene flow among populations and estimates of effective population sizes is still missing. All these studies are crucial for the understanding of past and ongoing evolutionary processes and their meaning for the conservation of such a spatially restricted endemic species.

Although there are several studies of species with very limited distribution ranges (e.g. Sunny *et al.* 2014; Wang 2009) and examples of amphibian species with highly structured populations (Blank *et al.* 2013; Blouin *et al.* 2010; Monsen & Blouin 2004; Savage *et al.* 2010), the critically endangered *C. arnoldi* represents an exceptional example to evaluate the positive or negative consequences of naturally driven species fragmentation in conservation. In this study, we aim at delineating the population

structure of *C. arnoldi* by using a set of 24 polymorphic microsatellite loci markers with a special focus on relevant conservation parameters such as effective population size and diversity indices and discuss the implications of natural fragmentation for the conservation of endemic species with restricted spatial ranges.

Material and methods

Sample collection and genotyping

A total of 160 individuals of *C. arnoldi* were analyzed, including samples from all known seven local populations of this species (Fig. 1). For conservation reasons, the three eastern populations are herein referred to as A1, A2, A3, and the four western populations as B1, B2, B3 and B4. Samples included 77 individuals from the eastern sector (23 from A1, and 27 from each A2 and A3 populations) and 83 individuals from the western sector (25 from B1, 28 from B2, 26 from B3 and 4 from B4). B4 is the less represented population as a result of the low abundance of individuals at this site. Therefore, results from this population should be treated with caution.

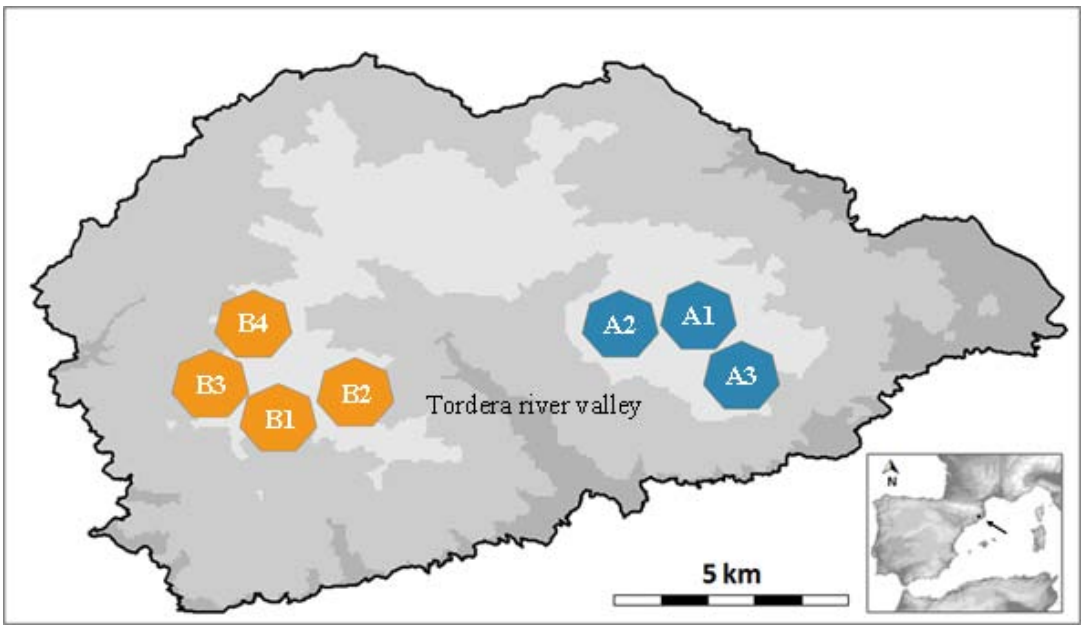

Figure 1. The distribution range of the Montseny brook newt, *Calotriton arnoldi*. All localities have been sampled for this study.

Tissue samples consisted of small tail or toe clips preserved in absolute ethanol. Genomic DNA was extracted using the QiagenTM (Valencia, California) DNeasy Blood and Tissue Kit following the manufacturer's protocol. Individuals were genotyped for a total set of 24 microsatellite loci: 15 specifically developed for *C. arnoldi* (Valbuena-Ureña *et al.* 2014 – Chapter V) and nine additional loci originally developed for the closely related sister species *C. asper* that cross-amplify successfully in *C. arnoldi* (Drechsler *et al.* 2013). Microsatellite loci were multiplexed in 5 mixes using the Type-it multiplex PCR (Qiagen). Primer combinations of the 5 mixes are provided in the supplementary material (Supp. Table 1). PCR conditions and genotyping of loci followed the descriptions provided in Drechsler *et al*. (2013).

Basic population genetic parameters

The MICRO-CHECKER software (Van Oosterhout *et al.* 2004) was used to check for potential scoring errors, large allele dropout and the presence of null alleles. Pairwise linkage disequilibrium between loci was checked using the software GENEPOP version 4.2.1 (Rousset 2008). The same program was used to calculate deviations from Hardy-Weinberg equilibrium in each population and for each locus, which provides an exact probability value (Guo & Thompson 1992). Genetic diversity was measured for each sampling site as the mean number of alleles (A), observed (H_O) and expected heterozygosity (H_E) and allelic richness (Ar) using FSTAT version 2.9.3.2 (Goudet 1995). The observed number of private alleles for each locus and each population was calculated with GDA (Lewis & Zaykin 2000), and a rarified measure of private allele richness (PAAr) was obtained with HP-RARE (Kalinowski 2005). Overall and intrapopulation subdivision coefficients (F_{IS}) for all markers and sampling sites were calculated using FSTAT, which calculates the estimator *f* of Weir & Cockerham (1984) for each marker as well as a multilocus estimate.

Population structure analysis

The differentiation between all pairs of populations (F_{ST}) and the correct *P* values for population differentiation among pairs of populations were tested with FSTAT. The estimator D (Jost 2008) was also calculated using the R package DEMEtics (Gerlach *et al.* 2010) with 1000 bootstrap iterations to obtain *P*-values and confidence intervals. In addition, the programs STRUCTURE version 2.3.4 (Pritchard *et al.* 2000) and GENETIX, version 4.05.2 (Belkhir *et al.* 2004) were used to analyze the population

structure of *C. arnoldi* across its distribution range. STRUCTURE uses a Bayesian clustering algorithm and assigns individuals to clusters without using prior information on their localities of origin. Used settings included an admixture model with correlated allele frequencies and the number of inferred clusters (K) ranging from one (complete panmixia) to eight (i.e. the number of sample locations plus one), with10 runs for each K with one million Markov chain Monte Carlo (MCMC). Iterations were performed after a "burn in" period of 100,000. We also ran STRUCTURE with the same parameters for each sector (eastern and western) separately to check for possible genetic substructure within sectors. The optimal number of clusters was inferred using the ΔK method described by Evanno *et al.* (2005) as implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2012). The average from all the outputs of each K was obtained with CLUMPP version 1.1.2 (Jakobsson & Rosenberg 2007) and then plotted with DISTRUCT version 1.1 (Rosenberg 2004). The degree of genetic differentiation of population k from the ancestral population was assessed by the estimator F_k obtained with STRUCTURE for each population cluster k. GENETIX software was also used to examine the population structure by performing a factorial correspondence analysis (FCA) on the allelic frequencies obtained for the seven Montseny brook newt populations. This analysis was performed across the distribution range of *C. arnoldi*, as well as in each sector separately to examine the existence of substructure within them. Analysis of molecular variance (AMOVA) was performed based on the results of STRUCTURE and grouping populations in ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010) based on 2000 permutations. Furthermore, isolation by distance (IBD) was evaluated by analyzing the relationship between geographical and genetic distances between populations with a Mantel test (Mantel 1967). Analyses were carried out separately among all sampled populations and for each sector with the software ARLEQUIN. Since the life style of *C. arnoldi* is strictly aquatic (Amat & Carranza 2009; Carranza & Amat 2005) distances were calculated following the watercourse and log-transformed to linearize the relationship between geographic distances and F_{ST} values (see Rousset 1997). Genetic distances were calculated as F_{ST} /(1- F_{ST}) and the significance of matrix correlation coefficients was estimated by 2000 permutations.

Analysis of recent gene flow

Recent gene flow events between sectors and among populations within sectors were assessed using three programs: GENECLASS 2.0 (Piry *et al.* 2004),

STRUCTURE and BIMr (Faubet & Gaggiotti 2008). The program GENECLASS uses Bayesian criteria for calculating the probability that a certain individual comes from a particular population (Rannala & Mountain 1997). A Monte Carlo resampling algorithm (10000 simulated individuals, $\alpha = 0.01$) following the recommendations by Paetkau *et al.* (2004) was used for the probability computation. Detection of first-generation migrants was based on the ratio $L = L_h/L_{max}$, where L_h describes the likelihood to find an individual in its original population and L_{max} describes the maximum likelihood of assignment to any sampled population. This ratio is more powerful compared to other estimators such as L_h and is the most appropriate if all source populations have been sampled (Paetkau *et al.* 2004). The program STRUCTURE was reran with the option USEPOPINFO (i.e. prior sample site information). This program detects migrants by calculating a Q value that is the proportion of that individual's ancestry from a population. An individual is a putative migrant when its Q value for its origin site (Q_o) is significantly lower than the Q value for its site of assignment (Q_a). BIMR was used to estimate migration rates of recent gene flow ($N_{gen} \leq 2$) between populations within sectors. BIMR uses a Bayesian assignment test algorithm to estimate the proportion of genes derived from migrants within the last generation and assuming linkage equilibrium and allowing for deviation from Hardy-Weinberg equilibrium. We estimated migration rates among populations within sectors separately. For each analysis, a Markov chain using a burn-in period of 50,000 iterations, followed by 50,000 samples collected using a thinning interval of 50 was run. Convergence of the Markov chain was assessed by repeating the analyses independently five times. Pairwise migration rates between and within populations across runs were averaged.

Estimation of effective population size (N_e)

The effective population size (N_e) for each cluster resulting from STRUCTURE based on one-sample N_e estimator method was calculated with three programs: ONeSAMP (Tallmon *et al.* 2008), COLONY version 2.0.4.4 (Jones & Wang 2010), and LDNe version 1.31 (Waples & Do 2008). ONeSAMP employs approximate Bayesian computation and calculates eight summary statistics to estimate N_e from a sample of microsatellite loci genotypes. The analyses were submitted online to the ONeSAMP 1.2 server (http://genomics.jun.alaska.edu/asp/Default.aspx), which uses an Approximate Bayesian Computation method. After using different priors and obtaining similar results, values were specified to a minimum N_e value of 2 and a maximum N_e value of

100 and 500 for populations or clusters, respectively. COLONY implements a maximum likelihood method to conduct sibship assignment analyses, which are used to estimate N_e. COLONY results were calculated assuming random mating. Finally, N_e with the one-sample linkage disequilibrium method (Hill 1981) was calculated as implemented in LDNe obtaining confidence intervals with a jackknife method. The criterion P_{crit}=0.02 (alleles with frequency lower than 0.02 are excluded) was used as following the recommendations of Waples & Do (2010) to avoid bias caused by rare alleles but still getting a high precision.

Relatedness of individuals

In order to test the levels of endogamy, the software MLRELATE (Kalinowski *et al.* 2006) was used to estimate the relatedness among individuals within each population. This program is appropriate as it is designed for microsatellite loci, is based on maximum likelihood tests, and considers null alleles. Furthermore, GenAlEx v. 6 (Peakall & Smouse 2006) was used to obtain pairwise relatedness among individuals in each population separately using the r_{qg} estimator (Queller & Goodnight 1989). Mean pairwise relatedness values were calculated and compared with the 95% confidence interval (CI) estimates determined by 999 bootstrap resampling, at a sector level. Significant differences among mean population relatedness were tested using a permutation test (Peakall & Smouse 2006), and genotypes from all populations by sector were permuted 999 times and derived upper and lower 95% intervals for the expected range of r_{qg} based on all populations. These intervals represent the range of r_{qg} that would be expected under random mating across all populations within sectors. Population r_{qg} values that fall above the 95% expected values from permutations indicate increasing relatedness due to processes such as reproductive skew, inbreeding, or drift among populations within the same sector.

Results

Genetic diversity for each sampled population and cluster obtained from the genetic structure analyses is given in Table 1 (see Supp. Table 2 for locus-specific results). Loci Us3 and Us7 were monomorphic for populations within the western sector. We further found that some alleles were fixed for some populations: Calarn15906 was found to be monomorphic in population B2, seven loci (Calarn 29994, Calarn06881, Calarn36791, Calarn52354, Calarn31321, Calarn15136 and Us2) were fixed in population B3 and loci

Calarn15906 and Ca32 showed no polymorphisms in individuals of population B4. Observed number of alleles per locus ranged from four to 12 with a mean of 7.08 and mean number of alleles in the eastern and western populations were 5.50 and 3.96, respectively.

Table 1. Estimates of genetic parameters for each *Calotriton arnoldi* population and cluster. Values represent averages across 24 loci. N, sample size; A, number of alleles per locus; Ar, allelic richness; PA, number of private alleles; PAAr, allelic richness of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. Values in bold indicate statistical significance after Bonferroni correction.

Grouping	N	A	Ar	PA	PAAr	H_O	H_E	F_{IS}
Population								
A1	23	4,167	4,167	7	0,311	0,545	0,538	0,017
A2	27	4,042	3,954	5	0,214	0,526	0,516	-0,015
A3	27	4,292	4,222	5	0,215	0,560	0,559	-0,007
B1	25	3,542	3,500	3	0,137	0,467	0,469	-0,005
B2	28	2,917	2,860	2	0,087	0,371	0,380	0,028
B3	26	1,792	1,768	2	0,079	0,230	0,197	-0,121
B4	4	2,333	-	0	-	0,438	0,433	-0,023
Clusters								
Eastern	77	4,167	4,112	75	3,157	0,544	0,538	**0,090**
A1-A2	50	4,099	4,052	19	0,746	0,535	0,526	0,029
Western	83	2,724	2,703	38	1,646	0,359	0,352	**0,184**
B1-B2-B4	57	3,150	3,162	19	0,750	0,418	0,423	**0,073**

There was no sign of linkage disequilibrium between any pair of loci with the only exception of Calarn02248 and Calarn50748 in population B3 after Bonferroni correction (P>0.00018). Only two loci in two different populations showed signs of null alleles (Us7 in A1 and Ca22 in B1). Private alleles (PA) – defined here as alleles exclusively found in a single population throughout the study site, i.e. the species range – are also listed in Table 1. Populations of the eastern sector had 75 PAs, while the western populations had 38 PAs. Allelic richness (AR) per population ranged from 1.77 to 4.22, and expected heterozygosity ranged from 0.197 to 0.559 (weighted average: 0.441) with the lowest value found in B3 and the highest in A3. No significant departures from Hardy-Weinberg equilibrium (P>0.0003) were found after applying Bonferroni correction. Overall, F_{IS} was estimated to 0.380 (P=0.0021), but this parameter did not show values significantly different from zero for each population after applying Bonferroni correction (see Table 1).

Determining population structure

Population differentiation was significant for each pair of population combination (P<0.001) for any of the parameters used (Table 2).

Table 2. Genetic differentiation among populations. Pairwise F_{ST}, below the diagonal; D estimator values, above the diagonal. All P values were significant ($P < 0.001$).

F_{ST}/D	A1	A2	A3	B1	B2	B3	B4
A1	-	0,131	0,243	0,814	0,816	0,852	0,782
A2	0,086	-	0,299	0,855	0,868	0,877	0,822
A3	0,151	0,178	-	0,806	0,801	0,858	0,762
B1	0,457	0,473	0,443	-	0,100	0,249	0,122
B2	0,509	0,524	0,491	0,096	-	0,248	0,146
B3	0,614	0,617	0,599	0,336	0,372	-	0,305
B4	0,443	0,460	0,419	0,109	0,145	0,488	-

F_{ST} values between populations of the eastern *versus* the western sector ranged from 0.443 to 0.617 and estimator D values ranged from 0.801 to 0.877. Pairwise comparisons among populations within sectors were much lower, with F_{ST} and D values within sectors ranging from 0.086 to 0.372 and 0.100 to 0.299, respectively. Population B4 was not included in the F_{ST} and D estimations due to its low sample size (only four individuals). Populations A3 of the eastern and B3 of the western sector were the most differentiated populations when compared with their respective sector populations.

STRUCTURE analysis revealed two highly distinct genetic clusters corresponding to populations included in the eastern and the western sectors (Fig. 2), and the degree of genetic differentiation of each sector from the ancestral population (F_k estimates) was 0.229 and 0.479, respectively.

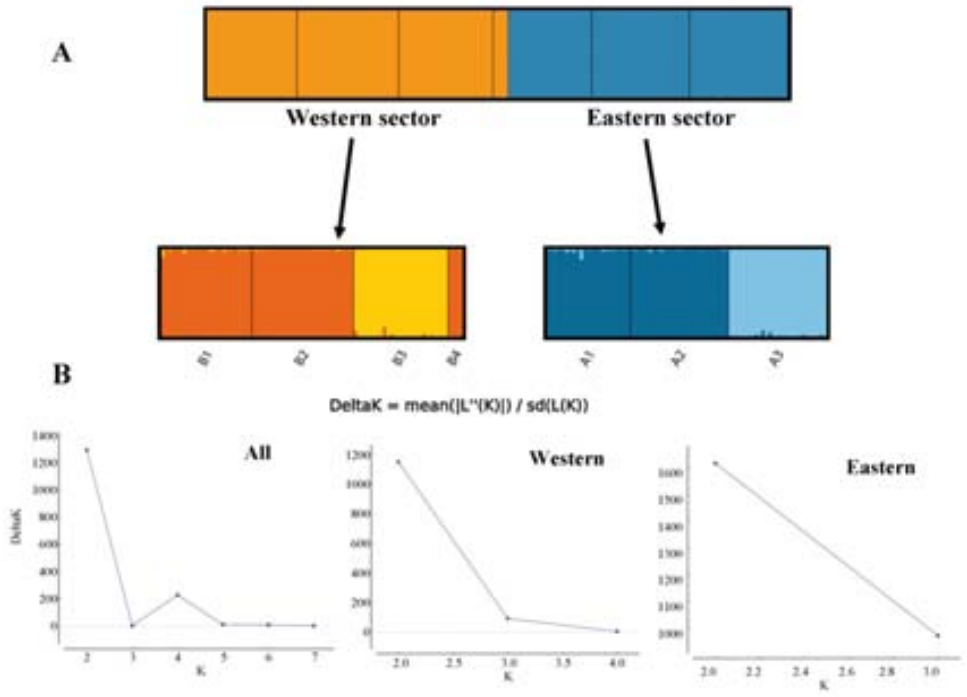

Figure 2. A, Results of Bayesian clustering and individual assignment analysis obtained with STRUCTURE after running the program with all populations (above) and by sector (below); vertical bars delimit sampling locations. B, inference for the best value of K based on the ΔK method among runs for all populations and by sector.

These values indicate that populations of the western sector have differentiated more from the potential ancestral population than the ones of the eastern sector. The existence of two clusters was highly supported by the analysis of ΔK values corresponding to K = 2 (Fig. 2b). Some evidence for additional substructure is also observed by a second weak peak at K = 4. When each sector was analyzed independently, two genetic clusters were identified for each sector, grouping A1-A2 separately from A3, and B1-B2-B4 separately from B3 (Fig. 2a). Within the western sector, population B3 presented relatively high values of differentiation from the ancestral population (F_k = 0.640). The same general results were also found with the FCA (Fig. 3), with the clear separation between the two sectors.

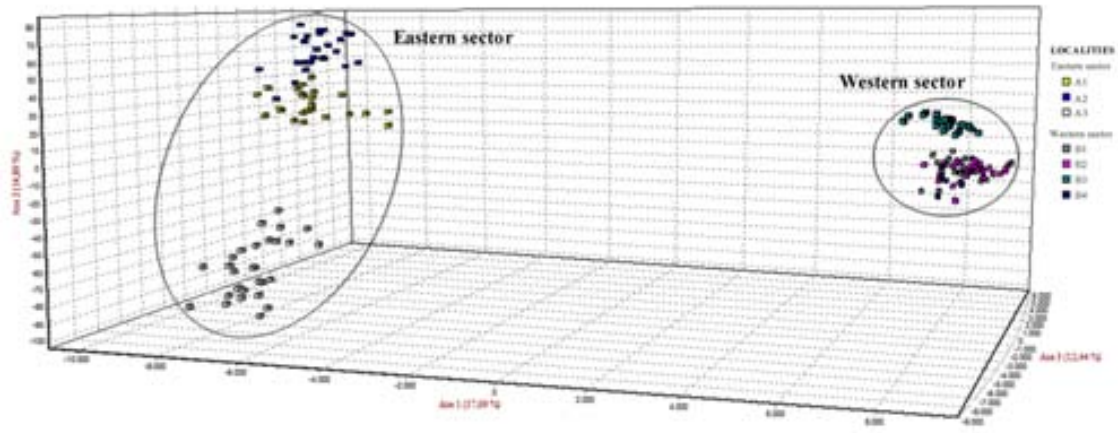

Figure 3. Population structure based on factorial correspondence analysis of all populations. Each square represents an individual multilocus genotype colored according to the location from where it was sampled.

In these results, the A3 and B3 populations also appeared as the most distinct ones in their respective sectors. The results of the a posteriori AMOVA revealed that the clusters resulting from STRUCTURE (K=2) explained 40.81% of the molecular variance, 11.61% was explained by among populations within groups and 48.31% by within population variation. These results agree with the population differentiation analysis (F_{ST} values; Table 2).

A relationship between genetic differentiation and geographical distance (Supp. Table 3) is found among all sampled populations (Fig. 4, r=0.735, P = 0.020), suggesting a strong isolation by distance at the level of all populations. However, at a

finer scale, when both sectors were analyzed independently, no isolation by distance was observed.

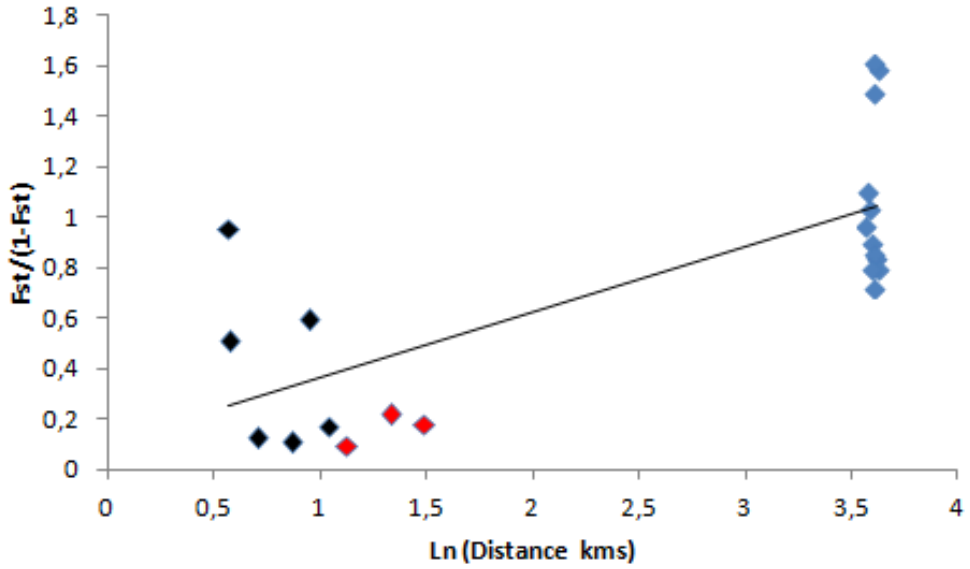

Figure 4. Isolation by distance between populations. Blue symbols represent among sectors comparisons; red and black symbols represent among populations comparisons of eastern and western sectors, respectively.

Recent gene flow and migration rates

No migration between sectors could be detected by any of the three programs. Individuals of the eastern sector were assigned by GENECLASS with more than 90% or higher probability to their population of origin. In the western sector, 96% of individuals originating from B1 and 88% of individuals originating from B3 were correctly assigned to their population of origin. A total of 10.7% of individuals from population B2 were assigned to B4, 17.9% to B1 and the rest (71% of individuals) to their population of origin. Some 8.7% or less of the individuals were not assigned to any of the populations sampled. No first-generation migrants were detected among populations from the eastern sector, and in the western sector only one individual from B2 was detected to be migrant from B1 ($P = 0.001$). Although this individual had a low probability to be migrant from B1 (probability of migration of 0.008 regarding the results of STRUCTURE), the estimated membership coefficients calculated by STRUCTURE showed a probability of 0.231 to have a single parent from population B1. STRUCTURE results were similar to GENECLASS assignments, with over 98% of the sampled individuals being assigned to their population of origin. Estimates of recent gene flow using BIMr were consistent among the five independent runs suggesting that convergence of the Markov chain had been reached. Recent migration rates resulted in

no detectable recent gene flow among populations in either eastern or western sector (Suppl. Table 4). The overall output of recent gene flow and migration rates are according to the population structure detected above.

Effective sample size, *Ne*

All three independent methods used to estimate the effective population sizes (N_e) of the two genetic clusters (i.e. sectors) and populations provided rather low and critical values (see Table 3). Estimation of the 95% CI upper limit for populations A1 and A3 was problematic (estimated at infinity or incongruence values) in the linkage disequilibrium method (LDNe). Despite slight differences between methods, all showed narrow confidence intervals thus supporting the consistence of received estimates. Effective population sizes were particularly low in population B3, with a 95% CI estimate of N_e = 2-30.

Relatedness of individuals

The proportion of individual relatedness within each population was similar (Table 3), with most individuals being highly unrelated with each other with percentages higher than 80%. The only exception was population B3 with a low percentage of the specimens unrelated (68.3%). Full sibling and parent-offspring relations in B3 represented the 12.31% and 11.38%, respectively, while the other populations showed much lower percentages not exceeding 4%. The estimated Queller & Goodnight (1989) index of relatedness calculated among individuals in each population separately indicated random mating among individuals within each population (A1, r_{qg} = -0.045; A2, r_{qg} = -0.038; A3, r_{qg} = -0.038; B1, r_{qg} = -0.042; B2, r_{qg} = -0.037; B3, r_{qg} = -0.067; B4, r_{qg} = -0.333). Instead, at a sector level, most populations had significantly higher degrees of relatedness than expected from the null distribution if sectors were in panmixia, which corroborated the observed genetic substructure within each sector (see Fig. 5), suggesting that there is no random mating among individuals from distinct populations within sectors. Although r_{qg} estimates within most populations were statistically higher than expected, values among most of them were similar and not exceedingly high (Fig. 5), with the only exception of population B3, which showed an average pairwise relatedness (r_{qg}) of 0.745 (upper and lower CI estimates at 95% of 0.759 and 0.730, respectively). Values for the other populations ranged from 0.150 to 0.206 and from 0.019 to 0.231 within the eastern western sectors, respectively.

Table 3. Estimates of effective population size (N_e) for each population and cluster calculated with three programs: LDNe, ONeSAMP and COLONY; estimations of the upper and lower 95% CI estimates for each method are indicated. Relationship indicates the percentage of individual relatedness within each population and cluster.

Grouping	LDNe			OneSamp			COLONY			Relationship			
Population	N_e	95% CIs		N_e	95% CIs		N_e	95% CIs		Unrelated	Half Siblings	Full Siblings	Parent Offspring
A1	342.30	77.00	infinite	27.65	24.51	34.81	46.00	26.00	90.00	91.70	7.51	0.40	0.40
A2	49.40	32.20	93.20	33.94	29.94	41.89	40.00	25.00	71.00	90.31	7.12	0.85	1.71
A3	142.10	61.80	infinite	36.85	33.33	43.20	44.00	28.00	80.00	91.17	7.98	0.28	0.57
B1	55.80	34.10	126.40	31.59	27.77	40.69	35.00	20.00	68.00	86.33	12.00	0.67	1.00
B2	62.20	27.50	15091.10	36.39	30.46	53.44	31.00	18.00	57.00	81.75	13.23	1.85	3.17
B3	7.30	2.40	21.70	14.97	12.61	19.61	13.00	7.00	30.00	68.31	8.00	12.31	11.38
B4	infinite	infinite	infinite	5.54	4.87	6.64	-	-	-	100	-	-	-
Clusters													
A1-A2	44.50	36.00	56.50	85.95	66.52	127.10	60.00	41.00	92.00	84.16	14.37	0.57	0.90
B1-B2-B4	30.00	23.60	39.00	80.14	55.44	157.01	42.00	27.00	66.00	80.89	15.91	1.50	1.69

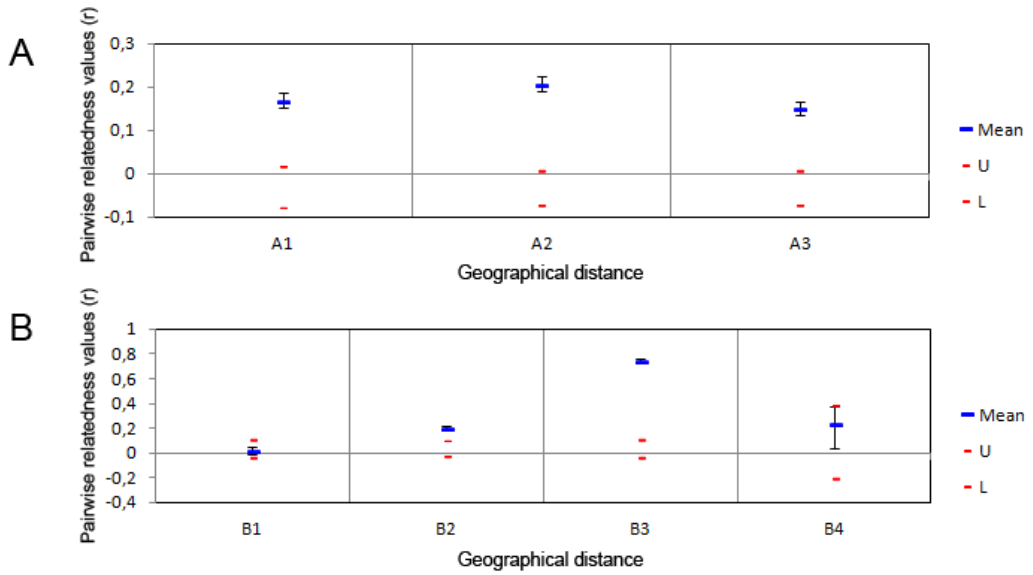

Figure 5. Mean within-population pairwise relatedness values (r_{qg}) between populations of eastern (A) and western (B) sectors. Red bars represent the upper (U) and lower (L) confidence intervals with 95 % confidence with a null distribution generated with 999 permutations. Blue bars represent the observed kinship mean conducted with 999 bootstraps.

Discussion

In general, habitat fragmentation leading to the subdivision of a species range into smaller subunits is considered to have a negative effect on species survival (Blank *et al.* 2013; Casas-Marce *et al.* 2013; Martínez-Cruz *et al.* 2007). However, according to Fahrig (2003), neutral or even positive effects can arise as a result of habitat fragmentation. In terms of genetic diversity, habitat fragmentation should lead to a decrease in genetic diversity due to random processes (e.g. genetic drift) that have a stronger effect in small populations (Leimu *et al.* 2006). Therefore, species restricted to small geographic areas should experience a high danger of extinction if populations become fragmented and isolated from each other. We are here studying an extreme case of population subdivision due to natural habitat fragmentation in an endangered endemic newt species, *Calotriton arnoldi*, and ask whether observed strong population subdivision due to habitat fragmentation really has an overall negative effect on this species. The fact that subpopulations of *C. arnoldi* show signs of morphological as well as genetic divergence, even for more slowly evolving genes, indicates that fragmented subpopulations must have existed already over a long evolutionary time scale. Accordingly, this specific system also allows further conclusions on the long-term effect of habitat fragmentation in an endemic and restricted species. We discuss below

our empirical data, which shows that species may have persisted with long-term fragmentation of habitat despite small effective population sizes.

Patterns of genetic diversity in *C. arnoldi*

Overall, it appears that across its distribution range the Montseny brook newt is characterized by moderate levels of genetic diversity and high genetic differentiation among sites. Genetic diversity of this species along its distribution range (weighted average H_E=0.441) tends to be similar to other salamanders and temperate amphibians, which show H_E averages ranging from 0.4-0.6 (Chan & Zamudio 2009 and references therein). Given that small populations tend to have low values of genetic variation due to random genetic drift, inbreeding or the accumulation of deleterious mutations (Frankham *et al.* 2010), a low observed genetic diversity was expected. The overall observed heterozygosity of *C. arnoldi* has been compared to some *C. asper* populations by performing a Wilcoxon ranked test, and no significant differences were detected (Drechsler *et al*. 2013; Valbuena-Ureña *et al*. unpublished data). Therefore, although *C. arnoldi* has a much smaller distribution range than *C. asper*, values of genetic diversity are similar and higher than expected for a critically endangered and isolated amphibian species with a very small population size (see Storfer *et al.* 2013 for an overview).

Our results clearly show that *C. arnoldi* populations are highly structured over short geographic distances and differentiated into an eastern and a western sector (Fig. 2). This finding is also supported by differences in morphology as well as mitochondrial and nuclear coding genes (Valbuena-Ureña *et al.* 2013 – Chapter IV), suggesting that the fragmentation into subpopulations is not a recent event and that it is likely the result of natural processes. Sectors are genetically highly different with no gene flow between them as indicated by a high number of private alleles in each sector (75 and 38 in the eastern and western sectors, respectively) and by the AMOVA results, high F_{ST} values, unambiguous genetic assignment of individuals, and observed isolation by distance. Since *C. arnoldi* is exclusively aquatic (Carranza & Amat 2005), dispersal can only occur along watercourses. This is in agreement with the levels of genetic differentiation observed, which are notably higher than values typically found in studies of other amphibians that use both aquatic and terrestrial habitats (Spear *et al.* 2005). Accordingly, sectors are effectively isolated for *C. arnoldi* by a 37 km long watercourse, whereas distance by air is only 6 km. The fact that the watercourse between the two sectors passes through a completely adverse environment for this

species, including a low altitude wide river with potential predators (Tordera River; Carranza & Amat 2005), has resulted into a strong migration barrier for these aquatic newts. Accordingly, we can assume that in this system natural fragmentation has a strong impact on observed and associated microevolutionary processes (see Sarasola-Puente *et al.* 2012; Templeton *et al.* 1990; Wang 2009).

The high number of private alleles and the high degree of allelic fixation (PAAr) within each sector (Table 1) suggests the role of long-term isolation between sectors and small-effective population size (Milá *et al.* 2010). Values of genetic variability (observed heterozygosities) were significantly different between sectors. Genetic variability of the populations within the eastern sector is generally higher than genetic variability in the populations from the western sector. Also, the number of alleles per locus as well as allelic richness is almost twice in the eastern sector for these parameters. Moreover, all 24 loci were polymorphic for the eastern sector while some of them were monomorphic (fixed) for the western populations. These results match with the higher level of genetic variability for mitochondrial and nuclear DNA sequences within the eastern sector (Valbuena-Ureña *et al.* 2013 – Chapter IV). Differences in their recent population history may be a reason for the differences in genetic variability between sectors (Jensen *et al.* 2013). Time since colonization and effective population size after colonization are important to consider when interpreting differences in the levels of genetic variation among populations (Jensen *et al.* 2013). Depletion of genetic variation occurs when a new population is established by a very small number of individuals from a larger population, i.e. founder effect (Nei *et al.* 1975) and genetic drift (Jensen *et al.* 2013). It is also considered that low allelic richness, low heterozygosity, and increased numbers of monomorphic loci to be an indicator of a bottlenecked population, as excess heterozygosity diminishes over time if the population size remains small (Richmond *et al.* 2013). Our results suggest that the western sector has differentiated more from the potential ancestral population than the eastern sector, as low genetic diversity values for the western populations may indicate that the isolation process is ancient enough to be detected (Sarasola-Puente *et al.* 2012). Additionally, the higher degree of genetic differentiation from the ancestral population (F_k) obtained for the western sector agrees with its lower population size and its probable longer isolation (Casas-Marce *et al.* 2013).

Estimates of genetic clusters and PCA analysis were consistent with each other regarding intra-sector differentiation. Two clusters are detected in each sector, being

populations A3 and B3 isolated from the other populations, each forming a different cluster (Fig. 2). Moreover, dispersal among populations within sectors is very low, not only on the basis of the migration tests. The significant F_{ST} values indicate that dispersal among populations is low and confirm the differentiation of populations A3 and B3 from the other populations within their respective sectors. Moreover, most populations showed significantly higher degrees of relatedness (r_{qg}) than expected if sectors were in panmixia. This pattern is expected when migration among populations is not sufficiently high to counteract relatedness that results from nonrandom mating among populations.

Advantage or burden – how to interpret the impact of natural fragmentation in *C. arnoldi*

It is broadly accepted that habitat fragmentation will result into the subdivision of populations and, if migration of individuals is not possible, subpopulations will start to diverge genetically (Frankham *et al.* 2010; Templeton *et al.* 1990). It is also important to differentiate between human induced (i.e. non-natural) and naturally caused (as it is the case in *C. arnoldi*) processes of habitat fragmentation. We would like to focus our discussion here on the effects of natural habitat fragmentation. In their influential paper, Templeton *et al.* (1990) pointed out the genetic consequences of natural habitat fragmentation for a set of quite distinct taxa in a restricted mountain range. Highlighting mainly the possible negative consequences of species fragmentation, they also concluded that, on the positive side, genetic variation of a fragmented species is not totally lost but often present as fixed differences between local populations (Templeton *et al.* 1990). Although this aspect might be rather relevant for species conservation, the scientific community has never considered it seriously. In our view, natural habitat fragmentation and its consequences on population structure in *C. arnoldi* provide an excellent system to discuss this so far overlooked aspect.

It is quite obvious that the census as well as effective population sizes observed in *C. arnoldi* ranks it as a critically endangered species; N_e values for all *C. arnoldi* populations were critically low (<50) and are consistent with the small found census size (Amat & Carranza 2005). In conservation biology, a N_e of 500 has been suggested as a minimum value for the long-term survival of a species, whereas N_e values below 50 in isolated populations are of major concern, as these populations have an increased probability of extinction as a result of genetic effects (Allendorf & Luikart 2007;

Hurtado *et al.* 2012) and stochastic processes. However, despite the critically low estimated values of N_e for all *C. arnoldi* populations (Table 3), the lack of gene flow between sectors and the extremely low level of gene flow between populations within sectors (Fig. 2), this species has probably survived for a long period of time in this area. Without considering the evolutionary history of a certain species, one should expect that small N_e estimates in a population may induce to high inbreeding coefficients as well as a high level of relatedness among individuals of a given population. However, F_{IS} values for populations were not significant and estimates of relatedness (r_{qg}) among individuals at each population were unexpectedly low. Also, the percentages of unrelated relationships exceed 80% in all populations but population B3 (68%). One possible explanation for this phenomenon could be that *C. arnoldi* has evolved mating strategies to maximize outbreeding of individuals within a population, i.e. a preference for mating with genetically more dissimilar partners. That female of distinct amphibian species are capable of mating preferentially has been already shown for distinct species. In newts, it has been shown that females mate with genetically more dissimilar males in condition-dependent choice situations (Garner & Schmidt 2003), whereas in terrestrial salamanders, females preferred genetically more similar males under complete natural conditions (Caspers *et al.* 2014) in the context of habitat adaptation (Steinfartz *et al.* 2007). Females of all salamandrid species, including also *C. arnoldi*, can store the sperm from multiple matings in special cloacal glands (so called *receptaculum seminis*) for later fertilization. Sperm storage and multiple paternities are common among salamanders and newts (Caspers *et al.* 2014; Kühnel *et al.* 2010; Steinfartz *et al.* 2006). We can also assume that females of *C. arnoldi* do sperm storage (Alonso 2013) and therefore, have the potential to increase the genetic diversity of their offspring by mating with multiple and maybe preferentially genetically dissimilar males. Thus, in terms of maintaining genetic diversity, small effective population sizes do not necessarily impose a problem, as there may be other reproductive or behavioral mechanisms that counteract the effect of genetic drift (Allentoft & O'Brien 2010). Therefore, a better knowledge of the species' biology, such as these reproductive strategies, will help to understand how the genetic diversity of this species is maintained across generations.

Our results indicate that the overall genetic diversity of *C. arnoldi* across its distribution range has been maintained relatively high despite the small area of occupancy. Therefore, species fragmentation in this case should be regarded as

advantageous. Populations of *C. arnoldi* do not show low levels of intrapopulation genetic diversity and signs of inbreeding, which are a typical by-product of habitat fragmentation. Also, one might further speculate that if a species consists of completely independent and isolated populations, as being the case here, a bet hedging strategy of isolated populations in the face of local extinction might prevent the extinction of the whole species. Especially in the event of epidemic infections, which have been shown to be particularly dangerous for amphibians by driving complete populations and even species to extinction (Bosch *et al.* 2001; Earl & Gray 2014; Martel *et al.* 2013; Skerratt *et al.* 2007; Spitzen-Van Der Sluijs *et al.* 2013). Small populations are more likely to go extinct due to stochastic processes and genetic effects. However, if populations are completely isolated and the contact and exchange between individuals through migration does not take place, single populations might survive an epidemic outbreak enabling the survival of the species.

Implications for the conservation

Impacts of habitat fragmentation must be measured independently from effects of habitat loss or degradation. Population isolation caused by the breaking apart of habitat might have either positive or negative effects depending on the species. As stated before, *C. arnoldi* seems to have coped with natural habitat fragmentation very well. However, the effects of habitat loss may outweigh the effects of habitat fragmentation and have important implications for conservation. Habitat loss has been widely recognized to have large and consistently negative effects on biodiversity, affecting negatively species richness, population abundance and distribution and genetic diversity (Fahrig 2003 and references therein). The low effective population sizes entail that habitat loss or degradation could drive very rapidly such small populations to extinction. Stochastic factors could cause high mortalities when species have very small distribution ranges. Although natural fragmentation has been demonstrated to involve neutral or positive effects to the survival of a certain species, the effects of habitat loss may be detected when the habitat is highly and rapidly fragmented. Intensifying habitat fragmentation causes an increase in the amount of edge in the landscape. That enlarges the probability of individuals leaving the breeding habitat and increases the habitat needed for persistence (Fahrig 2001). This implies that one main question that should be considered for the conservation of a certain species is probably “how much habitat is enough?” Different species require distinct amounts of habitat for persistence.

Therefore, the conservation of a vulnerable or endangered species requires estimating the minimum habitat required for persistence of the given species. In addition, many species require more than one kind of habitat within a life cycle. Therefore, landscape patterns that maintain the required habitat amounts should be covered (Fahrig 2003).

Species surviving in fragmented habitats are, overall, likely to suffer genetic erosion, compared to populations living in continuous habitats (Rivera-Ortiz *et al*. 2014). This study is an example of species that have evolved beneficial strategies to cope with habitat fragmentation. Therefore, it is crucial to determine the level of evolutionary potential of a given species and to characterize the specialized ecological requirements and life history traits of a given species.

Understanding the current genetic population structure and gene flow among populations contribute valuable information in the guidance and management of conservation programs (Boessenkool *et al.* 2009; Leonard 2008; Shepherd & Lambert 2008). The definition of appropriate conservation units are crucial for maintaining the distinct evolutionary lineages and the species' evolutionary potential (Frankham *et al.* 2010). In *C. arnoldi,* the evolutionary potential is not only manifested within the species as a whole but rather within each sector. Therefore, conservation strategies should be adopted to make sure that the evolutionary potential and the genetic diversity within the distinct groups are not lost (Hoffman & Blouin 2004). Outcrossing among them is not a recommended management strategy in order to avoid outbreeding depression (Frankham *et al*. 2011, Sagvik *et al*. 2005, Sherman *et al*. 2008). Furthermore, before any translocations are done it is important to evaluate whether individuals have evolved local adaptation and to clarify at what degree they show ecological exchangeability (Blank *et al.* 2013; Kraaijeveld-Smit *et al.* 2005). Thus, for all these reasons expressed above, conservation strategies should focus on habitat preservation and restoration in each evolutionary group with the aim of maintaining the strong population structure highlighted in this study.

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Supplementary Table 1. Characterization of the full set of 24 applied microsatellite loci for *Calotriton arnoldi*. Loci are grouped by multiplex combinations used for amplification. Locus name, primer sequence, direction (F is forward, R is reverse), annealing temperature of the primer for PCRs, microsatellite motif, amplified fragment size range, number of alleles, labeling dye, and references are provided.

Locus name	Primer sequence (5' 3')	Annealing temp. (°C)	Repeat motif of cloned allele	Size range of amplification product		Number of alleles	Fluorescence labeling	Reference
Mix1								
Calarn_02248	F: CACAACAACAGGCGAATGAC	59	$(TATC)_{18}$	169	209	9	FAM	Valbuena-Ureña *et al.* 2014
	R: ACTTTAGGTCTTGCGTTGGC							
Ca3	F: CCATGCATTCTTGGAGGTTT	59	$(AGAT)_{15}$	233	265	9	FAM	Drechsler *et al*. 2013
	R: TTCAAAGGCAGTGTTTCAGG							
Calarn_29994	F: ACCAGCTGCACTCTGCTATC	59	$(TATC)_{8}$	169	185	5	VIC	Valbuena-Ureña *et al.* 2014
	R: GTGCTGCTCATCAAATAGTCAAC							
Ca21	F: AGCGTGTGCAGCAGTATCC	59	$(AGAT)_{12}$	232	257	7	VIC	Drechsler *et al*. 2013
	R: GCAATGTGCCATTCATTACC							
Calarn_37825	F: CATCCTTGTAGCAGGCCTTTG	59	$(AGAT)_{18}$	211	255	12	NED	Valbuena-Ureña *et al.* 2014
	R: CTACCAGGGGTTGATCTCAGG							
Calarn_14961	F: TTGAGAAATGCAAGGTCGCC	59	$(TATC)_{11}$	201	225	7	PET	Valbuena-Ureña *et al.* 2014
	R: GTCAGGATGACGCGTTTCG							
Mix2								
Calarn_15906	F: TCAATCAAGGGCAAGATGATGAC	59	$(CTAT)_{14}$	105	129	7	FAM	Valbuena-Ureña *et al.* 2014
	R: ACCAATGACCTATCACAGCC							
Calarn_12022	F: CTCTCACGGAAAAGCTCAGG	59	$(TCTA)_{14}$	220	252	9	FAM	Valbuena-Ureña *et al.* 2014
	R: GCGTGGCCCAATACATATTCC							
Calarn_06881	F: AGCGCATTGCTGCCTGTG	59	$(AGAT)_{15}$	154	190	10	VIC	Valbuena-Ureña *et al.* 2014
	R: TACAGAGGGAGTGGGAGGAG							
Ca7	F: ACCCTTACACACCCCAAACC	59	$(AGAT)_{16}$	224	244	6	NED	Drechsler *et al*. 2013
	R: GTTCCCTGCATGGCTCTAAA							
Ca22	F: CTTCAGACTGCCGAGTGTTG	59	$(AGAT)_{13}$	126	146	6	PET	Drechsler *et al*. 2013
	R: ACCTTGTCACGGTGTAGGAAG							
Calarn_50748	F: ATTGGGGTATATTGGGGCTC	59	$(AGAT)_{13}$	201	217	5	PET	Valbuena-Ureña *et al.* 2014
	R: GGCATCCATCACCGATTATCTATC							
Mix3								
Ca8	F: AGAAGGGAGTCAGGCAGACA	59	$(AGAT)_{13}$	180	204	7	FAM	Drechsler *et al*. 2013
	R: GGAGGATCAAATGTGTTTGGA							
Calarn_36791	F: TTGGAGGTGTCATCAGTGGG	59	$(TCTA)_{18}$	120	148	8	VIC	Valbuena-Ureña *et al.* 2014
	R: AACCACAGAAATTCACCAGTC							
Calarn_59202	F: GTAGGTTTGGTGCGAAGTGG	59	$(AGAT)_{15}$	212	236	7	VIC	Valbuena-Ureña *et al.* 2014
	R: GTACGAGATCTTCTCAGTGGC							
Ca32	F: ACAGGGCAAGAGAGTCAACG	59	$(ACAG)_{10}$	185	197	4	NED	Drechsler *et al*. 2013
	R: CAGCCTATTGGCTTGTCAGC							
Calarn_52354	F: AAACTGTGGCATCTTGTGGC	59	$(ATCT)_{12}$	220	240	6	PET	Valbuena-Ureña *et al.* 2014
	R: AGACAGCATCTGTGTCCTCTG							
Mix4								
Calarn_30143	F: AGGTTAGGTTTAGGTTTACTGCAC	59	$(TCTG)_{7}$	186	238	9	FAM	Valbuena-Ureña *et al.* 2014
	R: AGCTTCGTCATTCTTGTACCC							
Calarn_31321	F: GCTTACATCCATCCTTCTCGTC	59	$(ATCT)_{19}$	148	193	10	VIC	Valbuena-Ureña *et al.* 2014
	R: AGGCAGATGTTTTGATGGGTG							
Calarn_15136	F: GCTAGTTTGCTTTGGCAGTTC	59	$(TGTA)_{15}$	165	177	4	NED	Valbuena-Ureña *et al.* 2014
	R: CTGCCTTTTGGCTAGGTTCG							
Mix5								
Calarn_37884	F: GGGGCGCAAGTTACAGTTAG	59	$(ATAG)_{10}$	240	267	7	FAM	Valbuena-Ureña *et al.* 2014
	R: ATGTAGTGTGGCAGGTGAGG							
Us2	F: TGGGCTGAAGGATTGAAAAA	59	$(AGAT)_{17}$	219	239	6	VIC	Drechsler *et al*. 2013
	R: CTCAGCTGCAGTGGTGTGTT							
Us3	F: AAGTTTGTAGGTATGCATAATAGCC	59	$(AGAT)_{16}$	143	167	5	NED	Drechsler *et al*. 2013
	R: GGAAGTCCAGGCCTGTAGAC							
Us7	F: CTGCACCGATTAATTGCAGA	59	$(ACAT)_{16}$	222	246	5	PET	Drechsler *et al*. 2013
	R: CTGCACCACTCGCTCCTC							

Supplementary Table 2a. Estimates of genetic parameters for each eastern population and locus. N, sample size; A, number of alleles per locus; Ar, allelic richness; PA, number of private alleles; PAAr, allelic richness of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient

Population	Locus	Ca1arm 02248	Ca3	Ca1arm 29994	Ca21	Ca1arm 37825	Ca1arm 14961	Ca1arm 15906	Ca1arm 12022	Ca1arm 06881	Ca7	Ca22	Ca1arm 50748	CA8	Ca1arm 36791	Ca1arm 59202	Ca32	Ca1arm 52354	Ca1arm 30143	Ca1arm 31321	Ca1arm 15136	Ca1arm 37884	US2	Us3	Us7	Overall
A1	N	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000	23.000
	A	6.000	3.000	2.000	2.000	8.000	4.000	4.000	4.000	4.000	2.000	4.000	4.000	4.000	5.000	4.000	4.000	5.000	6.000	4.000	5.000	4.000	4.000	4.000	3.000	4.167
	Ar	6.000	3.000	2.000	2.000	8.000	4.000	4.000	5.000	4.000	2.000	4.000	4.000	4.000	5.000	4.000	4.000	4.000	5.000	6.000	4.000	5.000	4.000	4.000	3.000	4.167
	PA	2.000	-	1.000	-	1.000	-	-	-	-	1.000	-	-	-	-	1.000	-	-	-	-	-	-	-	-	1.000	7.000
	PAAr	2.148	0.000	1.000	0.000	1.002	0.000	0.000	0.004	0.012	0.000	1.000	0.148	0.000	0.000	1.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.002	1.148	0.311
	H_O	0.739	0.522	0.087	0.304	0.870	0.609	0.913	0.391	0.391	0.652	0.043	0.565	0.391	0.826	0.478	0.435	0.565	0.609	0.652	0.478	1.000	0.826	0.565	0.174	0.545
	H_E	0.642	0.614	0.162	0.372	0.814	0.649	0.702	0.430	0.490	0.724	0.043	0.579	0.380	0.729	0.411	0.509	0.467	0.690	0.757	0.506	0.710	0.623	0.530	0.382	0.538
	F_{IS}	-0.156	0.154	0.470	0.185	-0.071	0.064	-0.309	0.092	0.205	0.101	0.000	0.024	-0.031	-0.136	-0.169	0.149	-0.217	0.120	0.142	0.057	-0.421	-0.335	-0.067	0.550	0.017
	F_{IS} *P*-value	0.169	0.219	0.125	0.359	0.334	0.407	0.019	0.373	0.122	0.272	1.000	0.549	0.632	0.202	0.202	0.235	0.095	0.251	0.145	0.444	0.001	0.029	0.434	0.006	0.709
A2	N	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000
	A	4.000	5.000	2.000	4.000	5.000	6.000	5.000	4.000	4.000	3.000	2.000	3.000	4.000	6.000	2.000	3.000	5.000	5.000	6.000	3.000	5.000	5.000	3.000	3.000	4.042
	Ar	3.852	4.852	1.852	4.000	4.980	5.684	5.000	3.961	4.000	2.998	2.000	2.850	3.852	5.849	2.000	3.000	4.959	4.852	5.849	3.000	4.850	4.852	2.980	2.832	3.954
	PA		1.000	-	1.000	-	-	1.000		2.000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.000
	PAAr	0.000	1.000	0.000	1.148	0.000	0.000	1.000	0.000	2.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.214
	H_O	0.667	0.667	0.037	0.667	0.630	0.741	0.815	0.407	0.741	0.593	0.296	0.148	0.556	0.667	0.407	0.667	0.630	0.593	0.593	0.259	0.741	0.741	0.259	0.111	0.526
	H_E	0.543	0.719	0.037	0.651	0.674	0.719	0.790	0.495	0.716	0.560	0.257	0.142	0.514	0.633	0.484	0.604	0.518	0.565	0.615	0.297	0.643	0.722	0.388	0.108	0.516
	F_{IS}	-0.233	0.074	0.000	-0.025	0.067	-0.031	-0.032	0.179	-0.035	-0.060	-0.156	-0.045	-0.083	-0.054	0.161	-0.106	-0.221	-0.049	0.037	0.129	-0.156	-0.027	0.336	-0.026	-0.015
	F_{IS} *P*-value	0.087	0.338	1.000	0.521	0.377	0.489	0.494	0.168	0.490	0.435	0.551	0.892	0.392	0.435	0.341	0.316	0.073	0.457	0.473	0.315	0.150	0.510	0.055	0.942	0.266
A3	N	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000	27.000
	A	3.000	4.000	2.000	4.000	6.000	7.000	3.000	9.000	4.000	3.000	3.000	5.000	6.000	5.000	4.000	3.000	5.000	6.000	5.000	2.000	4.000	4.000	4.000	2.000	4.292
	Ar	3.000	3.852	1.852	3.852	5.980	6.956	2.852	8.978	3.852	2.852	2.980	4.850	5.852	4.980	3.998	3.000	4.830	5.850	4.998	2.000	3.980	3.980	3.998	2.000	4.222
	PA	-	-	-	-	-	-	-	2.000	-	-	-	-	-	1.000	1.000	-	1.000	-	-	-	-	-	-	-	5.000
	PAAr	0.000	0.000	0.000	0.000	0.000	0.005	0.000	1.998	0.000	0.000	0.000	0.000	0.000	1.000	0.998	0.000	0.998	0.148	0.000	0.000	0.000	0.000	0.000	0.020	0.215
	H_O	0.444	0.593	0.037	0.704	0.889	0.593	0.370	0.815	0.407	0.222	0.556	0.741	0.704	0.778	0.519	0.630	0.481	0.815	0.704	0.185	0.741	0.444	0.556	0.519	0.560
	H_E	0.616	0.579	0.037	0.670	0.797	0.680	0.314	0.879	0.514	0.262	0.544	0.651	0.732	0.737	0.481	0.486	0.586	0.785	0.707	0.171	0.638	0.495	0.593	0.453	0.559
	F_{IS}	0.282	-0.023	0.000	-0.051	-0.117	0.131	-0.185	0.074	0.210	0.154	-0.021	-0.142	0.039	-0.056	-0.079	-0.304	0.182	-0.039	0.005	-0.083	-0.165	0.105	0.064	-0.148	-0.007
	F_{IS} *P*-value	0.037	0.528	1.000	0.447	0.150	0.163	0.358	0.215	0.103	0.357	0.522	0.177	0.741	0.407	0.405	0.027	0.154	0.467	0.555	0.820	0.165	0.281	0.401	0.362	0.482

Supplementary Table 2b. Estimates of genetic parameters for each western population and locus. N, sample size; A, number of alleles per locus; Ar, allelic richness; PA, number of private alleles; PAAr, allelic richness of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient

Population	Locus	Calarn 02248	Ca3	Calarn 29994	Ca21	Calarn 37825	Calarn 14961	Calarn 15906	Calarn 12022	Calarn 06881	Ca7	Ca22	Calarn 50748	CA8	Calarn 36791	Calarn 59202	Ca32	Calarn 52354	Calarn 30143	Calarn 31321	Calarn 15136	Calarn 37884	Us2	Us3	Us7	Overall
B1	N	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000	25.000
	A	3.000	4.000	2.000	3.000	8.000	5.000	3.000	4.000	6.000	4.000	4.000	3.000	3.000	3.000	4.000	2.000	3.000	4.000	4.000	4.000	4.000	1.000	1.000	1.000	3.542
	Ar	3.000	4.000	2.000	2.995	7.755	4.910	3.000	4.000	5.840	3.915	3.840	3.000	2.920	3.000	3.920	2.000	3.000	3.995	4.000	3.920	3.000	4.000	1.000	1.000	3.500
	PA	-	-	-	-	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	1.000	-	-	-	-	-	3.000
	PAAr	0.000	0.000	0.000	0.000	0.001	0.000	1.000	0.000	0.920	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	1.178	0.000	0.179	0.000	0.000	0.000	0.137
	Ho	0.240	0.600	0.560	0.480	0.720	0.760	0.320	0.720	0.680	0.600	0.280	0.440	0.240	0.480	0.560	0.280	0.600	0.440	0.600	0.520	0.560	0.520	0.000	0.000	0.467
	HE	0.223	0.589	0.509	0.467	0.793	0.602	0.344	0.638	0.602	0.533	0.520	0.631	0.220	0.427	0.691	0.301	0.613	0.523	0.569	0.479	0.448	0.533	0.000	0.000	0.469
	FIS	-0.079	-0.020	-0.102	-0.029	0.093	-0.270	0.070	-0.132	-0.132	-0.129	0.467	0.307	-0.095	-0.127	0.193	0.072	0.022	0.162	-0.056	-0.087	-0.256	0.025	-	-	-0.005
	FIS P- value	0.727	0.560	0.449	0.569	0.252	0.066	0.457	0.217	0.216	0.305	0.009	0.036	0.705	0.318	0.116	0.580	0.519	0.188	0.445	0.398	0.074	0.521	-	-	0.585
B2	N	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	27.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	28.000	27.958
	A	3.000	4.000	4.000	2.000	5.000	4.000	1.000	3.000	4.000	3.000	4.000	3.000	2.000	3.000	3.000	2.000	3.000	3.000	2.000	2.000	3.000	6.000	1.000	1.000	2.917
	Ar	2.971	3.996	3.966	2.000	4.999	3.792	1.000	2.821	3.999	3.000	3.998	3.000	1.821	2.000	3.000	1.821	3.000	2.999	1.821	2.000	2.821	5.817	1.000	1.000	2.860
	PA	-	-	1.000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	-	-	-	-	-	-	2.000
	PAAr	0.000	0.000	1.000	0.000	0.080	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.999	0.000	0.000	0.000	0.000	0.000	0.000	0.087
	Ho	0.214	0.571	0.357	0.464	0.679	0.607	0.000	0.429	0.571	0.464	0.407	0.821	0.036	0.250	0.357	0.036	0.714	0.607	0.036	0.143	0.464	0.679	0.000	0.000	0.371
	HE	0.257	0.610	0.393	0.493	0.711	0.524	0.000	0.527	0.646	0.451	0.572	0.646	0.036	0.223	0.389	0.036	0.634	0.571	0.036	0.249	0.371	0.742	0.000	0.000	0.380
	FIS	0.469	0.064	0.092	0.059	0.046	-0.162	-	0.189	0.117	-0.029	0.292	-0.278	0.000	-0.125	0.083	0.000	-0.130	-0.064	0.000	0.432	-0.258	0.087	-	-	0.028
	FIS P- value	0.293	0.383	0.330	0.538	0.415	0.222	-	0.194	0.250	0.536	0.026	0.032	1.000	0.645	0.357	1.000	0.240	0.440	1.000	0.076	0.166	0.269	-	-	0.240
B3	N	26.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	25.000	25.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	26.000	25.958
	A	2.000	2.000	1.000	3.000	4.000	2.000	2.000	3.000	1.000	2.000	2.000	2.000	2.000	1.000	2.000	1.000	1.000	2.000	1.000	1.000	2.000	1.000	1.000	1.000	1.792
	Ar	2.000	1.885	1.000	2.885	4.000	1.885	1.885	3.000	1.000	2.000	2.000	2.000	2.000	1.000	1.885	2.000	1.000	2.000	1.000	1.000	2.000	1.000	1.000	1.000	1.768
	PA	-	-	-	1.000	1.000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.000
	PAAr	0.000	0.000	0.000	0.885	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.079
	Ho	0.654	0.038	-	0.538	0.731	0.038	0.038	0.769	0.000	0.462	0.520	0.654	0.154	0.000	0.038	0.192	0.000	0.154	0.000	0.000	0.308	0.000	0.000	0.000	0.230
	HE	0.491	0.038	-	0.429	0.661	0.038	0.038	0.673	0.000	0.510	0.393	0.491	0.145	0.000	0.038	0.177	0.000	0.145	0.000	0.000	0.265	0.000	0.000	0.000	0.197
	FIS	-0.341	0.000	-	-0.261	-0.107	0.000	0.000	-0.145	-	0.096	-0.333	-0.341	-0.064	-	0.000	-0.087	-	-0.064	-	-	-0.163	-	-	-	-0.121
	FIS P- value	0.083	1.000	-	0.088	0.263	1.000	1.000	0.209	-	0.466	0.118	0.087	0.877	-	1.000	0.805	-	0.886	-	-	0.531	-	-	-	0.001
B4	N	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
	A	2.000	3.000	3.000	2.000	4.000	3.000	1.000	3.000	4.000	2.000	3.000	2.000	2.000	2.000	1.000	2.000	2.000	2.000	2.000	3.000	2.000	3.000	1.000	1.000	2.333
	Ar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.000
	PAAr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ho	0.750	0.500	1.000	0.250	0.750	0.750	0.000	0.750	0.500	0.750	0.750	0.500	0.250	0.250	0.250	0.000	0.500	0.250	0.250	1.000	0.250	0.250	0.000	0.000	0.438
	HE	0.536	0.679	0.679	0.250	0.750	0.607	0.000	0.750	0.607	0.607	0.536	0.679	0.536	0.536	0.250	0.000	0.429	0.250	0.250	0.750	0.250	0.464	0.000	0.000	0.433
	FIS	-0.500	0.294	-0.600	0.000	0.000	-0.286	-	-0.286	-0.286	-0.500	0.294	0.571	0.000	-	-0.200	0.000	0.250	0.000	0.000	-0.42	0.000	0.500	-	-	-0.023
	FIS P- value	0.580	0.432	0.243	1.000	1.000	0.579	-	0.571	0.210	0.566	0.577	0.439	0.441	0.421	1.000	-	0.861	1.000	1.000	0.343	1.000	0.141	-	-	0.507

Supplementary Table 3. Values used in isolation by distance (IBD) analysis. Measure of genetic differentiation F_{ST} /(1- F_{ST}), below the diagonal; logarithmic geographic distances in km, above the diagonal.

F_{ST}/(1-F_{ST}) / Ln (dist)	A1	A2	A3	B1	B2	B3	B4
A1	-	1,12914149	1,49357803	3,61005267	3,58482364	3,61552889	3,6221786
A2	0,09349371	-	1,33500107	3,59223088	3,56654231	3,59780529	3,60457351
A3	0,17730162	0,21684108	-	3,59035662	3,56461924	3,59594146	3,60272226
B1	0,84060372	0,89861401	0,79629962	-	0,86919912	0,577175	0,70754306
B2	1,03748981	1,10260723	0,96270854	0,10619469	-	0,95088538	1,04239461
B3	1,58866166	1,60824204	1,49314385	0,5051174	0,59159637	-	0,57210885
B4	0,79468772	0,85116623	0,72176309	0,12233446	0,16890707	0,95465207	-

Supplementary Table 4. Recent migration rate estimations between *Calotriton arnoldi* populations within eastern and western sectors.

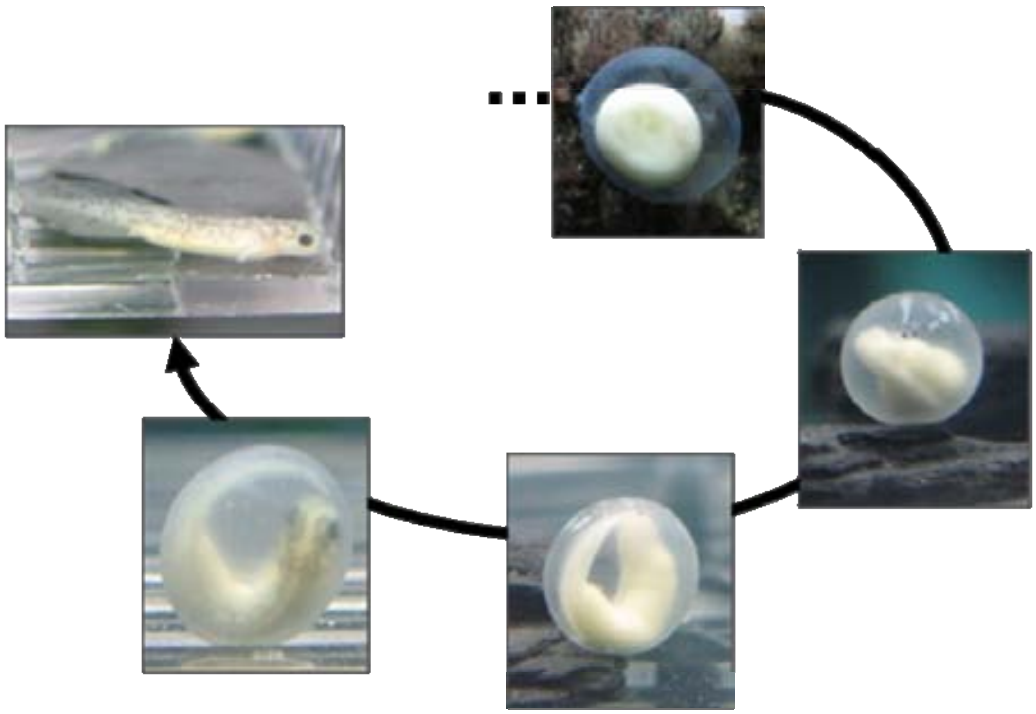
Eastern

	A1	A2	A3
A1	0,99868 (0,00518)	0,00068 (0,00314)	0,00064 (0,00289)
A2	0,00063 (0,00303)	0,99880 (0,00486)	0,00057 (0,00260)
A3	0,00077 (0,00339)	0,00066 (0,00283)	0,99857 (0,00535)

Western

	B1	B2	B3	B4
B1	1 (0,05838)	0,00105 (0,08827)	0,00204 (0,14776)	0,00157 (0,09339)
B2	0,00020 (0,00088)	1 (0,03856)	0,00020 (0,00038)	0,00021 (0,00091)
B3	0,00160 (0,08970)	0,00181 (0,06574)	1 (0,20118)	0,00187 (0,03317)
B4	0,00081 (0,01540)	0,00095 (0,01771)	0,00092 (0,01224)	1 (0,03283)

Valbuena-Ureña E, Soler-Membrives A, Steinfartz S, Alonso M, Carbonell F, Larios R, Obon E, Carranza S (to submit) Evaluating the ex situ conservation project of the critically endangered Montseny brook newt (*Calotriton arnoldi*): are we going in the right direction? Conservation genetics.



CAPÍTOL VII

Evaluating the ex situ conservation project of the critically endangered Montseny brook newt (*Calotriton arnoldi*): are we going in the right direction?

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Keywords

Calotriton arnoldi, captive breeding program, ex situ management, founders, genetic diversity, inbreeding, effective population size

Abstract

Ex situ management strategies play an important role for the conservation of threatened and endangered species. Two main objectives should be priority when planning breeding programs: the maintenance of the maximum neutral genetic diversity, and to obtain "self-sustaining" captive populations. In this study, we evaluate the genetic quality of the captive breeding stock of the Montseny brook newt, *Calotriton arnoldi*. This critically endangered species is geographically, morphologically and genetically differentiated into two disconnected sectors (eastern and western) in the wild. We test if the level of genetic diversity (allelic diversity and heterozigosity) detected in the two current captive breeding lines is comparable to that observed in the wild. Relatedness and the estimation of captive effective population sizes (N_e) are also evaluated to infer if the actual captive genetic diversity is sufficient for its endurance over time. The results show that the captive stock has retained 82% of the alleles present in the wild populations, and estimates of H_E of the captive stock were generally lower than their wild counterparts, though more evident in the eastern sector. Moreover, the genetic differentiation values between the captive and wild populations were around 5-7%. In agreement with standard conservation guidelines, we conclude that the genetic diversity in the captive stock is good but not optimal. New genetic material should be incorporated by introducing new unrelated individuals or their sperm. We suggest maintaining two distinct breeding lines, and we do not recommend crossing between them. Low N_e values were estimated (around 18-39) and not all founders were fully unrelated as assumed in a captive breeding program. These facts raise the possibility of inbreeding depression, and fail the goal of maintaining the 90% of the initial genetic variation within a long-term period. Therefore, we suggest implementing an open system that allows the continuous gene flow from in situ to captive populations. Captive stock and subsequent cohorts should be long-term monitored in order to preserve genetic variation.

Introduction

Many scientists agree that we are achieving the midst of the sixth great mass extinction. Anthropogenic pressure is affecting both directly or indirectly natural environments (Wake and Vredenburg 2008). The amphibians are one of the most severely affected groups, and are the only ones catalogued globally at risk. Therefore, currently and within the near future, the application of ex situ breeding attempts might become very important (Griffiths and Pavajeau 2008). The development of well-designed captive populations can be crucial for species recovery and management of threatened or endangered species with wild populations declining precipitously (Ralls and Ballou 1986; Frankham 1995). Lacy (2009) reported the difference between a well-designed and a non-designed captive breeding program with two bovid mammals (arabian oryx and markhor) concluding a very different future for them. The number of initial founders and their correct selection will be key factors worth considering.

Genetic diversity present in a captive population is determined by its founders (Mace et al. 1992). Captive populations often present small sizes due to spatial or financial limitations of the organizations that manage them. Moreover, captive populations are frequently created when the species at risk has dwindled to small numbers in the wild (Conde et al. 2011; Lyles and May 1987; Traylor-Holzer 2011; Williams and Hoffman 2009). For these reasons, individuals may represent only a small fraction of the genetic diversity of the wild population (Ballou and Lacy 1995; Mace 1986; Ralls and Ballou 1992) and therefore, limited genetic diversity may be present among ex situ founders. The number of founders, and their relatedness to each other, determines the amount of genetic diversity that can be retained in the captive population (Frankham et al. 2010; Mace et al. 1992; Senner 1980; Willis and Willis 2010). Loss of genetic variation may be a serious threat to its long-term viability in such populations (Lacy 1997; Willis 1993). Captive populations may encounter distinct types of genetic change similar to small closed populations, such as the loss genetic diversity and the accumulation of new mildly deleterious alleles which could drive into inbreeding depression. Moreover, genetic adaptation to captivity should be also considered when managing populations ex situ (Ballou and Lacy 1995; Frankham 1995; Frankham 2008; Princée 1995).

Carefully planned breeding programs are therefore required to minimize these threads. Two main aspects are important to be considered for the creation of a well-designed program. On the one hand, there is broad agreement among wildlife management officials that the retention of 97.5% of the genetic diversity from wild populations should be a sufficient goal for the long-term viability of founder populations (Edwards et al. 2014). On the other hand, it is estimated that a "self-sustaining" ex situ population must keep up to 90% of the founding population's genetic variability for the time of the ex situ program duration, equivalent to the time required for habitat recovery (Soulé et al. 1986; Ballou et al. 2006). Therefore, the capture of the maximum genetic diversity from wild and its conservation for a long-time period are the two main goals of ex situ breeding programs. However, in some cases these values cannot be achieved due to the realistic situation in the wild. In these cases, an accurately design should be planned to ensure the conservation of the highest amount of genetic diversity.

Genetic diversity is measured in terms of allelic diversity and heterozigosity (Ballou and Foose 1996; Ballou et al. 2010). Allelic diversity may represent the evolutionary potential of a population and plays an important role in the process of adaptation to to environmental change; it is, therefore, an important value to consider for a population's long-term ability (Ballou et al. 2010; Briscoe et al. 1992; Frankham 2003; Princée 1995). Allelic diversity may be affected by population bottlenecks, such as those which may arise as a result of the creation of breeding stock (Amos and Balmford 2001). Heterozygosity is described either as observed or expected heterozigosity. The former is defined by the proportion of genetic loci for which the average individual in a population is heterozygous; the latter is referred to the probability that two

homologous genes randomly drawn from the population are distinct alleles (Höglund 2009; Lacy 1994), i.e. the mean heterozigosity that would exist in a population if it were in Hardy-Weinberg equilibrium. Therefore, the measurement of the heterozigosity may be an indicator of individual health and its capability to response to selection (Lacy 1994; Reed and Frankham 2003).

To sum up, the number of founders that started a breeding program should be a balance between genetic diversity captured from the wild and its sustainability, and the number of individuals to be extracted from the wild population without compromising them, as well as the capability of maintaining these numbers of breeders and descendants.

The Montseny brook newt (*Calotriton arnoldi*) is one of the most endangered vertebrates in Europe and it is classified as critically endangered by the International Union for Conservation of Nature (IUCN) (Carranza and Martínez-Solano 2009). The current distribution range is limited to an area of only 8 km^2 in the Montseny Natural Park, NE Iberian Peninsula. Furthermore, their disconnected populations are found in seven geographically close brooks, fragmented into two main sectors (eastern and western) on both sides of the Tordera river valley and separated by unsuitable habitat (Valbuena-Ureña et al. 2013 – Chapter IV). With an estimated population size of less than 1500 mature individuals (Amat and Carranza 2005), the major threats affecting this species is habitat degradation and disturbance, such as large amounts of water extracted for commercial purposes, deforestation and the existence of tracks and roads that disrupt the brook continuity (Amat et al. 2014).

Contrary to what was expected, the previous genetic study focused on *C. arnoldi* concluded that genetic diversity in this species is comparable to populations of amphibian species with much larger distribution ranges (Valbuena-Ureña et al. – Chapter VI). This study asserted that natural habitat fragmentation stated above has not produced a negative effect on this species on an evolutionary time scale, and strongly suggested both sectors should be considered as separate management units for conservation. The strong population structure detected in this species, together with the low Ne estimated supported the current status catalogued by the IUCN.

Although the conservation plan is not yet approved for this species, as a consequence of its fragile status, some in situ management measures have been developed by the Barcelona Provincial Council, such as habitat restoration and field monitoring (Amat et al. 2014). Moreover, the Catalonian Government established in 2007 a captive breeding program for purposes of maintenance of representative refugee populations as well as for research and re-introduction or reinforcement of the wild populations (DAAM in progress). In order to maintain the maximum genetic diversity, the IUCN recommends in its guidelines to start a captive breeding program when a species still numbers in thousands in the wild. Considering the low numbers of individuals estimated for this species, was fully justified the need for a captive breeding program for this species, and it became evident the urgency to start immediately with

the breeding program. Individuals from eastern A1, and western B1 and B2 wild populations were sampled randomly ensuring a good body condition, pending detailed genetic results. These founders from each sector were kept separately as two distinct breeding lines, as a result of preliminary data on the genetic and morphological variability between the two sectors (Carranza and Amat 2005) that has been corroborated in posterior studies (Valbuena-Ureña et al. 2013 – Chapter IV; Valbuena-Ureña et al. – Chapter VI). Since its beginning in 2007 and after seven years operating, this program has successfully reared more than 1500 individuals (Carbonell et al. 2014). Though demonstrating the viability of the breeding program, no analyses have yet been performed to determine the levels of genetic diversity among captive individuals. Taking into account the vulnerability of the species – i.e. the low number of individuals present, the fragmentation into two sectors and the peculiar genetic characteristics in their natural environment (Valbuena-Ureña et al. – Chapter VI) – the correct design of the captive breeding stock will be crucial for ensuring its long-term viability. Moreover, this program offers an excellent opportunity for evaluating its utility by studying the selection and quantifying the founders needed and to state recommendations for similar species.

The aim of this study is to evaluate the genetic quality present in the captive stock of Montseny brook newts using 24 microsatellite loci. These will be assessed by evaluating if the level of genetic diversity (allelic diversity and heterozigosity) observed in the captive stock is comparable to that observed in the wild. Moreover, it is also evaluated if the actual captive gene diversity is sufficient for its endurance over time. For this aim the relatedness among individuals and the estimation of captive effective population sizes will be assessed. Recommendations suggested from these results are crucial to ensure the genetic quality of the breeding members and thus will help to successfully establish a "self-sustaining" captive population, and therefore the long-term endurance of the ex situ conservation program.

Materials and methods

Sample collection and genotyping

A total of 42 individuals (founders and descendents) from the captive breeding facility were analyzed, including samples from both breeding lines. All founders from the eastern sector belonged to population A1, and founders from the western captive breeding line were from populations B1 and B2 (see Valbuena-Ureña et al. (2013) – Chapter IV, for population codification). The samples contained 9 out of the 13 eastern founders and 10 out of the 11 western founders, which represented 79% of the 24 initial founders. Nine of them were female while 10 were male. Only 5 founders (4 from the eastern and 1 from western breeding lines) could not be sampled because they died before sampling. The remaining 23 samples correspond to F1 descendents, 17 from the eastern breeding line and 6 from the western one. In total, 26 captive individuals from the eastern breeding line were analyzed and compared to 77 genotyped

wild animals from this sector and 16 captive individuals from the western breeding line were genotyped and compared to 83 wild animals from the western sector (see Valbuena-Ureña et al. – Chapter VI) for information about the genotyping of the wild populations). Tissue samples consisted of tail tips or fingers preserved in absolute ethanol. Genomic DNA was extracted using QiagenTM (Valencia, California) DNeasy Blood and Tissue Kit following the manufacturer's protocol. All 42 individuals' arrays were genotyped for 24 polymorphic microsatellite loci, as described previously (Drechsler et al. 2013; Valbuena-Ureña et al. 2014 – Chapter V). Details of the PCR-multiplex combinations of loci and specific amplification procedures are given in Valbuena-Ureña et al. – Chapter VI.

Data analysis

The MICRO-CHECKER software (Van Oosterhout et al. 2004) was used to check for potential scoring errors, large allele dropout and the presence of null alleles. Pairwise linkage disequilibrium between loci was checked using the software GENEPOP version 4.2.1 (Rousset 2008). The same program was used to calculate deviations from Hardy-Weinberg equilibrium in each grouping and for each locus, which provides an exact probability value (Guo and Thompson 1992). Genetic diversity was measured for each grouping as mean number of alleles (A), observed (H_O) and expected heterozygosity (H_E) using FSTAT 2.9.3.2 (Goudet 1995). Formal analyses of raw allelic richness values were not performed because the number of alleles detected at a locus can be influenced by sample sizes. Therefore a rarefied estimate of allelic richness (Ar) was obtained with HP-RARE (Kalinowski 2005). The nonparametric Wilcoxon signed-rank tests (Wilcoxon 1945) was used to test for differences in diversity levels between captive and wild groups using locus-specific values of H_E or allelic richness as paired replicates. In analyses based on allelic richness, separate rarefactions were performed for each sector groupings to account for the smallest sample size from a particular group. The observed number of private alleles (PA), defined here as alleles found in a single population throughout the study region, for each locus and each grouping was calculated with GDA (Lewis and Zaykin 2000) and the ratio of presence compared to wild PA was computed. Moreover, overall and intrapopulation subdivision coefficients (F_{IS}) for all markers and groupings were calculated using the same program as above, which calculates the estimator f of Weir and Cockerham (1984) for each marker as well as a multilocus estimate. Genetic differentiation between the captive and wild population was derived from the F_{ST} values obtained in ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010).

A Bayesian clustering algorithm using the program STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used not only to analyze the structure of the captive stock comparing it to the wild populations but also to confirm the origin of founders and to corroborate the assignment of their descendants. The software assigns individuals to clusters without using prior information

on their localities of origin. The admixture model with correlated allele frequencies was assumed using a "burn in" period of 100000 iterations followed by one million Markov chain Monte Carlo (MCMC) iterations. Number of cluster (K) values evaluated ranged from one (panmixia) to eight (the number of sample locations plus one), and 10 runs for each K value were performed. The inference for the best value of K was based on the ΔK method described by Evanno et al. (2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012). The program uses a Markov chain Monte Carlo (MCMC) procedure to estimate $P(X|K)$, the posterior probability that the data fit the hypothesis of K clusters. The program also calculates the fractional membership of each individual in each cluster (Q). The probability that a certain individual comes from a particular population was calculated using Bayesian criteria in GENECLASS 2.0 (Piry et al. 2004). Assignment probabilities are computed based on a Monte Carlo resampling method (Paetkau et al. 2004). A total number of 10000 individuals were simulated and a threshold of 0.001 was used.

In order to test the levels of endogamy, the relatedness among individuals within each grouping was evaluated using MLRELATE (Kalinowski et al. 2006). This program is appropriate as it is designed for microsatellites, is based on maximum likelihood tests, and considers null alleles. Furthermore, GenAlEx v. 6 (Peakall and Smouse 2006) was used to obtain pairwise relatedness among individuals in each population using the r_{qg} estimator (Queller and Goodnight 1989).

The effective population size (N_e) for each breeding line based on one-sample N_e estimator method was calculated with ONeSAMP (Tallmon et al. 2008). ONeSAMP employs approximate Bayesian computation. The analyses were submitted online to the ONeSAMP 1.2 server (http://genomics.jun.alaska.edu/asp/Default.aspx), specifying a minimum N_e value of 2 and a maximum of 100.

To detect evidence for recent episodes of bottlenecks we tested for significant heterozygosity excess or deficit with the program BOTTLENECK, version 1.2.02 (Cornuet and Luikart 1996). This approach is effective for detecting bottlenecks that have occurred recently, within the past 0.2-4.0N_e generations (Luikart and Cornuet 1998). The infinite allele (IAM), stepwise mutation (SMM), and two-phase mixed (TPM) models were tested; the latter with the assumption that microsatellites follow a 95% stepwise-mutation model with a variance of 12.00, following the recommendations of Piry et al. (1999). To test whether a population contained a significant number of loci with heterozygosity excess, the Wilcoxon signed rank test with 2000 iterations was used. Second, a mode shift test was used to detect distortion of the L-shape expected under equilibrium for the frequency distribution of allele classes for each of the captive breeding lines (Luikart et al. 1998).

Results

A total of 140 alleles were identified for the 24 microsatellite loci in the 42 individuals of the captive stock analyzed (19 founders and 23 descendents), compared to the 170 alleles found in the 160 specimens analyzed from the wild populations. Regarding the presence of alleles by sector, 98 alleles were detected in the eastern captive breeding line in contrast to the 132 observed in the wild, and 74 alleles were detected in the western captive breeding line in contrast to the 95 observed in the wild. Like in the wild populations, all 24 microsatellites were polymorphic for the eastern captive breeding line but Us3 and Us7 were monomorphic throughout the western captive population (Suppl. Table 1). No evidence of null alleles was detected except for Ca8 from the eastern sector. No significant departures from Hardy-Weinberg equilibrium (HWE) were found in the captive breeding lines after applying Bonferroni correction (P>0.00104). Only one locus (Calarn 52354) in the eastern captive population deviated from HWE. There was no sign of linkage disequilibrium between any pair of loci except between Ca07 and Calarn52354 in the eastern captive group after applying a Bonferroni correction (P>0.00018).

The genetic diversity measures are shown in Table 1. Similar values of mean number of alleles (A) are found in the captive and wild individuals from the eastern sector, while captive individuals from the western sector showed slightly higher values of A than wild specimens. Regarding differences among groups taking into account sample size, rarefied allelic richness was lower in the eastern captive individuals than in the wild ones, though the difference was not significant, and allelic richness was similar between western captive and wild individuals. Generally, higher allelic richness values were observed in the eastern captive and wild groups compared to the western ones. Also similar values were found among founders and descendants. The number of alleles per locus ranged from one to a maximum of seven (Suppl. Table 1). Each breeding line was found to contain private alleles (PA) that are only present in a single sector. A total of 57 PA were found in the eastern captive individuals (two of them not found before in the wild) compared to 75 present in wild samples (i.e. the 76% of the PA are present in captivity). Thirty-one PA were found in the western captive samples out of 38 found in wild individuals (81.5%). One allele found in the eastern line captive population was considered a PA from the wild populations of the western sector, and vice versa (one captive western allele considered a wild PA from the eastern sector). Regarding F1, 11 and 2 new PA were found in the eastern and western sectors, respectively.

Table 1. Genetic diversities of the captive breeding lines compared to wild population sectors of the Montseny brook newt. Values represent averages across 24 loci. N, sample size; A, number of alleles per locus; Ar, allelic richness; PA, number of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. Values in bold indicate statistical significance after Bonferroni correction.

Group	N	A	Ar	PA	H_O	H_E	F_{IS}
Eastern							
Founders	9	3,417	3,420	46	0,574	0,501	-0,088
F1	17	3,750	3,330	55	0,522	0,513	0,012
Total captive	26	4,083	3,380	57	0,540	0,519	-0,022
Wild	77	4,167	4,112	75	0,544	0,538	**0,090**
Western							
Founders	10	2,917	2,650	28	0,413	0,421	0,072
F1	6	2,458	2,460	24	0,507	0,387	-0,227
Total captive	16	3,083	2,590	30	0,448	0,418	-0,039
Wild	83	2,724	2,610	38	0,359	0,352*	**0,184**

* H_E = 0.423 when population B3 is excluded from wild analysis (Valbuena-Ureña et al. – Chapter VI).

The mean expected heterozygosities (H_E) were 0.519 and 0.418 in the eastern and western captive breeding lines respectively. On a locus by locus basis, H_E of the captive eastern line was significantly lower than their wild counterparts (p = 0.0082). No differences were observed in the west between captive and wild western individuals, although a tendency towards smaller average heterozygosity was observed in the wild populations. Overall, F_{IS} did not show values significantly different from zero in each breeding line after applying Bonferroni correction. The F_{ST} between the captive and wild populations was 0.061 ($p<0.001$) for the eastern sector and 0.044 ($p<0.01$) for the western sector.

Captive and wild allele frequencies are given in Suppl. Table 2. Regarding the eastern sector, all but three of the alleles found in the captive sample were present in the wild sample, whereas 37 alleles found in the wild sample were not found in the captive stock. Instead, in the western sector only one allele found in captivity was not present in the wild, while 22 alleles present in the wild specimens were not found in the captive stock.

Genetic structure and population assignments

Calculation of ΔK from the structure output of the captive stock produced a modal value of the statistic at K = 2. All runs at K = 2 produced identical clustering solutions with similar values of cluster membership Q for all individuals within sectors (Table 2).

Table 2. Performance of the exclusion simulation test (GENECLASS) and Bayesian assignment test (STRUCTURE) used to assign individuals to a population of origin.

Sample	Geographic origin	STRUCTURE Q (E/W clusters; K=2)	GENECLASS locality of highest probability assignment-exclusion test	GENECLASS highest assignment probability
SPM10032701	Eastern-A1	0,902/0,098	Eastern-A1	0,091
SPM12030501	Eastern-A1	0,999/0,001	Eastern-A1	0,531
SPM12030502	Eastern-A1	0,999/0,001	Eastern-A1	0,683
SPM12030503	Eastern-A1	0,999/0,001	Eastern-A1	0,312
SPM12030504	Eastern-A1	0,999/0,001	Eastern-A1	0,710
SPM12030505	Eastern-A1	0,999/0,001	Eastern-A1	0,925
SPM12030506	Eastern-A1	0,999/0,001	Eastern-A1	0,185
SPM12030507	Eastern-A1	0,999/0,001	Eastern-A1	0,428
SPM12030508	Eastern-A1	0,999/0,001	Eastern-A1	0,794
SPM12050801	Captive F1 - A1	0,999/0,001	Eastern-A1	0,202
SPM12050802	Captive F1 - A1	0,999/0,001	Eastern-A1	0,566
SPM12050803	Captive F1 - A1	0,999/0,001	Eastern-A1	0,488
SPM12050804	Captive F1 - A1	0,998/0,002	Eastern-A1	0,152
SPM12050805	Captive F1 - A1	0,999/0,001	Eastern-A1	0,338
SPM12050806	Captive F1 - A1	0,999/0,001	Eastern-A1	0,285
SPM12050807	Captive F1 - A1	0,999/0,001	Eastern-A1	0,076
SPM12050808	Captive F1 - A1	0,999/0,001	Eastern-A1	0,103
SPM12050809	Captive F1 - A1	0,999/0,001	Eastern-A1	0,157
SPM12050810	Captive F1 - A1	0,999/0,001	Eastern-A1	0,123
SPM12050811	Captive F1 - A1	0,999/0,001	Eastern-A1	0,040
SPM12050812	Captive F1 - A1	0,999/0,001	Eastern-A1	0,227
SPM12050813	Captive F1 - A1	0,998/0,002	Eastern-A1	0,228
SPM12050814	Captive F1 - A1	0,999/0,001	Eastern-A1	0,154
SPM12050815	Captive F1 - A1	0,999/0,001	Eastern-A1	0,129
SPM13012501	Captive F1 - A1	0,999/0,001	Eastern-A1	0,568
SPM13012502	Captive F1 - A1	0,998/0,002	Eastern-A1	0,485
SPM13012509	Western-B1	0,001/0,999	Western-B1	0,267
SPM12030509	Western-B1/B2	0,001/0,999	Western-B1	0,866
SPM12030510	Western-B2	0,001/0,999	Western-B2	0,538
SPM12030511	Western-B1/B2	0,001/0,999	Western-B2	0,172
SPM12030512	Western-B2	0,001/0,999	Western-B2	0,310
SPM12030513	Western-B1	0,001/0,999	Western-B1	0,690
SPM12030514	Western-B1	0,001/0,999	Western-B1	0,019
SPM12030515	Western-B1	0,001/0,999	Western-B1	0,895
SPM12030516	Western-B1	0,001/0,999	Western-B1	0,711
SPM12030517	Western-B1	0,001/0,999	Western-B1	0,797
SPM13012503	Captive F1 - B2	0,001/0,999	Western-B1	0,265
SPM13012504	Captive F1 - B1	0,001/0,999	Western-B1	0,801
SPM13012505	Captive F1 - B1&B2	0,001/0,999	Western-B1	0,563
SPM13012506	Captive F1 - B1	0,001/0,999	Western-B1	0,717
SPM13012507	Captive F1 - B1&B2	0,001/0,999	Western-B1	0,802
SPM13012508	Captive F1 - B1&B2	0,001/0,999	Western-B1	0,850

The two clusters correspond exactly to geographical sectors, with all the individuals from each sector clustering together. An eastern cluster is present, with all founders and descendents from population A1 assigned to this cluster. All of the captive individuals from B1 and B2 were assigned unambiguously to the western cluster. Only one eastern founder has rather little partial inferred ancestry (mean $Q = 0.098$) to the western sector. The rate at which individuals correctly assign to their sampled locality can also be used as an assessment of population genetic structure (Manel et al. 2005). GENECLASS assigned all individuals to their population of origin when the locality of highest probability was considered (Table 2). Only one misassignment was detected for localities belonging to the same cluster: a descendent of parents from B2 with a highest probability of assignment to population B1, though the probabilities were low (probability of assignment to B1 = 0.265, and B2 = 0.159). The population of origin of the two founders is uncertain, because individuals from B1 and B2 were mixed into the same aquaria.

Relatedness of individuals

The proportion of individual relatedness within each captive breeding line was similar (Table 3). Most individuals were unrelated, with percentages higher than 80%. Full sibling and parent-offspring relations represented percentages not exceeding the 6% except for the descendents of the western sector which presented the 13% of full-siblings. Finally, 11% of the founders from the western sector were half-siblings related. The estimated Queller and Goodnight (1989) index of relatedness among all founders and among descendants from each breeding line were similar and near 0 (mean r_{qg} eastern founders = -0.125, western founders = -0.111, eastern F1 = -0.063, western F1 = -0.200).

Effective population size and bottleneck

The estimated N_e for the eastern captive breeding line was 38.96 (median, 95% CI: 34.27-52.25), and N_e = 17.62 (median, 95% CI: 15.30-24.05) for the western captive breeding line (Table 3). Founders N_e estimates from both breeding lines were roughly 10, while effective population sizes estimated from offsprings were 17.03 and 7.97 for the eastern and western populations, respectively.

No captive population showed any signs of a recent bottleneck under neither the TPM (one tailed test for heterozygote excess) nor the SMM models (Suppl. Table 3). Instead, under the IAM model the eastern captive breeding line including founder and descendent groups as well as founders from the western sector showed a signature of excess of heterozygosity. In addition, the mode shift test showed a normal L-shape for the eastern captive breeding line, while a shifted mode was detected for the western breeding line when analyzing founders and F1 independently but not for the total captive stock.

Table 3. Estimates of effective population size (N_e) for each breeding line calculated with ONeSAMP, with estimations of the upper and lower 95% CI, and the proportion of individual relatedness within each breeding line. Values are compared to wild results obtained from Valbuena-Ureña et al. – Chapter VI.

	Effective population size			Relatedness			
Group	N_e	95% Cis		Unrelated	Half Siblings	Full Siblings	Parent Offspring
Eastern							
Founder	10,20	9,20	12,18	97,22	2,78	0,00	0,00
F1	17,03	15,07	22,12	83,09	11,03	3,68	2,21
Total captive	38,96	34,27	52,25	85,85	8,31	2,77	3,08
Western							
Founder	10,93	9,38	15,07	86,67	11,11	0,00	2,22
F1	7,97	6,93	10,22	80,00	6,67	13,33	0,00
Total captive	17,62	15,30	24,05	83,33	9,17	1,67	5,83

Discussion

The main aim of the *Calotriton arnoldi* ex situ breeding program is to produce specimens for the reintroduction program into the restored habitat of the species. As has been shown in similar projects, the success of the program will depend on the genetic variability of the captive stock (Lacy 1994; Mitchell et al. 2011; Tzika et al. 2009). Decisions based on erroneous or incomplete data may lead to a decrease of genetic diversity, jeopardizing the long-term survival of the captive stock and affecting negatively the wild populations if captive specimens are reintroduced in the wild (Frankham 1995; Saccheri et al. 1998; Hedrick and Kalinowski 2000; Spielman et al. 2004). This is the first approach to the evaluation of the actual captive breeding program of the critically endangered Montseny brook newt.

Estimation of the genetic diversity present in the captive stock

Generally, 82% of the alleles present in the wild populations are also present in the captive stock, the percentages by sectors being 74% and 78% for the eastern and western sectors, respectively. Medium to high-frequency alleles detected in the wild populations are also observed within the set of captive individuals (Suppl. Table 2). Also, there is an acceptable representation of PA in the captive groups (76% and 81.5% of the wild private alleles are present in the eastern and western captivity groups, respectively). The results above, together the generally lower estimates of H_E of the captive stock than their wild counterparts, indicate that the individuals that conform the actual captive program are good enough but not optimal representatives of the genetic diversity present in the wild. Moreover, genetic theory indicates

that modest numbers of founders, namely at least 20 unrelated wild individuals, are sufficient to capture 97.5% of the genetic diversity of the wild population within the founder population (Leus 2011). The current genetic differentiation values between the captive Montseny brook newts and wild populations is around 5-7%, and exceed the maximum 2.5% desired. Therefore, new genetic material should be incorporated by introducing new unrelated individuals or their sperm. Taking into account that this species present sperm storage, an ideal strategy to maximize the amount of genetic pool to the captive population causing the minimum impact to the wild populations could be the incorporation of wild-caught gravid females. Thus, descendants may retain the genetic diversity from individuals not kept in captivity.

Maintaining two distinct ESU's

According to the results of the Bayesian clustering algorithm and pairwise F_{ST}, a high genetic differentiation is observed between both breeding lines. This agrees with the significant genetic differentiation found between the eastern and western wild populations using both mitochondrial and nuclear markers. Also, the higher allelic richness observed in the eastern captive stock compared to the western one agrees with what has been found for the wild populations for both mitochondrial and nuclear makers, in which the eastern sector appears to be genetically richer than the western one (Valbuena-Ureña et al. 2013 – Chapter IV; Valbuena-Ureña et al. – Chapter VI). Therefore, the maintenance of the two breeding lines separately is strongly suggested. Although outcrossing the two conservation units would be an action that may help to increase the genetic diversity (Frankham et al. 2010), we do not recommend it at this point for two main reasons: a) the importance of maintaining the two evolutionary groups, and b) the potential threat to the existing populations due to outbreeding depression (OD) (Allendorf and Luikart 2007). Existing conservation guidelines might focus on maintaining high levels of genetic diversity within the two distinct breeding lines. As being done so far, in case of re-introduction of individuals into brooks not occupied by this species at current time, it is important to keep in mind the origin of each larvae reared in order to avoid mixture of sectors.

Enlarging the captive breeding population

Although r_{qg} estimates among founders and descendents are near 0, relatedness percentages indicate that not all founders are unrelated, as it is generally assumed in a captive breeding program. Moreover, a drop of the effective population size is detected in the eastern captive group (N_e = 39) compared to the wild populations (N_e = 86; Valbuena-Ureña et al. – Chapter VI), as well as in the western captive group (N_e = 18) compared to the wild populations (N_e = 80; Valbuena-Ureña et al. – Chapter VI). This low value of N_e raises the possibility of inbreeding depression. The theoretical loss of genetic variation is $^1/_{2Ne}$ per generation (Montgomery et al. 2000; Allentoft and O'Brien 2010). The current N_e estimated based on these

genetic results for each captive breeding line was 39-18 individuals. With an estimated generation time of 5 years per generation (Carbonell et al. 2013), the calculated proportion of initial heterozygosity retained in 100 years (20 generations) would be 74.36% and 44.44% for the eastern and western captive breeding lines, respectively. Although generation time could be expanded by manipulating the age of breeding, this loss of heterozigosity could compromise the traditionally accepted goal in captive breeding programs to maintain 90% of the initial genetic variation within 100 years.

Following the Amphibian Ark guidelines, at least 20 unrelated founders by sector to survive and breed with an equal sex ratio would be required. The minimum genetic target population size, defined as the minimum population size needed to meet the genetic goals of a 100 years program is 320 individuals by sector, assuming individual management, age to maturity over 5 years and reproductive lifespan of 15 years or less (Shad 2008). In order to achieve the target of self-sustainability of the captive population during a long period of time, very variable descendants should be included into the program. Nonetheless, the non-optimal representation of the total genetic diversity present in the wild seen above elucidate the need of introducing additional individuals into the two ex situ breeding lines.

The expected heterozygosity (H_E) of the captive eastern stock is significantly lower than that of the wild populations (p = 0.0082), indicating that the eastern captive stock only represents part of the existing gene pool of the eastern wild populations. Captive individuals of this breeding line originate from a single population (A1). As suggested by previous analyses of both mitochondrial and nuclear data, the eastern populations are grouped into two clusters, being population A3 the most differentiated and populations A1 and A2 the less differentiated, clustering together in the microsatellite analysis (Valbuena-Ureña et al. 2013 – Chapter IV; Valbuena-Ureña et al. – Chapter VI). Therefore, it is recommended to introduce further individuals, mainly from the wild population A3.

No differences were observed between captive and wild western populations, although a tendency towards smaller average heterozygosities was observed in the wild. Nonetheless, in order to reduce the 11% of half-siblings relatedness detected among the western founders and to avoid the possibility of inbreeding, an increase of breeder individuals from this sector is also recommended. According to microsatellite data, the western sector clusters into two groups, the main one formed by populations B1, B2 and B4, and the other one by population B3, which is separated into a distinct cluster (Valbuena-Ureña et al. – Chapter VI). Whereas overall western H_E is 0.352, the heterozygosity increases up to 0.423 when population B3 is excluded. The low genetic diversity (H_E =0.197) detected in this population biases the mean western wild H_E value. Currently there are no founders from B3, but as a result of its low genetic diversity it is unadvisable to have further breeders from this site. Therefore, in case of incorporating additional founders to this breeding line, individuals from B1, B2 and B4 should be prioritized.

So, with the aim of minimizing relatedness among breeders and avoiding the inbreeding of low effective size breeding groups it is recommended to incorporate additional specimens to the breeding stock. However, in order to properly identify the best candidates and to clarify their origin and relatedness with other captive individuals, specimens must be genotyped before being incorporated into the captive population.

A need for periodic re-evaluation

Similar values of rarefied allelic richness are found among founders and descendants, a fact that indicates that the descendants inherited almost all the microsatellite alleles from the founders. Eleven and two new PA were found in the descendent group not detected in the founders, regarding the eastern and western sectors respectively. This fact could be explained by many reasons; the first, there are five founders that could not be included in the present analyses, as individuals died before they could be sampled; second, some founder females were already gravid when they were caught in the wild, so the genotype of the males was unknown; and third, some females could fertilize their eggs with sperm from wild males stored in their reproductive apparatus using sperm storage, a strategy suggested for *C. arnoldi* (Alonso 2013) and present in many urodel species (Kühnel et al. 2010). Therefore it is highly recommended that further descendents in subsequent offspring's are genotyped in other to ensure that genetic diversity is maintained over time.

The use of molecular data has been useful to investigate the relatedness among individuals for the design of optimal mating groups and has allowed the identification of the natural population from which the founders originated. It is thus underlined the importance of using molecular markers to evaluate genetic management of captive breeding programs. Moreover, it is crucial to detect population bottlenecks in managed species because a reduction in N_e may enhance the rate of inbreeding, loss of genetic variation and fixation of deleterious alleles considerably and thereby increase the risk of population extinction (Luikart et al. 1998). Nevertheless, as the captive breeding program of the Montseny brook newt was founded very recently (less than 10 years have passed) it can be difficult to identify losses of genetic variation or a possible founder effect due to the short period of time. Therefore, the captive stock and subsequent cohorts should be long-term monitored in order to preserve genetic variation. Moreover, further analyses focusing on the resolution of the pedigree to avoid possible mating of closely related individuals, deterministic parental assignment of offspring and the suggestion of optimal mating groups for maximizing diversity are needed. The current breeding protocol, which has been started using in this program, and which is widely used by many captive breeding programs to minimize loss of genetic diversity is pairing individuals according to the mean kinship (MK) (Willoughby et al. 2015), which is based on the number and degree of relatives that the individual has in the captive stock.

Finally, a problem associated to small founder populations is their significant rapid adaptation to captivity (Griffiths and Pavajeau 2008; Gilligan and Frankham 2003; Frankham 2008; Witzenberger and Hochkirch 2011). A possible solution would be to regularly renew the captive populations with previously genotyped individuals from the wild, thereby reducing adaptation to captivity to a minimum. In fact, we suggest building an open system that allows the continuous gene flow from wild to captive populations. As a consequence of open population management, the minimum size of the captive populations needed to achieve the standard current goal of being successful and "self-sustainable" could be slightly smaller.

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Supplementary table 1a. Estimates of genetic parameters for the eastern breeding line by locus. N, sample size; A, number of alleles per locus; Ar, allelic richness; PA, number of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. Values in bold indicate statistical significance after Bonferroni correction.

	Locus	Calarn 02248	Ca3	Calarn 29994	Ca21	Calarn 37825	Calarn 14961	Calarn 15906	Calarn 12022	Calarn 06881	Ca7	Ca22	Calarn 50748	CA8	Calarn 36791	Calarn 59202	Ca 32	Calarn 52354	Calarn 30143	Calarn 31321	Calarn 15136	Calarn 37884	Us2	Us3	Us7	Overall
Eastern founders	N	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	A	5,000	2,000	2,000	2,000	5,000	4,000	4,000	4,000	3,000	4,000	1,000	2,000	4,000	4,000	3,000	4,000	4,000	4,000	4,000	3,000	4,000	5,000	4,000	3,000	3,417
	Ar	4,617	1,976	1,902	2,000	4,471	3,799	3,662	3,333	2,642	3,897	1,000	2,000	3,471	3,892	2,333	3,564	1,902	3,642	3,569	2,666	3,642	4,000	3,657	2,662	3,100
	PA	5	2	1	1	1	1	3	2	2	2	1	0	3	3	1	2	1	2	4	0	3	0	4	2	46
	H_O	1,000	0,333	0,222	0,556	0,889	0,667	0,889	0,556	0,444	0,444	0,000	0,667	0,444	0,889	0,222	0,667	0,222	0,889	0,667	0,444	0,778	0,778	0,778	0,333	0,574
	H_E	0,753	0,278	0,198	0,475	0,710	0,617	0,685	0,574	0,364	0,716	0,000	0,444	0,451	0,691	0,204	0,562	0,198	0,642	0,623	0,475	0,660	0,630	0,648	0,426	0,501
	F_{IS}	-0,274	-0,143	-0,067	-0,111	-0,196	-0,021	-0,243	0,091	-0,164	0,429	-	-0,455	0,072	-0,231	-0,032	-0,129	-0,067	-0,333	-0,011	0,123	-0,120	-0,179	-0,143	0,273	-0,088
Eastern F1	N	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17
	A	6,000	3,000	2,000	2,000	6,000	3,000	4,000	5,000	3,000	4,000	2,000	4,000	5,000	5,000	3,000	3,000	4,000	4,000	6,000	3,000	3,000	5,000	3,000	2,000	3,750
	Ar	4,508	2,579	1,353	2,000	3,646	2,874	3,643	4,372	2,687	3,864	1,353	2,941	3,446	3,830	1,941	2,811	3,203	3,430	4,221	2,840	2,742	3,679	2,825	1,982	3,030
	PA	6	3	1	1	3	1	3	2	2	2	1	1	4	4	2	2	2	2	6	0	2	0	3	2	55
	H_O	0,882	0,529	0,059	0,529	0,647	0,412	0,706	0,765	0,412	0,706	0,059	0,471	0,353	0,706	0,176	0,471	0,353	0,588	0,824	0,471	0,647	0,706	0,588	0,471	0,522
	H_E	0,697	0,469	0,057	0,493	0,619	0,517	0,663	0,683	0,420	0,720	0,057	0,580	0,606	0,621	0,164	0,458	0,528	0,644	0,687	0,569	0,555	0,621	0,512	0,360	0,513
	F_{IS}	-0,237	-0,099	0,000	-0,043	-0,014	0,233	-0,035	-0,089	0,051	0,050	0,000	0,217	0,442	-0,107	-0,043	0,004	0,358	0,116	-0,170	0,202	-0,135	-0,107	-0,119	-0,280	0,012
Eastern total captive	N	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26
	A	6,000	3,000	2,000	2,000	7,000	4,000	4,000	5,000	3,000	4,000	2,000	4,000	6,000	5,000	4,000	4,000	4,000	4,000	6,000	3,000	4,000	5,000	4,000	3,000	4,083
	Ar	4,543	2,385	1,553	2,000	4,016	3,234	3,582	4,132	2,672	3,829	1,231	2,642	3,420	3,858	2,015	3,017	2,903	3,412	4,103	2,746	3,040	3,621	3,382	2,204	3,060
	PA	6	3	1	1	3	1	3	2	2	2	1	1	4	4	2	2	2	2	6	0	3	0	4	2	57
	H_O	0,923	0,462	0,115	0,538	0,731	0,500	0,769	0,692	0,423	0,615	0,038	0,538	0,385	0,769	0,192	0,538	0,308	0,692	0,769	0,462	0,692	0,731	0,654	0,423	0,540
	H_E	0,739	0,411	0,109	0,488	0,659	0,557	0,674	0,663	0,406	0,729	0,038	0,550	0,572	0,652	0,179	0,499	0,434	0,649	0,675	0,582	0,597	0,625	0,576	0,384	0,519
	F_{IS}	-0,231	-0,103	-0,042	-0,084	-0,089	0,122	-0,122	-0,024	-0,022	0,175	0,000	0,040	0,345	-0,161	-0,055	-0,061	0,309	-0,047	-0,120	0,226	-0,141	-0,150	-0,115	-0,083	-0,022

Supplementary table 1b. Estimates of genetic parameters for the western breeding line by locus. N, sample size; A, number of alleles per locus; Ar, allelic richness; PA, number of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. Values in bold indicate statistical significance after Bonferroni correction.

	Locus	Calarn 02248	Ca3	Calarn 29994	Ca21	Calarn 37825	Calarn 14961	Calarn 15906	Calarn 12022	Calarn 06881	Ca7	Ca22	Calarn 50748	CA8	Calarn 36791	Calarn 59202	Ca 32	Calarn 52354	Calarn 30143	Calarn 31321	Calarn 15136	Calarn 37884	US2	Us3	Us7	Overall
Western founders	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	A	3,000	4,000	3,000	3,000	6,000	3,000	1,000	4,000	3,000	3,000	3,000	3,000	3,000	3,000	2,000	3,000	3,000	3,000	3,000	3,000	1,000	4,000	1,000	1,000	2,917
	Ar	2,551	3,848	2,600	2,600	5,000	2,600	1,000	3,803	2,936	2,596	2,596	2,853	2,705	2,985	3,547	1,853	2,985	2,200	2,705	2,838	1,000	3,803	1,000	1,000	2,650
	PA	3	4	2	1	3	0	0	0	2	1	2	0	1	0	2	0	0	0	4	0	1	1	1	0	28
	Ho	0,200	0,900	0,400	0,600	0,600	0,400	0,000	0,800	0,500	0,400	0,400	0,700	0,300	0,500	0,600	0,200	0,800	0,200	0,300	0,500	0,000	0,600	0,000	0,000	0,413
	He	0,335	0,715	0,515	0,535	0,740	0,545	0,000	0,675	0,515	0,445	0,445	0,585	0,340	0,620	0,610	0,180	0,635	0,185	0,340	0,460	0,000	0,675	0,000	0,000	0,421
	F_{IS}	0,446	-0,209	0,273	-0,069	0,239	0,314	-	-0,134	0,082	0,153	0,153	-0,145	0,169	0,244	0,069	-0,059	-0,210	-0,029	0,169	-0,034	-	0,163	-	-	0,072
Western F1	N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	A	2,000	4,000	3,000	2,000	5,000	3,000	2,000	2,000	4,000	2,000	2,000	2,000	2,000	3,000	4,000	1,000	3,000	2,000	2,000	2,000	2,000	3,000	1,000	1,000	2,458
	Ar	2,000	4,000	3,000	2,000	5,000	3,000	2,000	2,000	4,000	2,000	2,000	2,000	2,000	3,000	4,000	1,000	3,000	2,000	2,000	2,000	2,000	3,000	1,000	1,000	2,460
	PA	2	4	2	1	2	0	0	0	3	1	1	0	1	0	2	0	0	0	2	0	2	2	1	0	24
	Ho	0,167	0,667	1000	1000	1000	0,833	0,167	0,333	0,833	1000	1000	0,500	0,333	0,500	0,667	0,000	0,833	0,167	0,333	0,167	0,167	0,500	0,000	0,000	0,507
	He	0,153	0,681	0,611	0,500	0,792	0,625	0,153	0,444	0,653	0,500	0,500	0,486	0,278	0,403	0,653	0,000	0,569	0,153	0,278	0,153	0,153	0,542	0,000	0,000	0,387
	F_{IS}	0,000	0,111	-0,579	-1000	-0,176	-0,250	0,000	0,333	-0,190	-1000	-1000	0,063	-0,111	-0,154	0,070	-	-0,389	0,000	-0,111	0,000	0,000	0,167	-	-	-0,227
Western total captive	N	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
	A	3,000	4,000	3,000	3,000	6,000	3,000	2,000	4,000	4,000	3,000	3,000	3,000	3,000	4,000	2,000	3,000	3,000	3,000	4,000	3,000	2,000	4,000	1,000	1,000	3,083
	Ar	2,387	3,89	2,770	2,375	4,953	2,770	1,375	3,384	3,290	2,374	2,374	2,617	2,482	2,934	3,551	1,617	2,971	1,992	2,851	2,540	1,375	3,479	1,000	1,000	2,590
	PA	3	4	2	1	3	0	0	0	3	1	2	0	1	0	2	0	0	0	4	0	2	1	1	0	30
	Ho	0,188	0,813	0,625	0,750	0,750	0,563	0,063	0,625	0,625	0,625	0,625	0,313	0,500	0,500	0,625	0,125	0,813	0,188	0,313	0,375	0,063	0,563	0,000	0,000	0,448
	He	0,275	0,711	0,576	0,525	0,779	0,580	0,061	0,607	0,576	0,490	0,490	0,555	0,320	0,564	0,631	0,117	0,623	0,174	0,328	0,361	0,061	0,633	0,000	0,000	0,418
	F_{IS}	0,348	-0,111	-0,053	-0,401	0,070	0,063	0,000	0,003	-0,053	-0,245	-0,245	-0,095	0,057	0,146	0,042	-0,034	-0,275	-0,047	0,080	-0,006	0,000	0,143	-	-	-0,039

Supplementary table 2. Microsatellite allele frequencies in wild and captive populations of the Montseny brook newt. Sample sizes at each locus are also provided. Data from wild specimens were obtained from Valbuena-Ureña et al. – Chapter VI.

		Eastern		Western	
Locus	**Allele size (bp)**	**Wild**	**Captive**	**Wild**	**Captive**
Calarn02248	**169**	-	-	0,072	0,094
	173	-	-	0,711	0,844
	177	-	-	0,217	0,063
	185	0,006	0,038	-	-
	193	0,045	0,212	-	-
	197	0,357	0,192	-	-
	201	0,487	0,404	-	-
	205	0,097	0,115	-	-
	209	0,006	0,038	-	-
Ca3	**233**	-	-	0,096	0,125
	237	-	-	0,681	0,375
	241	-	-	0,066	0,188
	245	-	-	0,157	0,313
	249	0,201	0,038	-	-
	253	0,435	0,731	-	-
	257	0,273	0,231	-	-
	261	0,084	-	-	-
	265	0,006	-	-	-
Calarn29994	**169**	0,026	0,058	-	-
	173	0,961	0,942	0,018	-
	177	0,013	-	0,735	0,500
	181	-	-	0,199	0,406
	185	-	-	0,048	0,094
Ca21	**232**	-	-	0,572	0,531
	236	0,117	-	0,410	0,438
	240	0,266	0,423	0,012	0,031
	244	0,487	0,577	-	-
	248	0,097	-	-	-
	252	0,032	-	-	-
	257	-	-	0,006	-
Calarn37825	**211**	-	-	0,030	0,094
	215	-	-	0,229	0,250
	219	-	-	0,036	-
	223	-	-	0,012	0,031
	227	0,019	-	0,006	-
	231	0,071	0,038	0,157	0,125
	235	0,149	0,058	0,392	0,188
	239	0,149	0,038	0,127	0,313
	243	0,240	0,019	-	-
	247	0,208	0,462	0,012	-
	251	0,149	0,346	-	-
	255	0,013	0,038	-	-

Calarn14961	**201**	0,416	0,212	-	-
	205	0,084	-	0,012	-
	209	0,045	0,135	0,386	0,438
	213	0,266	0,615	0,560	0,469
	217	0,039	-	0,018	0,094
	221	0,130	0,038	0,006	-
	225	0,019	-	0,018	-
Calarn15906	**105**	-	-	0,018	-
	108	0,006	-	0,934	0,969
	113	0,156	0,442	0,048	0,031
	116	0,481	0,173	-	-
	121	0,221	0,308	-	-
	125	0,110	0,077	-	-
	129	0,026	-	-	-
Calarn12022	**220**	0,019	-	-	-
	224	0,039	0,077	0,024	0,094
	226	0,506	0,519	-	-
	232	0,065	0,192	0,006	-
	236	0,058	0,077	0,337	0,313
	240	0,091	-	0,470	0,531
	244	0,136	-	0,163	0,063
	248	0,052	0,135	-	-
	252	0,032	-	-	-
Calarn06881	**154**	0,026	-	-	-
	158	0,097	-	-	-
	162	0,175	0,135	-	-
	166	0,571	0,750	-	-
	170	0,019	-	0,006	-
	174	0,110	0,115	0,108	0,188
	178	-	-	0,181	0,188
	182	-	-	0,639	0,594
	186	-	-	0,060	0,031
	190	-	-	0,006	-
Ca7	**224**	0,305	0,250	-	-
	228	0,532	0,288	-	-
	232	0,117	0,327	0,241	0,031
	236	0,045	0,135	0,133	0,344
	240	-	-	0,608	0,625
	244	-	-	0,018	-
Ca22	**126**	-	-	0,500	0,625
	130	0,006	-	-	-
	134	0,753	0,981	-	-
	138	0,227	0,019	0,414	0,344
	142	0,013	-	0,043	-
	146	-	-	0,043	0,031
Calarn50748	**201**	0,013	-	-	-
	205	0,117	0,038	-	-

	209	0,675	0,519	0,319	0,063
	213	0,169	0,423	0,295	0,500
	217	0,026	0,019	0,386	0,438
CA8	**180**	-	-	0,319	0,125
	184	0,026	0,019	0,651	0,813
	188	0,175	0,019	0,030	0,063
	192	0,506	0,596	-	-
	196	0,110	0,250	-	-
	200	0,091	0,096	-	-
	204	0,091	-	-	-
	208	-	0,019	-	-
Calarn36791	**120**	0,110	-	-	-
	124	0,013	-	0,036	0,219
	128	0,058	0,058	0,861	0,594
	132	0,006	-	0,102	0,188
	136	0,201	0,519	-	-
	140	0,422	0,192	-	-
	144	0,156	0,192	-	-
	148	0,032	0,038	-	-
Calarn59202	**212**	-	-	0,169	0,219
	216	-	-	0,705	0,531
	220	0,019	-	-	0,063
	224	0,688	0,904	-	-
	228	0,201	0,058	0,120	0,188
	232	0,065	0,019	0,006	-
	236	0,026	-	-	-
	240	-	0,019	-	-
Ca32	**185**	0,175	0,173	-	-
	189	0,636	0,673	-	-
	193	0,175	0,135	0,910	0,938
	197	0,013	0,019	0,090	0,063
Calarn52354	**220**	0,071	0,019	-	-
	224	0,643	0,731	0,241	0,281
	228	0,149	0,115	0,235	0,500
	232	0,019	-	0,524	0,219
	236	0,019	-	-	-
	240	0,097	0,135	-	-
Calarn30143	**186**	0,097	-	-	-
	190	0,084	0,135	-	-
	198	0,396	0,365	-	-
	202	0,032	-	-	-
	222	0,136	-	0,036	0,063
	226	0,240	0,442	0,157	0,031
	230	0,013	0,058	0,699	0,906
	234	-	-	0,084	-
	238	-	-	0,024	-
Calarn31321	**148**	-	-	0,066	0,063

	152	-	-	0,873	0,813
	157	-	-	0,042	0,063
	161	-	-	0,018	0,063
	173	0,110	0,038	-	-
	177	0,052	0,077	-	-
	181	0,442	0,481	-	-
	185	0,156	0,269	-	-
	189	0,182	0,115	-	-
	193	0,058	0,019	-	-
Calarn15136	**165**	0,032	0,096	0,428	0,156
	169	0,812	0,442	0,024	-
	173	0,123	0,462	0,512	0,781
	177	0,032	-	0,036	0,063
Calarn37884	**240**	-	-	0,036	0,031
	244	-	-	0,783	0,969
	248	0,279	0,346	0,006	-
	252	0,143	0,019	-	-
	256	0,305	0,115	-	-
	260	0,188	0,519	-	-
	267	0,084	-	0,175	-
US2	**219**	-	-	0,054	0,063
	223	0,045	0,077	0,024	-
	227	0,292	0,519	0,620	0,500
	231	0,377	0,308	0,133	0,313
	235	0,188	0,058	0,163	0,125
	239	0,097	0,038	0,006	-
Us3	**143**	-	-	1,000	1,000
	155	0,104	0,077	-	-
	159	0,662	0,596	-	-
	163	0,208	0,231	-	-
	167	0,026	0,096	-	-
Us7	**222**	0,006	-	-	-
	226	0,071	0,231	-	-
	234	0,792	0,750	-	-
	238	0,130	-	-	-
	246	-	0,019	1,000	1,000

Supplementary table 3. Test for detection of recent bottlenecks implemented in BOTTLENECK following the infinite allele (IAM), stepwise mutation (SMM), and two-phase mixed (TPM) models (values in bold indicate statistical significance). Results for the Mode-shift test (modes are indicated by - for normal L-shaped and + for shifted mode).

Group	IAM	TPM	SMM	Mode-shift
Eastern				
Founder	**0,033**	0,710	0,857	-
F1	**0,009**	0,855	0,940	-
Total captive	**0,007**	0,952	0,980	-
Western				
Founder	**0,035**	0,663	0,715	+
F1	0,052	0,406	0,568	+
Total captive	0,099	0,759	0,797	-

VIII. RESUM DELS RESULTATS I DISCUSSIÓ GENERAL

The more you know about a species, the more you understand about how better to help protect them

Alan Clark

1. RESUM DELS RESULTATS

L'objectiu d'aquesta tesi va ser proporcionar dades genètiques rellevants i assessorament per ajudar i millorar la gestió contínua de *C. arnoldi*. La biogeografia de l'espècie (Capítol IV), el disseny de marcadors moleculars polimòrfics (Capítol V), la caracterització i estructura genètica de les poblacions (Capítol VI) i l'avaluació del programa de la conservació ex situ de l'espècie (Capítol VII) es van dur a terme per assolir aquest objectiu.

Els resultats obtinguts en el Capítol IV confirmen que, tot i que les dues espècies del gènere *Calotriton* s'han originat recentment i són dues espècies ecològicament similars i geogràficament molt properes, no hi ha cap senyal d'hibridació entre elles. Per tant, es confirma la validesa taxonòmica de les dues espècies i refuta que hi hagi hagut contacte entre *C. asper* i *C. arnoldi* posterior al procés d'especiació. A més, en aquest capítol es revela una alta estructura genètica en el tritó del Montseny, tot i el seu reduït rang de distribució, en comparació amb el tritó pirinenc. Les anàlisis moleculars i morfològiques indiquen l'existència de dos grups poblacionals geogràficament diferenciats, amb absència de flux genètic entre ells, el sector oriental i el sector occidental.

Els quinze *loci* polimòrfics descrits al Capítol V juntament amb els nou *loci* desenvolupats per *C. asper* i que s'han amplificat amb èxit en *C. arnoldi* (Drechsler et al. 2013), proporcionen una eina poderosa per abordar els estudis de genètica de conservació desenvolupats en els capítols posteriors.

El Capítol VI conclou que la fragmentació de l'hàbitat natural d'aquesta espècie s'ha traduït en una forta divisió genètica de les poblacions en dos sectors, sense migració detectable entre aquests. Encara que les estimacions de mida poblacional efectives suggereixen valors críticament baixos per a totes les poblacions, no s'ha trobat evidència d'alts nivells de consanguinitat entre els individus dins de les poblacions. Així, els nivells de diversitat genètica de *C. arnoldi* són comparables als d'altres espècies d'amfibis amb rangs de distribució molt més grans. En aquest capítol es planteja que la fragmentació de l'hàbitat natural no va tenir efectes negatius en una escala de temps evolutiu, i que l'espècie ha pogut evolucionar gràcies a estratègies reproductives (per exemple, la selecció de parella o *mate choice*) per fer front a les poblacions de mida petita. No obstant això, en el capítol s'adverteix que la fragmentació natural de l'hàbitat ha de ser avaluat com un factor diferent de la pèrdua o degradació de l'hàbitat. L'efecte de tots dos factors ha de ser considerat de forma independent en la planificació de les estratègies de conservació d'una espècie en perill d'extinció. Els resultats d'aquest capítol, juntament amb els obtinguts al capítol 1, suggereixen que ambdós sectors han de ser considerats com a dues unitats de gestió independents per a la conservació, tant in situ com ex situ.

A nivell ex situ, l'avaluació detallada en el Capítol VII mostra que l'estoc captiu actual s'ha iniciat i conté un percentatge mitjà-alt de la freqüència al·lèlica de les poblacions salvatges. Així, s'arriba a la conclusió que la diversitat genètica i representativitat de les poblacions captives és bona però no òptima. Es proposa que ha de ser incorporat nou material genètic a través de la introducció de nous individus no relacionats o del seu esperma. D'acord amb les unitats de maneig anteriorment definides (oriental i occidental), es suggereix mantenir dues línies de cria en captivitat diferents, recomanant evitar la fecundació creuada entre elles. A més, es recomana mantenir un programa ex situ obert que permeti un flux genètic continu de les poblacions salvatges a les poblacions captives per tal d'evitar en les successives generacions l'adaptació al captiveri.

2. DISCUSSIÓ GENERAL

El coneixement aportat en aquesta tesi doctoral és rellevant per a la conservació i gestió del tritó del Montseny (*Calotriton arnoldi*). El coneixement de la biologia d'aquesta espècie és escàs, degut a la dificultat d'observar-la i estudiar-la en estat salvatge. Aquesta informació, però, és necessària per avaluar el risc d'extinció i determinar així una correcta gestió. En aquest cas, l'investigació genètica és molt útil per tal d'augmentar aquest coneixement, ajudant a omplir algunes llacunes importants de la dinàmica de les poblacions de l'espècie.

En moltes espècies amenaçades, les anàlisis genètiques no són una eina molt valuosa ja que aquestes espècies acostumen a presentar baixos llindars de variabilitat genètica, fet que aportarà poca informació per a la seva gestió (Lawrence 2008). Generalment, a mesura que una població disminueix la seva mida, la variabilitat genètica també minva. Això limita avaluar en profunditat aspectes sobre la biologia de l'espècie a escala contemporània, així com la seva història evolutiva.

Afortunadament, tot i que *C. arnoldi* és una espècie amenaçada i restringida a una àrea de distribució molt reduïda amb pocs efectius, ha retingut la variabilitat genètica necessària per a fer possibles estudis poblacionals detallats. És, per tant, una oportunitat excepcional per a fer un estudi en profunditat i obtenir informació molt valuosa per aquesta espècie. El nivell de variació genètica trobat en aquesta espècie ens permet examinar les seves poblacions a diferents escales temporals, i ens permet extrapolar la demografia històrica i actual de l'espècie, així com la viabilitat de les poblacions (estat de salut segons les característiques genètiques de les poblacions), entre d'altres. Aquestes informacions tindran una gran implicació en el tipus de gestió a seguir per tal d'afavorir la supervivència de l'espècie a llarg termini, i s'han de tenir en

compte alhora de prendre decisions sobre la seva gestió in situ (per exemple, determinar les unitats de gestió), i ex situ (avaluar i dissenyar el programa de cria en captivitat).

La importància de conservar la variabilitat genètica d'aquesta espècie rau en poder conservar el seu potencial evolutiu així com permetre que investigacions futures puguin esbrinar encara més enigmes sobre el tritó del Montseny, allunyant-lo de l'extinció.

2.1. IMPLICACIONS EN LA CONSERVACIÓ

Els resultats moleculars donen suport a l'estatus de "en perill crític" establert per la IUCN per a aquesta espècie. La forta estructura de les poblacions, juntament amb les seves mides poblacionals efectives estimades, són resultats a tenir molt en compte a l'hora de definir les línies de gestió i conservació.

Definició de les unitats de conservació/maneig en l'espècie

Un dels objectius de la conservació d'espècies amenaçades és mantenir el seu potencial evolutiu (Frankel 1970; Frankel i Soulé 1981; Frankham 2010), mitjançant la priorització de les unitats mínimes de conservació, assegurant que la història evolutiva (i potencial evolutiu) de l'espècie es maximitzi, protegeixi i mantingui. La delimitació precisa de les unitats mínimes de gestió per aconseguir aquest objectiu és de vital importància però és un criteri àmpliament discutit.

Les espècies són la unitat taxonòmica bàsica tant per a la conservació com pel maneig de la biodiversitat, i és el primer pas alhora de discutir futures qüestions sobre biogeografia, ecologia, conservació o evolució (Vasconcelos 2010). Existeixen múltiples definicions d'espècie, però en gairebé totes elles s'interpreten les espècies com a llinatges evolutius. No obstant, en molts casos la diferenciació intraespecífica és tan elevada que s'ha de tenir en compte nivells inferiors alhora de definir els diferents llinatges evolutius.

El concepte de subespècie es troba a la interfície entre la sistemàtica i la genètica de poblacions, i representa una unitat d'organització biològica en zoologia que s'utilitza àmpliament en les disciplines de la taxonomia i biologia de la conservació. Aquest concepte va ser originalment concebut per a taxons amb una variació geogràfica dins de les espècies (poblacions al·lopàtriques), i amb diferenciació morfològica, sense tenir en compte l'estructura genètica. En canvi, la definició d'Unitats Evolutives Significatives (ESUs) ja contemplava l'objectiu de capturar la història evolutiva dins d'una espècie. De fet Moritz (1994) postulava

que les poblacions primer divergien en ESUs, com a conseqüència de la deriva genètica, però presentaven poca o limitada diferenciació fenotípica. Quan aquesta diferenciació morfològica apareixia ja es definia com a subespècie.

En conclusió, les poblacions al·lopàtriques d'una espècie poden representar una etapa incipient de la diferenciació, és a dir, poden estar en un procés d'especiació independent, amb un mínim flux de gens entre elles, de manera que comprenen grups evolutius diferents (Gompert *et al.* 2006; Joyce *et al.* 2009; Zink 2004). Amb el temps evolutiu, aquestes esdevindran noves espècies. Les poblacions són dinàmiques i estan contínuament evolucionant. Per sota del llindar d'espècie, en un tall en la branca de l'arbre de la vida, les subespècies o ESUs es troben enmig d'un camí continu de diferenciació de les seves poblacions comprenent a dins grups reproductius dins d'una espècie. En el procés d'un llinatge d'especiació al·lopàtrica, les subespècies o ESUs representen l'estadi prematur de diferenciació amb l'adquisició d'unes o altres propietats (Figura 10).

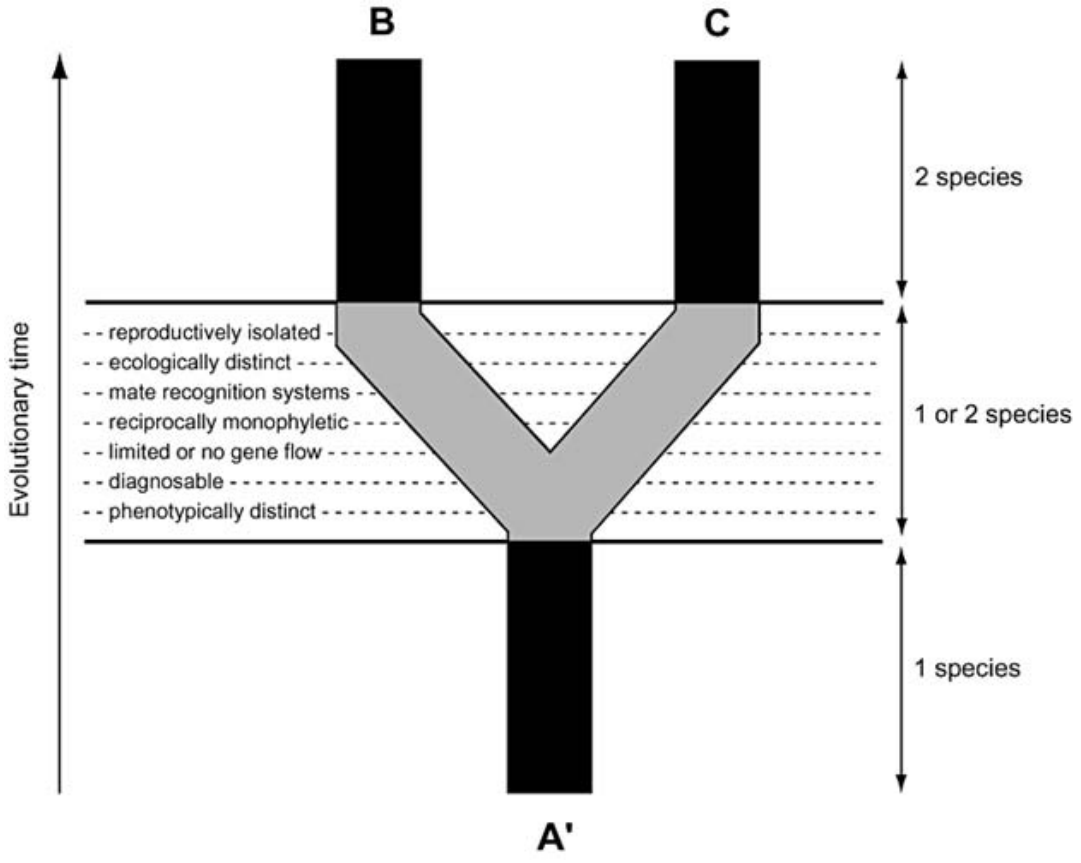

Figura 10. Procés de formació de dues espècies (B i C) a partir d'un únic llinatge ancestral (A) segons Braby *et al.* (2012). La zona grisa representa el temps durant el qual els llinatges fills adquireixen propietats diferents. El nombre d'espècies representats a dalt i a baix de les barres negres (zona negre) és clar, en el dos casos. En canvi, en la zona grisa, en els processos incipients de diferenciació de les dues espècies, hi ha més controvèrsia, i és on estaries reconegudes les subespècies o ESUS.

Avui en dia, l'actual definició de subespècies al·lopàtriques ja s'utilitza tenint en compta les dades moleculars. Així doncs aquest terme es solapa àmpliament amb els criteris per considerar ESUs: han d'estar reproductivament isolades d'altres unitats poblacionals, i han de representar un important component evolutiu per al llegat de l'espècie (Waples 1995).

En aquests moments, doncs, s'hauria de reconèixer les ESUs com a subespècie? La resposta a aquesta qüestió dependrà probablement del taxó concret, i l'interès final. Per a la biologia de la conservació, al final la qüestió rau en: aquestes poblacions diferenciades (i per tant el seu potencial evolutiu) mereixen estatus taxonòmic formal per al reconeixement en els programes de conservació?

Tot i que recentment en molts àmbits el dos conceptes es sinonimitzen, en la biologia de la conservació, la utilització del terme subespècie encara és molt controvertit:

- No hi ha un consens per a delinear les unitats infraespecífiques de conservació. Com s'haurien de definir els llindars? És molt difícil delimitar un llindar entre subespècie i espècie. Si una subespècie presenta diferències morfològiques i genètiques, perquè aleshores no és considerada espècie?
- Depèn molt del criteri dels taxònoms; hi ha dues tendències, el *splittitng* i el *lumping*: la tendència en separar l'espècie en dos o més subespècies (*splitting*), o bé la tendència en mantenir-los en una mateixa espècie (*lumping*) fins que hi hagi prou evidències per descriure-ho com a espècies diferents; és per tant una decisió taxonòmica. En alguns casos, a més, hi pot haver inconsistència entre experts.
- Tot i que les subespècies poden ser objecte de conservació (Braby *et al*. 2012), la falta de validació d'aquestes pot dificultar a l'administració i organitzacions conservacionistes la seva aprovació per a ser incloses en els llistats de conservació (Gippoliti i Amori 2007; Haig *et al.* 2006; James 2010; Stanford 2001). Per exemple, si bé les avaluacions de conservació de la IUCN és obligatori per a les espècies, la inclusió de subespècies depèn de l'actitud i el coneixement d'especialistes en diferents taxons i en diferents regions.

En aquesta tesi, que té un objectiu clar d'elaborar recomanacions per a la conservació i gestió d'aquesta espècie, s'ha optat per la terminologia ESU per definir la unitat de gestió, a la espera de la possibilitat que en un futur es pugui fer una avaluació de la situació i treure conclusions taxonòmiques. Si es té en compte aquestes línies evolutives alhora d'establir les estratègies de gestió per a aquesta espècie, s'afavorirà el manteniment de la variabilitat genètica d'aquesta espècie així com el seu potencial evolutiu.

Les poblacions de *C. arnoldi* estan genèticament molt estructurades, amb una diferenciació genètica significativa entre sectors (oriental més divers que l'occidental). Els dos sectors compleixen els criteris d'estar reproductivament aïllats (aïllament per distància): a) estan geogràficament aïllats, i b) no hi ha cap indici de flux genètic entre ells i aquests estan ben diferenciats genèticament. A més a més, hi ha diferències morfològiques clares entre individus dels dos sectors, fet que podria indicar una possible adaptació local a cadascun dels dos sectors.

Així doncs, segons l' estructura poblacional de *C. arnoldi*, s'haurien de considerar dues ESUs diferents: les poblacions del sector oriental i les poblacions del sector occidental.

Vulnerabilitat de l'espècie

És àmpliament reconegut que la determinació de la grandària efectiva de la població genètica (N_e) és més important que mesurar la mida del cens (N_c) en les poblacions salvatges (Beebee i Griffiths 2005). De fet, la mida efectiva de la població (N_e) és el paràmetre d'interès primari per als estudis de genètica de conservació, ja que és un bon indicador de la viabilitat d'aquesta a llarg termini perquè reflecteix amb precisió el potencial evolutiu d'una població. Les mesures de conservació d'espècies amenaçades han de tenir en compte aquest tipus de vulnerabilitat poblacional, sobretot en especies amb poblacions altament aïllades.

Generalment, les estimes de N_e amb mètodes demogràfics solen ser difícils; en canvi, les estimes per mètodes genètics permeten obtenir valors N_e fiables (Palstra i Ruzzante 2008). S'ha suggerit que per tal que una espècie o població sigui viable a llarg termini ha de tenir un N_e mínim de 500 (Franklin 1980; Soulé 1980). Valors de N_e de menys de 50 en poblacions aïllades són de gran preocupació, ja que aquestes poblacions tindrien una major probabilitat d'extinció com a resultat dels efectes genètics (Allendorf i Luikart 2007; Hurtado *et al.* 2012). No obstant, aquestes assumpcions no són aplicables a tots els organismes, ja que la N_e depèn àmpliament de la biologia de les determinades espècies. Així, N_e inferiors a 100 són comuns en amfibis (Beebee i Griffiths 2005; Funk *et al.* 1999).

En el cas de *Calotriton arnoldi*, les estimes demogràfiques realitzades per Amat i Carranza (2005) ja mostraven valors poblacionals petits. Els resultats genètics obtinguts en aquesta tesi doctoral corroboren els censos demogràfics estimats, amb valors d'estimes poblacions efectives molt reduïdes per a totes les poblacions conegudes d'aquesta espècie. Aquest fet suggereix que la situació d'aquestes és preocupant.

Generalment, les poblacions amb N_e molt reduïdes són susceptibles a l'esgotament genètic a través de la deriva i endogàmia, amb conseqüències adverses per a la seva viabilitat (Frankham

2005; Palstra i Ruzzante 2008). En aquest cas, no obstant, s'han obtingut nivells de diversitat genètica elevats tenint en compte l'extensió i demografia de l'espècie, i no s'ha detectat evidències d'endogàmia. Una possible explicació a la manca de correspondència entre la mida efectiva petita i la baixa endogàmia pot ser degut a l'estratègia reproductiva de l'espècie. Les femelles promíscues en diverses espècies, i entre ells els urodels i *C. arnoldi*, podrien evitar l'endogàmia mitjançant l'emmagatzematge d'esperma (*sperm storage*) (Alonso 2013; Bretman *et al.* 2009). Aquests mecanismes poden haver evitat que, tot i el reduït rang de distribució i la fragmentació de les seves poblacions, aquesta espècie presenti signes d'esgotament genètic. Així, pel que fa al manteniment de la diversitat genètica, les mides petites de població efectiva no necessàriament representen un problema, ja que pot haver-hi altres tipus de mecanismes de reproducció o comportament que poden contrarestar els efectes de la deriva genètica (Allentoft i O'Brien 2010).

No obstant, les N_e petites són potencialment més vulnerables al risc d'extinció, ja no tant com a resultat de la deriva genètica, sinó a causa de la impossibilitat de sobreviure esdeveniments estocàstics ambientals (per exemple, malalties o desastres naturals) o estocasticitat demogràfica (per exemple, variacions aleatòries en la proporció de sexes, la mortalitat o reproducció), i més encara en espècies amb poblacions isolades.

D'altra banda, en poblacions aïllades, però, cal diferenciar entre fragmentació natural de l'hàbitat i pèrdua d'aquest. En la majoria d'espècies, la fragmentació de l'hàbitat comporta una pèrdua d'aquest, provocant els signes d'esgotament genètic i endogàmia anteriorment esmenats. No obstant, aquests dos conceptes no són sinònims. En el cas de *C. arnoldi*, si bé la fragmentació de l'hàbitat sembla haver contribuït a mantenir els nivells genètics de l'espècie, la pèrdua d'hàbitat sí que pot comportar una greu amenaça per a la supervivència d'aquesta espècie.

La viabilitat a llarg termini de les poblacions del tritó del Montseny és molt preocupant ja que:

- Aquesta espècie està restringida a una àrea geogràfica molt petita (<8 km^2), susceptible a un alt risc d'extinció.
- El seu hàbitat és molt vulnerable a l'acció humana i canvi climàtic, fet que podria comportar una pèrdua d'hàbitat.
- Les poblacions estan fragmentades i isolades entre elles i presenten valors d'estimes poblacionals efectius molt petits, fets que, tot i que no comporten un problema de pèrdua de variabilitat genètica, deixa a cadascuna d'elles en una situació alarmant per a la supervivència.

Per tot això, es confirma la fragilitat de l'espècie i la seva classificació com a críticament amenaçada (IUCN).

Gestió de les poblacions salvatges i captives

L'encreuament de les dues unitats de conservació (outcrossing) seria una acció que podria ajudar a augmentar la diversitat genètica (Frankham et al. 2010). No obstant això, en aquest cas no es recomana per dues raons principals: a) la importància de mantenir els dos grups evolutius, i b) el potencial amenaçador de les poblacions existents a causa de la depressió per exogàmia (outbreeding depression, OD) (Allendorf i Luikart 2007). Sovint, les directrius de conservació existents se centren en mantenir un alt nivell de diversitat genètica en lloc de mantenir el potencial evolutiu de les poblacions (Allentoft i O'Brien 2010). Actualment, recents estudis de biologia de la conservació ja indiquen la importància de maximitzar el potencial evolutiu i el reconeixent de la microevolució com a factor de persistència de la població en entorns canviants (Rice i Emery 2003; Sgrò et al. 2011). El potencial evolutiu de C. arnoldi rau no només dins de l'espècie en el seu conjunt, sinó també dins de cada sector. Per tant, les estratègies de conservació a seguir s'han d'orientar en assegurar que no es perdi aquest potencial evolutiu i la diversitat genètica present (Hoffman i Blouin 2004), evitant l'outcrossing d'aquests dos grups evolutius. D'altra banda, les poblacions aïllades durant més de 500 anys poden presentar modestes o altes probabilitats d'OD (Frankham et al. 2011). Tenint en compte que l'aïllament dels dos sectors, probablement, es va dur a terme fa més de 250.000 anys (Amat i Carranza 2007a), es suggereix la possibilitat que aquesta espècie pugui presentar OD. Tenint en compte que altres autors van documentar OD per a altres amfibis i que representa una preocupació legítima (Sagvik et al. 2005; Sherman et al. 2008), no sembla prudent considerar l'outcrossing abans d'obtenir informació sobre l'OD. A més, abans de realitzar qualsevol actuació, és important avaluar si les poblacions orientals i occidentals han adquirit una adaptació local i estudiar-ne el potencial d'intercanviabilitat ecològica (Blank et al. 2013; Kraaijeveld-Smit et al. 2005), fet que podria estar passant en el tritó del Montseny al presentar diferències morfològiques patents entre sectors. Per tant, per totes aquestes raons expressades anteriorment, es suggereix que ambdós sectors han de ser considerats com a unitats de gestió independents per a la conservació, tan a nivell in situ com ex situ. A més a més, per al maneig genètic de poblacions captives, també es recomana tenir en compte l'estructura genètica trobada dins de cada sector.

2.2. FUTURES LÍNIES DE RECERCA

Aquest estudi ha millorat en gran mesura el coneixement sobre la diversitat genètica i estructura poblacional del tritó del Montseny. Esforços de mostreig genètics s'han de mantenir en els propers anys i durant un llarg període de temps per tal de detectar les fluctuacions demogràfiques i genètiques.

El seguiment i el manteniment d'altes mides efectives en les poblacions i de la variabilitat genètica és molt important per a la conservació d'espècies amenaçades, ja que aquests factors tenen el potencial de millorar l'adaptació evolutiva a llarg termini en resposta a l'estocasticitat ambiental (Frankham *et al.* 2010). És important assenyalar aquí que les estimacions de la grandària efectiva de la població obtinguda en aquest estudi han de ser tractats com aproximacions, ja que són estimes puntuals, d'una generació. És important mantenir aquests estudis d'estimes poblacionals efectives al llarg de diverses generacions per tal de detectar les fluctuacions temporals en la mida de la població i les tendències. L'estudi continuat de N_e ens ajudarà doncs a determinar si la població és estable, o bé està disminuint o augmentant. Aquesta informació serà particularment valuosa per al disseny del pla de gestió de la conservació d'aquesta espècie en perill crític.

En relació a la gestió ex situ, un problema important en que s'enfronten els programes de cria en captivitat és que les poblacions captives es poden veure afectades per fenòmens com ara l'adaptació genètica a la captivitat, ja que s'esbiaixa la selecció natural (es controlen les malalties, es seleccionen determinades parelles i s'evita la competència amb altres espècies entre d'altres). Per altra banda, a mesura que augmenta el número de generacions, poden aparèixer problemes de depressió per endogàmia (els individus estaran emparentats, i amb això, pot haver-hi un augment en la probabilitat que al·lels recessius deleteris es presentin i redueixin la fecunditat i supervivència). A més a més, en el moment de la creació de l'estoc fundador es dóna una pèrdua de diversitat genètica, ja que el pool genètic de la població salvatge es trobarà només representat pels individus fundadors, fet que podria provocar pèrdues del potencial evolutiu de l'espècie (Frankham *et al.* 2010). Si no es fa un maneig adequat de les poblacions captives, es podria donar una pèrdua de diversitat genètica existent en l'estoc captiu, fet que podria provocar una diferenciació entre la població captiva i la salvatge, que podria tenir efectes perjudicials en el moment d'una reintroducció. Per tot això és important que les cohorts posteriors siguin monitoritzades a llarg termini. Es suggereix la construcció d'un sistema obert que permet que el flux genètic continu de les poblacions salvatges a les poblacions captives. El coneixement previ de la seva aportació genètica dels nous individus salvatges que s'incorporin a l'estoc captiu és essencial, i per tant és convenient avaluar-los genèticament abans de ser

incorporats al programa ex situ, per tal de seleccionar els més òptims i optimitzar al màxim l'aparellament entre fundadors.

Per últim, l'objectiu principal de qualsevol programa de reintroducció d'espècies amb fins de conservació ha de ser establir una població salvatge autosostinguda, que es defineix com aquella població mínima viable (PMV) amb una elevada probabilitat de persistència fent front a l'extinció dels desastres naturals, demogràfics, o bé a l'estocasticitat genètica o ambiental. L'avaluació de l'èxit dels programes de reintroducció requereix bones dades de seguiment posterior a l'alliberament a llarg termini (Scott i Carpenter 1987), ja que aquests permeten modelitzar i fer l'anàlisi de viabilitat poblacional (PVA; Beissinger i Westphal 1998). Així doncs, també es suggereix fer el seguiment genètic de les reintroduccions del tritó del Montseny a les àrees òptimes seleccionades i calcular-ne les PMV, és a dir, el nombre mínim d'individus de les poblacions reintroduïdes que els permeti retenir la diversitat genètica i el potencial evolutiu per tal de sobreviure a mitjà i llarg termini, i adaptar-se a futurs canvi ambientals (Frankham *et al.* 2014).