UNIVERSITAT DE
BARCELONA

Estructura, distribución e historia evolutiva de las poblaciones de estrellas de mar *Echinaster sepositus* y *Coscinasterias tenuispina*

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Alex Garcia Cisneros - Tesis doctoral - 2016

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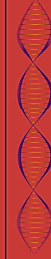

Alex Garcia Cisneros

Tesis doctoral

UNIVERSITAT DE BARCELONA

Facultad de Biología

Departamento de Biología Animal

Programa de doctorado en Biodiversidad

Estructura, distribución e historia evolutiva de las poblaciones de estrellas de mar *Echinaster sepositus* y *Coscinasterias tenuispina*

Memoria presentada por Alex Garcia Cisneros
para optar al grado de doctor por la
Universidad de Barcelona

Alex Garcia Cisneros

Abril de 2016

Directoras de tesis:

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Agradecimientos

Gracias a la confianza que depositaron en mi ambas directoras de tesis; primero pude obtener la beca para dedicarme a ella y después de más de cuatro años, acabarla. Me alegro de que se confundiesen y no lo pensasen mucho porque sino no podría haber disfrutado estudiando estas dos estrellas de mar. Gracias Creu, gracias Rocío, entre las dos habéis formado un buen equipo, compensado y equilibrado. Ambas habéis aportado un buen puñado de granos de arena, en algunos momentos un puñado reiterativo, pero al final he aprendido mucho de vosotras, no solo de ciencia y, encima, hemos llegado a buen puerto!

No tengo ninguna duda de que esta tesis no se parecería en nada a la que finalmente ha acabado siendo si no hubiese sido por las charlas y debates delante de un café con Owen Wangesteen, Enric Planas y Lucía Pita. Habéis sido unos grandes compañeros, aunque en este final de tesis se ha notado vuestra ausencia.

Sin la ayuda de Celia Bisbal, Judit Bellés, Marta Campos y Eva Guerrero, que estuvisteis procesando montones de muestras para vuestros trabajos de máster, no me seria posible estar escribiendo estas líneas ahora mismo y probablemente necesitaría mucho más tiempo para poder completarlas.

El despacho donde he estado todos estos años no habría sido el mismo y, hubiese sido mucho más duro trabajar sin la compañía de Mari Carmen Pineda, Kataisa López, Maria Casso, Vanessa Arranz, Núria Massana, Guillem Santamaria, Roger Esplugues, Manuel Orobitg, Sandra Ortiz, Beatriz Llorente, Marc Balsalobre, Cristina Martell, Marta Arjona, Marta Blanco… seguramente me estaré dejando a alguien, porque en este espacio siempre hay mucha vida y, en algunos momentos, un poco de overbooking.

Muchas gracias al equipo de la Laguna y en especial a Jose Carlos Hernández y Sabrina Clemente, que nos ayudásteis durante la campaña de Tenerife, tanto a buscar como a saber dónde había que ir a encontrar cada especie.

A las compañeras de representación de estudiantes de doctorado primero por vuestra compañía y luego por la labor de representación y organización de seminarios, que aunque no siempre tuvieron gran afluencia de gente, fueron muy útiles: Eli Mora, Lucía Pita (sí, repites), Marina Mitjans y Fabiana Saporiti.

Of course, I also have to thank the great welcome received in my different abroad stays. First of all, thanks to Helen Nilsson Sköld and Bethanie Carney Almroth; in few weeks we did a lot of work that it would have been impossible without you and besides, you made me feel very comfortable in Sweden. Também obrigado a Renato Ventura, quem era sempre pendente e disponível para qualquer coisa, a Paulo Paiva (Paulinho), eu lembro muito das conversações indo para o laboratório, eu aprendi mais da história e do operação do Brasil em unos poucos viagens a Ilha do Fundão que com qualquer leitura. Agradeço a gente do Museu e do laboratório da UFRJ, especialmente a Elinia Lopes, Barbara Feital, Mariana Contins, Carlos Barboza, Gustavo Mattos, Alanna Dahan, Marcela Rosa e Fernanda Viana, eu passei bom momentos com vocês.

A professores e investigadores, tanto de la Universidad, del CEAB o ya mudados a otros lugares, que han contribuido de diferentes maneras a lo largo de mi recorrido en los años de tesis: Xavi Turon (per facilitarme el temps necesari en aquesta última recta final), Gustavo Llorente, Carles Ribera, Eduardo Mateos, Susana López, Iosunne Uriz, Marta Pascual, Carles Carreras, Manuel Ballesteros, Mª José López Fuster, Santi Mañosa, Esther Esteban, Pedro Moral, entre otros.

Als amics de diferents llocs que hem compartit tertúlies i partits, cerveses, sopars, calçotades o el que fes falta i que formen un grup heterogeni de persones: Marc Jabardo, Eduard Piqueras, Sandra Garcès, Ariadna Nogué, Martha Florido, Mònica Sureda, Kati y Gena (que apellidos más complicados que tenéis...), Jaume Ruscalleda, Sergi Martínez, i tants altres que m'estic oblidant. En especial al Robert López i a l'Ivan Linares que sempre que ho necessitat, heu estat disponibles per fer la cervesa de rigor, tot i que crec que des de fa un temps que no podeu dir el mateix de mi. També, haig d'agrair en bloc a tot l'equip dels Ícars, sort a vosaltres i als partits dels dijous tinc una càrrega de bateria extra cada setmana. A Laura Teixans, gràcies per les recomanacions tipogràfiques per dissenyar la tesis.

Grazie alla famiglia Italiana: Ivana, Luigi, Corrado e Camilla; per farmi sentire come a casa ogni volta che ci siamo istallati a Sesto, per il supporto tecnico di pupino ogni volta che avevamo bisogno (oltretutto, venendo da così lontano), per ripristinare il parmigiano ogni tanto, e tante altre cose.

Gràcies als meus pares, que encara que a vegades no ho sembli, teniu molt a veure amb tot això. Difícilment hagués arribat aquí sense vosaltres, que sempre heu sigut els primers a animar-me a fer allò que m'agrada, pel vostre suport e interès continu en la meva recerca. També al meu germà, que he procurat deixar-te enrere sempre que he pogut, sigui amb bici, esquís o corrent, i sempre donarà satisfacció veure't darrera. A l'Ariadna Comella, que entre altres coses i juntament a l'Eric, esteu preparant al Nil Comella per a que doni la tabarra amb el Nicolò.

Per últim, a la meva petita família. Al Nicolò Garcia, que tot i ser ben petit i créixer com un bolet, m'alegres cada dia que arribo a casa; a la bola de pèl del Mao, que m'has servit de pilota anti-estrès mentre escrivia. Y como no, a Fabiana Saporiti, que me has soportado mis tiempos lentos, aguantado cada vez que me ponia a hablar de tesis, animado a ser un poco más eficiente y ordenado, que hacía falta. Pero sobretodo por estar ahí en el dia a dia ayúdandome un montón en todo.

Agradecimientos

Contenido

Introducción General

Introducción

El mar ha sido considerado clásicamente como un medio continuo y sin barreras aparentes. En este contexto, la distribución de las especies marinas vendría condicionada por la capacidad de dispersión de larvas y adultos, que posibilitaría la conexión entre poblaciones de áreas distantes, considerándolas como poblaciones "abiertas" (ver revisión en Cowen & Sponaugle, 2009). Sin embargo, durante las últimas décadas son muchas la evidencias que demuestran que el medio marino no es tan homogéneo como inicialmente se consideraba (Levin, 2006), y que la dispersión y distribución de las especies está influenciada por numerosos factores, como los procesos de circulación oceanográfica (Patarnello *et al.*, 2007), la morfología de la costa (Sköld *et al.*, 2003), los factores biológicos relacionados con el comportamiento de las larvas (Toonen & Pawlik, 2001), la depredación y la competencia, los factores físico-químicos, o la acción antropogénica (Hoegh-Guldberg & Bruno, 2010). El conocimiento de las fluctuaciones poblacionales de las especies, su nivel de estructuración, así como de sus migraciones más importantes y sus niveles de conectividad, permiten reconstruir parte de su historia evolutiva y entender los eventos geológicos y los procesos históricos que han influido en las mismas. De esta manera se pueden predecir también algunos efectos de futuros cambios sobre una especie en particular y sobre el ecosistema en general.

Los marcadores moleculares son en la actualidad una herramienta básica para determinar la estructura genética de las poblaciones y para inferir las características demográficas y la conectividad de las mismas. Cada suceso "dramático" o prolongado en el tiempo queda "marcado" en el ADN de las poblaciones y, por tanto, los marcadores genéticos son herramientas que permiten detectar y cuantificar estos sucesos (Hellberg, 2009; Putman & Carbone, 2014). Sin embargo, es necesario también el conocimiento de la biología y ecología de las especies para comprender la relación entre los cambios demográficos y los eventos bióticos o abióticos que les han podido afectar a lo largo de su evolución.

En la introducción de esta tesis doctoral se presentan conceptos básicos sobre genética de poblaciones y filogeografía, las particularidades biológicas y ecológicas de las dos especies objeto del trabajo, y las técnicas utilizadas para abordar los estudios realizados.

Herramientas moleculares y genética de poblaciones

Las herramientas moleculares se utilizaron inicialmente en trabajos de medicina, aunque en pocos años ya empezaron a aplicarse en estudios de ecología, concretamente en la década de los 60 del pasado siglo. Los primeros estudios se realizaron con alozimas (Lewontin & Hubby, 1966), que son variantes de diferente tamaño para una misma enzima provenientes de diferentes alelos. Así, sin llegar a conocer la secuencia de bases en el ADN, las diferencias de tamaño de los fragmentos obtenidos indican la presencia de alelos diferentes.

El primer fragmento secuenciado de ADN data de los años 70, y los primeros estudios ecológicos utilizando secuencias conocidas surgen a raíz de la publicación del trabajo de Sanger *et al.* (1977), en que desarrolla la técnica de amplificación del ADN mediante la reacción en cadena de la polimerasa (PCR). La mejora en las técnicas de PCR y la posterior secuenciación, permitieron que a finales de los 80 hubiese un fuerte incremento de trabajos moleculares aplicados a la conservación y a la ecología (Féral, 2002). Las herramientas moleculares permiten responder preguntas evolutivas, ayudar al manejo de stocks biológicos y de áreas protegidas,

aclarar problemas relacionados con la taxonomía y sistemática y, entre otras cuestiones, desarrollar estrategias para la conservación de la diversidad.

Actualmente existe la posibilidad de aislar un gran número de marcadores genéticos de diferentes regiones del ADN, tanto los considerados neutros (no sometidos a selección) como no neutros (sometidos a selección) y a un coste relativamente bajo. Cada marcador o región tiene unas características concretas que los hace más adecuados a los diferentes tipos de estudio.

Cuadro 1: Marcadores genéticos

Los marcadores genéticos son fragmentos de ADN (locus), que pueden tener o no una ubicación y/o función conocida, y que pueden estar en la parte codificante o no codificante de un gen. La herencia de los alelos puede rastrearse y, por tanto, sus diferencias entre individuos pueden ser utilizadas para identificar genealogías y determinar las relaciones evolutivas entre especies o entre poblaciones. Los marcadores pueden ser monoparentales, cuando provienen de cromosomas sexuales (como por ejemplo algunos genes localizados en ciertas partes del cromosoma Y, y que son de herencia exclusivamente paterna, como es el caso de los mamíferos), o de las mitocondrias (de herencia exclusivamente materna, en la mayoría de organismos superiores), o bien provenir de ambos progenitores, cuando el marcador se encuentra en regiones nucleares de cromosomas no sexuales.

Dos tipos de marcadores ampliamente utilizados en estudios de genética de poblaciones de organismos marinos son los genes mitocondriales, y entre ellos el gen de la Citocromo C Oxidasa subunidad I (COI), y los microsatélites nucleares (*Short Tandem Repeats*, STR). El gen COI se encuentra en el genoma mitocondrial, fuera del núcleo celular, mientras que los microsatélites están presentes a lo largo de todo el genoma nuclear y, por tanto, la herencia de ambos marcadores -en el caso de la reproducción sexual- es diferente: en la mayor parte de los casos, la mitocondria solo se transmite por vía materna, a través del óvulo, y tiene un único cromosoma circular (n); el núcleo tiene el material genético por duplicado (2n) y la siguiente generación hereda la mitad de cada progenitor, además de poder recombinar. En el caso concreto de los microsatéllites, la herencia de los alelos maternos y paternos es codominante, lo que permite conocer el legado genético de ambos progenitores.

El gen Citocromo C Oxidasa (COI)

La Citocromo C Oxidasa es una proteína transmembrana de las mitocondrias; está compuesta por diferentes grupos prostéticos y su función está relacionada con la respiración celular. El ADN mitocondrial se caracteriza porque se hereda, en general, por vía materna (es, por lo tanto, haploide), no recombina en cada generación, tiene una alta tasa de mutación y pocas regiones no codificantes. Parte de la secuencia de ADN que codifica para el primer complejo de la Citocromo C Oxidasa (la subunidad I) permite estudiar la variabilidad intraespecífica, y ha sido muy utilizada en estudios de genética de poblaciones y filogenias en una gran variedad de organismos, incluyendo equinodermos y otros invertebrados marinos (e.g. Dawson, 2001; Kelly & Palumbi, 2010). La gran cantidad de datos genéticos disponibles para COI permite comparar los resultados obtenidos entre especies cercanas y disponer de más información acerca de la evolución del gen. Las tasas de mutación varían dependiendo de la especie considerada, aunque éstas diferencias no superan el orden de magnitud. En estrellas de mar, por ejemplo, puede variar entre el 2.3 % y el 3.6 % (Wares & Cunningham, 2001; Crandall *et al.*, 2012; Foltz *et al.*, 2013).

Microsatélites

Los microsatélites (STR) son fragmentos de ADN compuestos por secuencias nucleotídicas cortas, de uno a seis pares de bases, que se repiten en tándem. Este tipo de secuencias repetitivas está ampliamente distribuido por el genoma, y se encuentran principalmente en regiones no codificantes del ADN y, por tanto, inicialmente se asume que no están sometidas a selección, aunque se ha demostrado que no siempre es así. Los diferentes alelos difieren en el número de repeticiones que contienen (normalmente entre 2 y 50 por locus). Los microsatélites tienen una elevada tasa de mutación y, por tanto, un elevado polimorfismo, lo que genera una gran variabilidad intraespecífica. Además, se trata de marcadores codominantes y de herencia mendeliana simple. Estas propiedades los convierten en buenos marcadores para realizar pruebas de paternidad, análisis de criminología, cartografía genética o estudios de genética de poblaciones. En los últimos años, los avances en las técnicas de secuenciación han dado lugar a una gran mejora en los métodos que permiten su caracterización y desarrollo. Así mismo, el refinamiento de los métodos de aislamiento y análisis han facilitando la utilización de éstos marcadores en organismos no modelo, como es el caso de las especies analizadas en el presente trabajo.

Por estas razones, los microsatélites se han convertido en uno de los marcadores más usados en genética de poblaciones en los últimos años. Actualmente, debido al desarrollo de las nuevas técnicas de secuenciación masiva, otros marcadores como los SNPs (*Single Nucleotide Polimorphisms*) están reemplazando el uso de los microsatélites debido a su mayor facilidad para ser genotipados, aunque los objetivos que se abordan con ambos tipos de marcadores son similares en muchos casos (Guichoux *et al.*, 2011; Putman & Carbone, 2014).

El estudio de los principios y procesos que gobiernan la distribución geográfica de los linajes genéticos a nivel intraspecífico es la base de la filogeografía (Avise, 2000). A través de la información obtenida del análisis de los marcadores moleculares, la filogeografía permite revelar los aspectos históricos y evolutivos de la distribución espacial de dichos linajes. Para interpretar esta distribución se requiere la contribución teórica, metodológica y/o conceptual de campos de conocimiento muy diversos, como son la genética de poblaciones, la demografía, la etología, la filogenia, la paleontología, la geología y la geografía histórica (Avise, 2000).

El estudio de la genética de poblaciones de una especie informa de su estructura genética a nivel poblacional, incluyendo la frecuencia y distribución de alelos, niveles de heterocigosidad, aislamiento, conectividad o flujo genético entre poblaciones; así mismo, permite conocer los eventos demográficos recientes de esta especie que han dejado huella en las poblaciones. La base teórica para estudiar la genética de poblaciones son los principios de herencia mendeliana. Además de estos patrones de herencia, es importante conocer cuán lejos -o no- están las frecuencias de alelos del equilibrio de Hardy-Weingberg; este equilibrio se da cuando las poblaciones se mantienen constantes, con tamaños efectivos infinitos, sin efectos de selección o migración, con apareamiento aleatorio y sin mutaciones, algo que en realidad no se cumple en poblaciones naturales (Waples *et al.*, 2008), pero que es un concepto teórico básico de la genética de poblaciones. Otro punto clave en los estudios de genética de poblaciones es definir y limitar lo que es una "población", un tema que puede generar cierta controversia. Desde un punto de vista evolutivo, una población sería aquella comunidad de individuos que tienen la capacidad potencial de reproducirse entre ellos, pero ecológicamente también se considera población al grupo de organismos que ocupa una área geográfica concreta durante el mismo periodo de tiempo (Waples & Gaggiotti, 2006).

Los estudios de estructura genética permiten inferir la capacidad de dispersión de una especie y, por tanto, son herramientas imprescindibles para entender el efecto que tienen las barreras

oceanográficas sobre el aislamiento de las poblaciones de las especies en determinadas áreas (Hellberg, 2009; Keever *et al.,* 2009). A lo largo de la historia evolutiva de una especie, ésta puede sufrir variaciones demográficas. Cuando éstas variaciones son bruscas, pueden ser detectables a través de los valores de diversidad genética de las poblaciones actuales por métodos como la coalescencia (Edwards & Beerli, 2000; Charlesworth, 2009). El conocimiento de las variaciones demográficas de las poblaciones es, en algunos casos, muy relevante, ya que puede utilizarse como mecanismo de gestión de stocks biológicos, como es el caso de los caladeros de pesca y cohortes de edad de determinadas especies; obviamente, este conocimiento no siempre se traduce en una adecuada gestión de los recursos (Waples *et al.,* 2008).

Eventos geológicos y oceanográficos en el Mediterráneo

Los factores hidrológicos, desde las grandes corrientes oceánicas hasta la circulación local, pueden influir profundamente en los patrones de distribución de las especies, favoreciendo la conexión entre zonas distantes y/o actuando como barreras físicas que limitan el contacto entre zonas cercanas (Benzie, 2000; Lessios *et al.,* 2001; Addison & Hart, 2004; Le Gac *et al.,* 2004; Pérez-Portela *et al.,* 2010). Las áreas marinas separadas por estrechos son muy interesantes desde el punto de vista filogeográfico, puesto que presentan cambios bruscos de profundidad y fuertes corrientes, con desconexiones y reconexiones recurrentes durante los eventos de glaciación, como ha sido el caso del Canal de la Mancha o del estrecho de Gibraltar, entre otros (Wares & Cunningham, 2001; Maggs *et al.,* 2008; Pérez-Portela *et al.,* 2010).

El mar Mediterráneo es un mar semi-cerrado, que comunica con el océano Atlántico a través del estrecho de Gibraltar, un paso de 12,8 km de longitud y con una profundidad entre 280 y 1000 metros. El Mediterráneo está formado por diferentes cuencas, con sus propios regímenes de circulación, salinidad y temperatura; las mareas son muy limitadas. Pero esto no siempre ha sido así. No fue hasta hace 200 millones de años (m.a) que la disgregación del continente Pangea permitió la formación de nuevos océanos. Entre las grandes placas tectónicas, que posteriormente formarían los actuales continentes, se formó una cuenca marina llamada Tetis. Inicialmente, el mar de Tetis estaba abierto en dirección este, conectando con las placas que delimitarían el océano Índico (Arabia, India, Australia) y con las que formarían el océano Pacífico. Hace unos 110 millones de años que el mar de Tetis se abrió hacia el actual Atlántico Norte. Posteriormente Arabia y Eurasia se unieron y cerraron el contacto hacia el este (35-50 m.a.). Las diferentes cuencas que se formaron sufrieron el evento paleo ambiental más importante de los últimos 20 m.a., cuando la placa de Alborán quedó calzada entre la península Ibérica y África, hace 6 m.a. (Hsü *et al.,* 1973). A partir de este momento y durante 640.000 años, el actual Mediterráneo quedó cerrado y se fue secando, provocando lo que hoy se conoce como la Crisis Salínica del Messiniense (MSC) (Krijgsman *et al.,* 1999).

Durante este período de MSC, se produjo la desaparición de la mayor parte de las especies a causa de la elevada salinidad marina y solo unos pocos taxones fueron capaces de sobrevivir (Patarnello *et al.,* 2007). La crisis salínica acabó con la apertura del Estrecho de Gibraltar y la posterior entrada masiva de agua desde el Atlántico al Mediterráneo, un evento que fue seguido por un periodo de elevada productividad, ya que durante el Plioceno (anterior al Cuaternario, entre 5,3 y 2,5 m.a aprox.) las aguas de los océanos fueron cálidas (Haywood *et al.,* 2000; Meyers & Arnaboldi, 2005). Conocer el efecto de la MSC es primordial para entender las naturaleza de las actuales comunidades marinas del Mediterráneo y su influencia Atlántica. Esto es especialmente importante en el caso de los equinodermos, ya que las características de su sistema ambulacral en relación con su capacidad de regulación osmótica, les proporciona poca capacidad para tolerar cambios de salinidad (Hamel & Mercier, 1996) y, por tanto, se cree que la mayoría de ellos pudieron desaparecer del Mediterráneo durante la MSC.

El segundo evento importante para comprender los grandes cambios demográficos en la historia evolutiva de algunas especies son los periodos glaciales (comenzaron aproximadamente hace 580, 200 y 80 miles de años) e interglaciares, que se han ido sucediendo a lo largo del Pleistoceno cercano. Actualmente nos encontramos en un periodo interglaciar que comenzó hace unos 11.500 años, después del llamado Último Máximo Glacial (siglas en inglés: LGM), que sucedió aproximadamente hace 20.000 años. Durante este periodo glacial, el nivel del mar bajó mucho, en algunas zonas hasta 130 m, debido a la retención de agua continental (Lambeck *et al.,* 2002), y buena parte del Atlántico Norte quedó bajo una capa de hielo. Aún así, la conexión entre Atlántico y Mediterráneo no cesó durante este frío periodo (Patarnello *et al.,* 2007; Maggs *et al.,* 2008), aunque las corrientes de conexión entre ambas cuencas pudieron verse altamente alteradas. Estas variaciones han tenido importantes implicaciones en la distribución de las especies, lo que ha afectado a su historia evolutiva, y ha favorecido numerosos procesos de especiación (Wares & Cunningham, 2001). Durante los grandes periodos de frío, con la bajada del nivel de mar en el Atlántico Norte a lo largo de la costa de Europa, las islas de la Macaronesia o el Mediterráneo podrían haber sido refugios para muchas especies costeras someras que vieron mermadas sus poblaciones de las localidades más septentrionales (Maggs *et al.,* 2008).

En la actualidad, la circulación general entre el Atlántico y el Mediterráneo está dominada por dos grandes corrientes. Las aguas del Atlántico, menos densas debido a una menor salinidad, fluyen hacia el este, penetrando en el Mediterráneo por la zona más superficial del estrecho de Gibraltar; las aguas Mediterráneas fluyen hacia el oeste, en dirección al Atlántico, a través de la zona profunda del estrecho (280 - 1000 metros), porque son más densas debido a su mayor
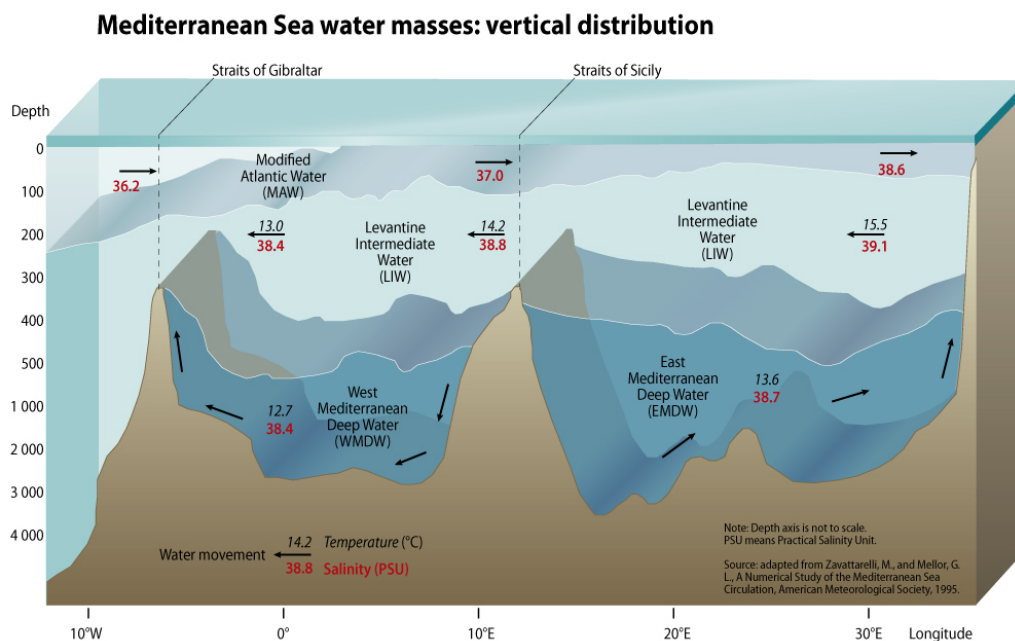
Figura 1. Masas de agua en las diferentes cuencas del Mediterráneo. Hay tres grandes masas de agua que forman este mar; la primera, proviene del Atlántico, es una corriente de superficie que entra en el Mediterráneo y que sufre una fuerte evaporación (sobre todo en la cuenca Este, MAW). Debido al aumento de la salinidad por la evaporación, esta masa de agua se hunde y forma una masa intermedia (LIW), que circula en dirección opuesta a la masa superficial. En el fondo de las cuencas podemos encontrar la masa de agua más fría y de gran densidad, que no llega a mezclarse entre las cuencas debido al estrecho Siculo Tunecio o de Sicilia (EMDW y WMDW). Imagen de GRIDA-Arendal (www.grida.no). Fuente: Zavattanelli & Mellor, (1995).

salinidad (Millot, 1999). Esta circulación explica que la verdadera barrera biológica y física para muchas especies no se encuentre geográficamente en el estrecho de Gibraltar, si no que se sitúe en el llamado frente Almería-Orán, en el extremo oriental del Mar de Alborán (al este del estrecho), donde confluyen las masas de agua atlántica y mediterránea creando dos grandes giros de circulación (Tintore *et al.*, 1988; Patarnello *et al.*, 2007) (Figura 2).

El Mediterráneo está actualmente formado por dos grandes sub-cuencas principales, la Oriental y la Occidental, separadas por el llamado estrecho Sículo-Tunecino, situado entre Sicilia y Túnez, con una anchura de unos 145 km y una profundidad máxima de 316 metros. Ambas cuencas tienen aguas profundas (1.400 m de promedio), con un máximo en la cuenca oriental de más de 5.200m (Figura 1). Las corrientes dominantes en ambas cuencas giran en sentido anti-horario; los pequeños remolinos y otros fenómenos hidrológicos locales pueden también condicionar la conectividad de las poblaciones (Figura 2, Millot, 1999; Millot & Taupier-Letage, 2005).

Patrones filogeográficos de equinodermos Atlanto – Mediterráneos

Las poblaciones, no los individuos, son consideradas las unidades básicas de evolución. El flujo de genes entre poblaciones, promovido por la migración, que depende de la dispersión de larvas y adultos (Barton & Hewitt, 1985), introduce nuevos polimorfismos en las poblaciones sobre las que la selección natural puede actuar (Oleksyk *et al.*, 2010). Por tanto, la capacidad de dispersión no sólo influye en el rango de distribución geográfico y en la estructura genética de las especies, sino que también juega un papel importante en los procesos de diferenciación y especiación de las poblaciones, con profundas consecuencias para filogeografía de la especie (Solé-Cava & Thorpe, 1991). Las diferencias en la capacidad de dispersión de las especies están determinadas por su ontogenia, y parcialmente correlacionadas con el tiempo que las larvas pasan en el plancton, aunque otros factores como el comportamiento larvario también pueden influir en su dispersión real (Pérez-Portela *et al.*, 2010; Selkoe & Toonen, 2011). De esta manera, algunas
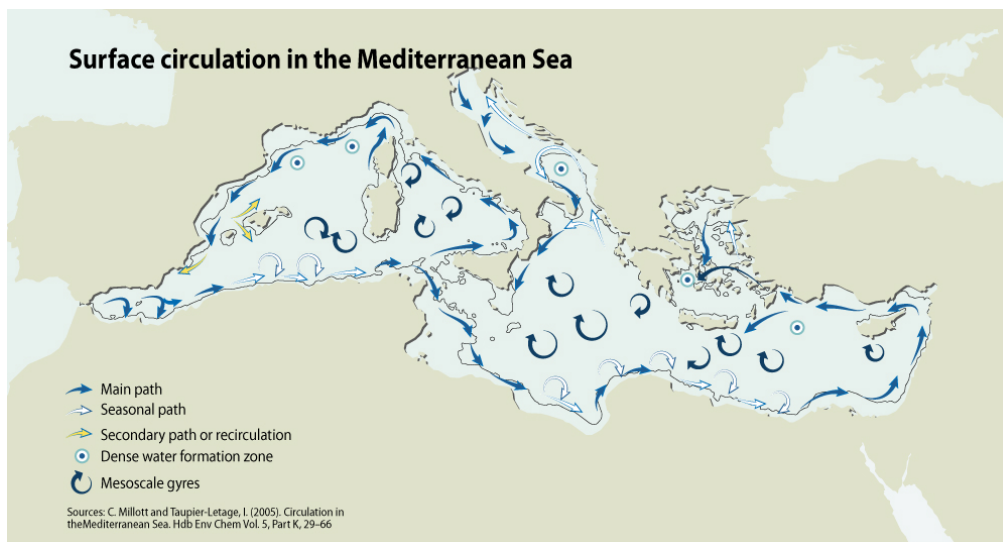

Figura 2. Representación de las corrientes principales (flechas en azul), temporales (flechas en blanco), corrientes de recirculación (flechas en amarillo), formación de aguas profundas (círculos cerrados) y giros de mesoescala (flechas circulares azules) a lo largo del mar Mediterráneo. Imagen de GRIDA-Arendal (www.grida.no). Fuente: Millot & Taupier-Letage (2005).

especies con una alta capacidad de dispersión potencial pueden presentar una estructura poblacional muy marcada, y al contrario (Zane *et al.*, 2000; Launey *et al.*, 2002). Los patrones de diversidad genética reflejan, además, la interacción histórica y contemporánea de los procesos ecológicos, demográficos, etológicos, genéticos, oceanográficos, geológicos y climáticos (Palumbi, 1995; Benzie, 1999). Como ya se ha comentado anteriormente, los estudios filogenéticos y de genética de poblaciones nos permiten analizar e inferir la conectividad, flujo genético y demografía de las especies, además de la variabilidad intra-específica (Hellberg, 2009).

Según Coll *et al.* (2010) en el Mediterráneo hay 154 especies de equinodermos y, de los cuales 33 especies son asteroideos. A pesar de tratarse de un grupo muy conocido, peculiar e incluso simbólico, no son muchos los estudios de filogeografía realizados hasta el momento con equinodermos. Aunque los patrones de discontinuidad genética entre el Mediterráneo y el Atlántico son frecuentes en muchos invertebrados (Quesada *et al.*, 1998; Zane *et al.*, 2000; Launey *et al.*, 2002; Pérez-Losada *et al.*, 2002; Duran *et al.*, 2004a, 2004b; Patarnello *et al.*, 2007), la mayoría de especies de equinodermos estudiadas muestran cierta permeabilidad y flujo genético entre ambos lados del estrecho (Duran *et al.*, 2004b; Zulliger *et al.*, 2009; Pérez-Portela *et al.*, 2010, 2013; Borrero-Pérez *et al.*, 2011; Wangensteen *et al.*, 2012). Únicamente la estrella de mar incubadora *Asterina gibbosa*, presenta unas diferencias muy marcadas entre las poblaciones atlánticas y mediterráneas (Baus *et al.*, 2005).

En los equinodermos atlanto-mediterráneos, al igual que en muchos otros invertebrados marinos litorales, se han observado patrones de expansión demográfica durante los últimos 200 mil años. En algunos casos se ha podido demostrar que estas expansiones demográficas permitieron la colonización reciente del Mediterráneo desde el océano Atlántico, como por ejemplo en el caso del equinoideo *Arbacia lixula* o la estrella *Marthasterias glacialis* (Pérez-Portela *et al.*, 2010; Wangensteen *et al.*, 2012), mientras que en otros, como en la holoturia *Holothuria polii* parece que el Mediterráneo sirvió como refugio durante épocas glaciares, y se expandió demográficamente en posteriores periodos interglaciares (Valente *et al.*, 2014).

Entre los equinodermos atlanto-mediterráneos estudiados hasta la actualidad desde un punto de vista genético, únicamente el equinoideo *Arbacia lixula* y el asteroideo *Coscinasterias tenuispina* presentan poblaciones a ambos lados del Atlántico, tanto en la costa de Brasil como en las islas Macaronésicas y norte de África. Wangensteen *et al.* (2012), en su trabajo sobre filogeografía de *A. lixula*, demostraron una diferenciación genética significativa entre el Atlántico oriental y Brasil, con las poblaciones brasileñas posiblemente divergiendo de un stock del Atlántico oriental. En lo que respecta a *Coscinasterias teniuspina*, el tema se aborda como uno de los objetivos de esta tesis doctoral, con el que se pretende conocer el grado de diferenciación genética entre las poblaciones de Brasil y las del Atlántico oriental, entre otros aspectos (ver capítulo 3).

Dos especies simpátricas con estrategias vitales diferentes

En esta tesis doctoral se estudian dos especies de asteroideos atlanto-mediterráneos, *Echinaster sepositus* y *Coscinasterias tenuispina*. Ambas estrellas tienen estrategias vitales diferentes, pero distribuciones geográficas parecidas. Las dos especies son muy comunes en el litoral rocoso del Mediterráneo pero, hasta el desarrollo de esta tesis doctoral, se desconocía su historia evolutiva y muchas de sus características biológicas.

Echinaster (*Echinaster*) *sepositus*: Sistemática y biología.

La estrella de mar, *Echinaster (E.) sepositus* (Retzius 1816), fue la primera estrella de mar que se mencionó en un trabajo científico, concretamente por Aristóteles en la antigua Grecia, hace más de 2.300 años (Turner, 2013). Sus características morfológicas la hacen muy conspicua: es roja, vistosa y puede llegar a ser muy común en algunas áreas, incluso en zonas costeras someras, ya que habita desde 5 m hasta más de 200 m de profundidad. Ha sido, además, una especie muy estudiada entre los años 1.800 y principios de 1.900, ya que se usó como modelo de anatomía animal y biología experimental (Turner, 2013). Además en los últimos años se ha utilizado como especie modelo para estudios de sistemática y morfología de asteroideos (Lafay *et al.,* 1995; Mah & Blake, 2012).

Echinaster sepositus (Figura 3) pertenece a la clase Asteroidea, superorden Spinulosacea, orden Spinulosida, familia Echinasteridae, género *Echinaster* y subgénero *Echinaster*. El género *Echinaster* ha sido clásicamente subdividido en dos subgéneros, *Echinaster (Othilia)* y *Echinaster (Echinaster)*. Según el registro fósil, el orden Spinulosida apareció durante el Cretáceo, hace 120 - 140 millones de años, más tarde que los otros cuatro órdenes de asteroideos (Astropectinidae, Forcipulatida, Valvatida y Velatida), que lo hicieron 100 millones de años antes (Lafay *et al.,* 1995). Es posible que este hecho haya contribuido a que el orden esté constituido por tan solo una familia (Echinasteridae). En cualquier caso, la radiación de especies de esta familia se debe principalmente a dos géneros: *Echinaster,* con un total de 27 especies y dos subgéneros, y *Henricia,* con 91 especies (Mah & Blake, 2012). Estos autores, basándose su propio trabajo y en otros estudios, apuntaron que buena parte de los caracteres para determinar las diferentes especies dentro de cada uno de ambos géneros son difíciles de reconocer y que muchos se solapan entre ellos (Madsen, 1987; Clark & Downey, 1992; Fontanella & Hopkins, 2001; Turner, 2013). De hecho, un trabajo en elaboración de Elinia Lopes y colaboradores (comunicación personal), ha

Figura 3. Individuos de *Echinaster sepositus* en Port Salvi, Sant Feliu de Guíxols.

cuestionado la monofilia (el origen evolutivo único a partir de un ancestro común) del género *Echinaster,* ya que las especies del subgénero *Othilia* son más distantes con el resto de especies del género *Echinaster* y del género *Henricia.*

Las diferentes especies del género *Echinaster* se encuentran distribuidas por toda la zona templada de mares y océanos, y buena parte de ellas viven en zonas poco profundas, de entre 0 – 200 m de profundidad (Turner, 2013). El subgénero *Echinaster,* del que forma parte *E. sepositus,* está formado por otras dos especies Atlánticas, *E. modestus,* que se encuentra en el Atlántico noroccidental, Golfo de México y Caribe, y *E. reticulatus*, presente en el Atlántico suroriental (Clark & Downey, 1992). Hasta donde conocemos, no hay datos biológicos ni poblacionales de estas dos especies, y su ausencia en trabajos de biología marina realizados en sus áreas de distribución indica la rareza y la dificultad de su localización.

Echinaster sepositus habita a lo largo de todo el Mediterráneo y áreas templadas del Atlántico, siendo sus límites norte y sur, respectivamente, las islas que se encuentran en el Canal de la Mancha y el archipiélago de Cabo Verde (Southward & Campbell, 2006). Sus individuos se concentran en determinadas zonas, siguiendo una distribución agregada (Villamor & Becerro, 2010). Del mismo modo que otras especies del género, se alimenta de detritos y pequeños organismos que se encuentran sobre el sustrato; para ello, su estómago se expande de forma característica hacia el exterior de la abertura bucal (Vasserot, 1961). También hay evidencias de que se alimenta de esponjas (Maldonado & Uriz, 1998), a pesar de que sus sistemas defensivos pueden afectar a la estrella (Waddell & Pawlik, 2000). Parece ser que *E. sepositus* tiene afinidad por áreas en las que predominan las algas incrustantes, caracterizadas por tener muchos pequeños invertebrados asociados, y ser un lugar donde reclutan larvas de muchas especies (Villamor & Becerro, 2010).

Esta especie es objeto de recolección para ornamentar acuarios privados y, por tanto, es susceptible de sufrir una pesca no controlada (Olivotto *et al.,* 2011). De hecho, en algunas zonas

Figura 4. *Echinaster sepositus* en el momento en que expulsa los gametos masculinos en la cueva de Ses Negres, Begur. Fotografía de Xavier Salvador, Coordinador del proyecto SILMAR.

del litoral ibérico, se ha constatado una mengua en la densidad de algunas poblaciones someras, sin que se conozcan sus causas (observación personal).

Biologia reproductiva de *Echinaster sepositus*

Echinaster sepositus se reproduce únicamente de forma sexual, mediante fecundación externa. Parece ser que en el Mediterráneo noroccidental la emisión de gametos se da durante los meses de verano, principalmente junio y julio, que es cuando las estrellas presentan el máximo peso gonadal (Villamor, 2010). No obstante, en septiembre de 2015, la Xarxa de Seguiment Ibèric del Litoral Marí (SILMAR) observó con pocos días de diferencia el desove de estrellas de esta especie (Figura 4) en tres lugares diferentes de la costa catalana (comunicación personal).

El resultado de la fecundación es una larva lecitotrófica, que no se alimenta durante su vida planctónica y que, el periodo previo al asentamiento y metamorfosis no supera, en la mayoría de casos, una semana (Turner, 2013). Las características de la larva difieren según los diferentes trabajos publicados. Nachsteim (1914), con ejemplares del mar Adriático, describió la larva como flotante y esta característica fue ratificada por Mitic (1992). Estudios posteriores realizados en acuarios mostraron que las larvas que se generaban eran densas y pegajosas, quedándose adheridas al fondo de los tanques (Villamor, 2010; Riesgo *et al.*, 2011). Estas diferencias de comportamiento larvario podrían explicarse por el hecho de que otras especies del mismo género son capaces de producir tanto larvas flotantes como larvas no flotantes, incluso presentando coloraciones diferentes (Atwood, 1973 ; Lopes, comunicación personal). Las diferencias en la capacidad de flotación podrían conferir diferente capacidad de dispersión a los dos tipos de larvas.

Las larvas lecitotróficas tienen una reserva energética que es consumida en el momento del asentamiento, lo que les confiere -respecto a las larvas planctotróficas- una mayor probabilidad de éxito en el reclutamiento y una mayor supervivencia de los asentados (Byrne *et al.*, 1999; Villinski *et al.*, 2002). Además, como su dispersión suele ser limitada, la probabilidad de asentarse cerca de las zonas habitadas por los adultos (progenitores) les asegura un área suficientemente buena para su crecimiento y desarrollo vital, aunque al mismo tiempo, este comportamiento puede tener grandes implicaciones en la estructura genética de sus poblaciones. Las especies con larvas lecitotróficas disponen, pues, de un "sistema tampón", por el que, a través de asumir menos riesgos en su vida larvaria, limitan los grandes cambios demográficos típicos de otros equinodermos, tanto en lo que respecta a la pérdida como al crecimiento poblacional (Uthicke *et al.*, 2009). Esta estrategia de asentamiento cerca de los progenitores sin embargo puede conllevar un aumento de la probabilidad de cruzamiento con individuos genéticamente emparentados, lo que aumenta la tasa de consanguinidad, con importantes implicaciones en la diversidad genética de las poblaciones.

Aunque se trata de una especie dioica, algunos autores han descrito hermafroditismo en esta especie en porcentajes relativamente elevados (4%), con óvulos y espermatozoides en la misma gónada (Cognetti & Delavault, 1962).

Coscinasterias tenuispina: Sistemática, distribución y biología

El género *Coscinasterias* pertenece a la clase Asteroidea, superorden Forcipulatacea, orden Forcipulatida y a la familia Asteriidae; este género está compuesto por cuatro especies, presentes en las zonas templadas de ambos hemisferios: *Coscinasterias tenuispina* (Lamark, 1816)*, C. acutispina, C. calamaria y C. muricata*. De hecho, dos de las cuatro especies, *C. tenuispina* y *C. acutispina,* se encuentran tanto en el hemisferio norte como en el sur. La primera, *Coscinasterias*

tenuispina (Figura 5), objeto de estudio en esta tesis, es una estrella espinosa de talla mediana y coloración variable. Es fisípara, por tanto se puede reproducir asexualmente, y se caracteriza por presentar un número de brazos normalmente superior a 5. Está ampliamente distribuida en el Mediterráneo, las islas de la Macaronesia (Canarias, Madeira, Azores), el Golfo de Vizcaya, Bermudas, el Golfo de México, Cabo Verde y Brasil (Clark & Downey, 1992), habitualmente a poca profundidad. El primer estudio filogenético del género *Coscinasterias* determinó que se trataba de un grupo monofilético, separándolo claramente del género *Astrostole*, posiblemente confundido en algún trabajo con el género *Coscinasterias* en las costas australianas (Dakin & Bennett, 1987; Waters & Roy, 2003). Una filogenia posterior de todo el orden Forcipulatida propuso el género *Coscinasterias* como un grupo parafíletico, compartiendo el clado con el género *Astrometis*, formado por una sola especie, *Astrometis serulifera* (Mah & Foltz, 2011); esta especie habita una zona de aguas templadas en la costa Americana del Pacífico, donde paradójicamente no hay citada ninguna especie de *Coscinasterias*.

Según Waters & Roy (2003) *C. muricata,* presente en las costas de Nueva Zelanda y Australia, es la especie basal del género *Coscinasterias*; las diferencias moleculares dentro de esta especie son mayores que las que existen entre las otras tres, formando posiblemente entre todas un complejo de especies. Además, la zona del océano Pacífico de dónde es originaria *C. muricata* es también el lugar donde hay más especies de la familia Asteriidae, de afinidades templadas, que comparten el mismo clado (Mah & Foltz, 2011).

Todas las especies del género *Coscinasterias* son depredadoras y se alimentan de otros invertebrados, como bivalvos, erizos, pequeños cangrejos, etc. De hecho, la especie japonesa *C. acutispina* es muy voraz con algunas lapas valor comercial elevado, provocando un impacto económico importante (Fujita & Seto, 1998, 2000).

Figura 5. Individuos de *Coscinasterias tenuispina* de diferentes tamaños de Llançà, en la costa Catalana.

Reproducción en *Coscinasterias tenuispina*

Los individuos del género *Coscinasterias* tienen capacidad para reproducirse sexualmente y asexualmente, por fisión. Durante la reproducción sexual, se produce una larva planctotrófica con capacidad de vivir varias semanas en la columna de agua (Barker, 1978; Shibata *et al.,* 2011). Como ya se ha comentado anteriormente, y aunque no siempre va relacionado, el tiempo de vida en la columna de agua permite a las especies tener una elevada dispersión potencial, lo cual puede tener gran impacto en la estructura de las poblaciones de la especie (Waters & Roy, 2003). La incidencia de la reproducción asexual en *Coscinasterias* es muy importante y puede ocurrir reiteradas veces en un mismo individuo, dependiendo de sus condiciones fisiológicas intrínsecas y de las condiciones ambientales. Los individuos más pequeños (que no más jóvenes, ya que el tamaño *a priori* no está relacionado con la edad) tienden a dividirse con mayor frecuencia (Crump & Barker, 1985). En *C. acutispina,* un aumento de la temperatura y una buena alimentación potencian la reproducción asexual (Haramoto *et al.,* 2007; Seto *et al.,* 2013), aunque, por otro lado, algunas infecciones parasitarias disminuyen la capacidad de fisión (Haramoto *et al.,* 2007). Contrariamente, en *C. calamaria* la disponibilidad de alimento disminuye la frecuencia de fisión de la especie (Crump & Barker, 1985). Por tanto, parece que en las diferentes especies, la incidencia de la reproducción asexual depende de factores ambientales diversos.

Sin embargo, en todas las especies del género, la reproducción asexual puede provocar desequilibrios en la proporción de sexos, que en la mayoría de los casos es a favor de los machos, lo que también condiciona los niveles de diversidad y estructura genética de las poblaciones. En *C. calamaria* todas las poblaciones analizadas por Crump & Barker (1985) presentaban desequilibrios genéricos, pero solo en una de cuatro poblaciones dominaban las hembras; unos años más tarde, la proporción de esta misma población cambió a favor de los machos (Sköld *et al.,* 2002). Por tanto, la dinámica en la proporción de los sexos no es estable en el tiempo y puede variar en pocos años. En *C. acutispina* se encontraron poblaciones formadas únicamente por machos (Seto *et al.,* 2000) de la misma manera que sucede en poblaciones de *C. tenuispina* de la costa de Brasil (Alves *et al.,* 2002).

Las primeras observaciones del ciclo biológico de *C. tenuispina* las realizó Crozier (1915, 1921) en estudios llevados a cabo en las islas Bermudas. Crozier, observó que esta especie se reproducía sexualmente en los meses más fríos, durante los cuales la proporción de individuos que se dividían disminuía significativamente; el autor correlacionó las bajas temperaturas con la disminución de la asexualidad. Congnetti & Delavault (1958), en la costa mediterránea del Tirreno, observaron que tan solo el 52 % de los individuos de *C. tenuispina* tenían gónadas y que, muy frecuentemente, éstas no se encontraban en todos los brazos. Finalmente, el único estudio exhaustivo sobre el ciclo reproductor de esta especie lo realizaron en Brasil Alves *et al.* (2002) en el que observaron máximos gonadales en los meses fríos (julio y agosto, en el hemisferio sur).

La edad de un clon

El resultado de la reproducción asexual por fisión, en ausencia de recombinación, es la formación de nuevos individuos idénticos a los progenitores, los clones. En el género *Coscinasterias,* la fisión da lugar directamente a individuos adultos, mientras que otras formas reproducción asexual, como por ejemplo la gemación o la partenogénesis, generan individuos no maduros a partir de un grupo de células germinales (de Meeûs *et al.,* 2007). En estos dos últimos casos, la edad de un individuo es diferente a la edad de un linaje clonal. Pero ¿Cómo se puede averiguar la edad de un clon generado por fisión? ¿Cuál de las dos mitades es el progenitor? ¿Qué edad tiene el "nuevo" individuo?

En especies de plantas con reproducción asexual, como el álamo, *Populus tremuloides*, o la fanerógama marina *Posidonia oceánica*, se ha inferido la edad de un linaje clonal mediante la acumulación de mutaciones somáticas, usando el reloj molecular, y la capacidad de expansión de un clon (Ally *et al.*, 2008; Arnaud-Haond *et al.*, 2012). Ambos métodos además se combinan para calibrarse entre ellos y analizar la convergencia de resultados. La estimación de la edad de un linaje clonal mediante el reloj molecular ha dado lugar a algunos trabajos con animales con resultados sorprendentes, donde, por ejemplo, se ha visto que algunos linajes de rotíferos u ostrácodos presentan una longevidad de varios millones de años (Judson & Normark, 1996). Sin embargo, estos métodos no están exentos de errores. Los cálculos se basan en un reloj molecular calibrado en especies parecidas, de reproducción sexual y, en algunos casos, las tasas de mutación entre organismos sexuales y asexuales pueden diferir significativamente, lo que puede introducir un error en las estimaciones (Fontaneto *et al.*, 2012).

En algunas especies de equinodermos, el crecimiento de las placas calcáreas del esqueleto dérmico puede relacionarse con la edad del individuo, ya que durante el proceso de crecimiento se van formando bandas de diferente densidad según la época del año (Gage, 1990, 1992; Turon *et al.*, 1995; Dahm & Brey, 1998; Ebert, 2008; Calderón & Turon, 2010). Este tipo de estructura de bandas de crecimiento no se detecta en *Coscinasterias tenuispina*, y tampoco existe un método bien desarrollado para relacionar crecimiento y edad, como ocurre en la mayor parte de los asteroideos. Sin embargo, existen otras técnicas para analizar la edad de los organismos, que se basan en análisis moleculares, como es el caso de la medida de la longitud de los telómeros (ver Cuadro 2), una técnica ampliamente usada y desarrollada para estudios en humanos, y que se ha aplicado en esta tesis doctoral.

Cuadro 2: Telómeros

Los telómeros son secuencias repetitivas $(5'\ TTAGGG\ 3')_n$ que se encuentran en los extremos de los cromosomas y que se encargan, entre otras funciones, de mantener la estabilidad de la estructura de los mismos (Hemann *et al.*, 2001); también mantienen inactivados sistemas de senescencia y apoptosis celular (Herbig *et al.*, 2006; Rando, 2006; Jaskelioff *et al.*, 2011). Los telómeros son secuencias muy conservadas en la mayoría de los metazoos (Gomes *et al.*, 2010). Durante la duplicación del ADN, previa a cada división celular, las proteínas polimerasas se encargan de duplicar todo el material genético de la célula. Una característica de las polimerasas es que solo pueden duplicar en una dirección, y que en dirección contraria tienen que ir duplicando por fragmentos, los llamados fragmentos de Okazaki. Otra característica es que la polimerasa se desliga del ADN y deja de duplicar cuando la proteína llega al final de la cadena de ADN, sin poder duplicar las últimas bases de ésta. Este hecho hace que los telómeros se vayan acortando cada vez que la célula se divide.

Como la longitud telomérica va disminuyendo con el tiempo, ésta se ha usado en diferentes estudios como marcador de edad y senescencia (Cawthon *et al.*, 2003; Rando, 2006; Shawi & Autexier, 2008; Watson & Riha, 2011), ya que se puede relacionar con la cantidad de divisiones celulares. También se ha demostrado que el estrés y el modo de vida pueden afectar a la longitud de los telómeros (Pauliny *et al.*, 2006; Horn *et al.*, 2010; Dunshea *et al.*, 2011) por lo que, aunque haya una relación longitud/edad, nunca podrá reflejar el valor absoluto y exacto de ésta, y se tiene que considerar en valores relativos.

A pesar de sus limitaciones, la técnica de medida de la longitud de los telómeros podría ser una herramienta para averiguar la edad de los individuos clonales, herramienta que por otra parte, ha sido ampliamente utilizada en estudios con humanos (Allsopp & Harley, 1995; Campisi *et al.*, 2001; Rando, 2006).

¿Por qué estas especies?

En el año 1968, el senegalés Baba Dioum, durante un mitin de la *International Union for Conservation of Nature* (IUCN), postuló: "*Al final, conservaremos sólo lo que amamos, amaremos sólo lo que entendemos y entenderemos sólo lo que nos enseñan*". Cualquier contribución al conocimiento es importante para entender el funcionamiento complejo de los océanos,de los mares y de las especies que los constituyen, permitiéndonos desarrollar más y mejores herramientas para conservar los ecosistemas y transmitir su interés a la sociedad. Pero siempre amaremos más aquello que sintamos más cercano y que, además, nos guste.

Las dos estrellas de mar objeto de estudio en esta tesis, habitan en sustratos rocosos litorales, entre los 0 y los 200 metros de profundidad (Crozier, 1921; Villamor & Becerro, 2010). Las poblaciones de ambas especies coinciden con zonas de buceo recreativo y baño. La estrella roja *Echinaster sepositus*, es una especie muy conspícua, presente en numerosas fotografías submarinas, que la han convertido en una especie simbólica y emblemática del Mediterráneo. Todo lo contrario sucede con *Coscinasterias tenuispina:* suele vivir bajo piedras y su mimetismo la hace menos evidente, aunque se encuentra muchas playas rocosas someras del litoral mediterráneo. La accesibilidad de ambas especies hace que potencialmente puedan ser conocidas por un gran número de personas y por lo tanto, que los resultados de su estudio puedan ser asimilados por la sociedad, recordados y transmitidos con mayor frecuencia.

A pesar de su accesibilidad, se trata de dos especies de las que se desconocen aspectos básicos de su biología, biogeografía e historia evolutiva. Ambas tienen una amplia distribución, aunque su biología reproductiva y su capacidad dispersiva es muy diferente. Las preguntas que por tanto se pueden plantear son: ¿Cómo dos especies tan lejanas desde el punto de vista biológico consiguen converger en zonas concretas y solapar en gran parte de su área de distribución? Desde un punto de vista evolutivo, ¿dónde se originaron sus poblaciones?, ¿cómo están conectadas entre sí?, ¿cuáles son sus linajes más importantes y dónde se localizan?, ¿cuáles son los atributos biológicos que han condicionado su distribución?

El conocimiento ayuda a sensibilizar a la sociedad sobre el cuidado y conservación de las especies y, fomenta una mejor comprensión del ecosistema en el que habitan.

Objetivos

El **objetivo principal** de esta tesis consiste en comparar patrones filogeográficos y de genética de poblaciones en dos especies de estrellas de mar con características biológicas distintas, *Echinaster (E.)sepositus* (Retzius, 1783) y *Coscinasterias tenuispina* (Lamark, 1816) pero que comparten gran parte de su área de distribución a lo largo del arco atlanto-mediterráneo. Esta comparación permitirá entender mejor como los factores históricos y actuales han afectado a cada una de las dos especies, y también incrementará el conocimiento sobre el efecto de las barreras oceanográficas en esta área marina, un tema que en la actualidad presenta controversia y no ha sido completamente resuelto.

Para ello se han planteado diferentes objetivos concretos, que se abordan en los 5 capítulos de esta tesis doctoral.

El objetivo del **capítulo 1** consiste en desarrollar marcadores microsatélite para equinodermos con distribución atlanto – mediterránea. Entre las especies candidatas se encuentran ambas especies de estrella de mar objeto de esta tesis, *Echinaster sepositus* y *Coscinasterias tenuispina*, y un equinoideo, *Arbacia lixula*, que no es objetivo de estas tesis.

Los objetivos del **capítulo 2** consisten en:

1. Analizar la estructura genética y la filogeográfia de las poblaciones de *Echinaster sepositus* a lo largo del Atlántico y del Mediterráneo, así como el efecto de las diferentes barreras oceanográficas, y entender cómo de determinante ha sido la presencia de una larva lecitotrófica en la estructura genética de las poblaciones.
2. Generar los conocimientos necesarios para entender la vulnerabilidad de esta especie, de acuerdo con sus características genéticas, que puedan servir de base para desarrollar herramientas de gestión.

Los objetivos del **capítulo 3**:

1. Explorar y determinar los factores históricos que han podido influir en la evolución de *Coscinasterias tenuispina* dada su capacidad de reproducirse tanto sexual como asexualmente, y establecer las barreras marinas contemporáneas más importantes para la especie.
2. Analizar la prevalencia de los diferentes sistemas de reproducción (sexual versus asexual) a lo largo del amplio rango de distribución geográfica de la especie, y si las mismas están relacionadas con factores ambientales.
3. Determinar si en los límites de distribución de la especie los factores ambientales pueden ser limitantes para completar el ciclo sexual.

Los objetivos del **capítulo 4**:

1. Determinar, en una población de *Coscinasterias tenuispina* que se mantiene monoclonal a lo largo del tiempo, los patrones temporales y la variabilidad individual de sus estrategias reproductivas (sexual y asexual), así como los diferentes factores que influyen en las mismas.
2. Probar que ambas estrategias reproductivas se conservan a lo largo del tiempo, en especial el potencial para la reproducción sexual.
3. Estudiar las variaciones de densidad y las estructuras de tallas de esta población a lo largo del tiempo, y como ambas pueden modular la estrategia reproductiva de la especie

Finalmente, ante la presencia de poblaciones monoclonales que son capaces de mantenerse en ausencia de reproducción sexual, el objetivo del **capítulo 5** fue determinar cómo *Coscinasterias tenuispina* puede evitar procesos de senescencia asociados a largos periodos de reproducción asexual, y en ausencia de recombinación de nuevas variantes genéticas.

Estructura

La estructura de la tesis pretende ir paso a paso para lograr responder a los objetivos planteados. Por ello, se ha estructurado en tres partes que integran los 5 capítulos de la misma. En la parte I se describe cómo se aislaron y optimizaron los marcadores nucleares microsatélites, imprescindibles para poder llevar a cabo los diferentes estudios de que consta esta tesis doctoral. La parte II se dedica a la especie *Echinaster sepositus*, y en ella se describe la filogeografía y la genética de poblaciones de la especie. Finalmente, en la parte III se analiza la especie *Coscinasterias tenuispina* desde varios puntos de vista y con metodologías muy diferentes, y por ello está dividida en tres capítulos donde se describen: la filogeografía y genética de poblaciones de la especie, su ciclo biológico y, finalmente, el enigma de cómo las poblaciones clonales sobreviven al paso del tiempo sin sufrir procesos de deterioro orgánico, genético y senescencia, o lo que es lo mismo, sin "envejecer".

PARTE I – Marcadores moleculares

Capítulo 1. Characterization of thirty two microsatellite loci for three Atlanto-Mediterranean echinoderm species [*Caracterización de treinta y dos loci microsatélite para tres especies de equinodermos atlanto-mediterráneos*]

- Autores: <u>Alex Garcia-Cisneros</u>, Claudio Valero-Jiménez, Creu Palacín y Rocío Pérez-Portela

PARTE II – *Echinaster sepositus*

Capítulo 2. Low genetic diversity and recent demographic expansion in the red starfish *Echinaster sepositus* (Retzius 1816). [*Valores bajos de diversidad genética y expansión demográfica reciente en la estrella de mar roja* Echinaster sepositus (Retzius 1816)]

- Autores: <u>Alex Garcia-Cisneros</u>, Creu Palacín, Yousra Ben Khadra, Rocío Pérez-Portela

PARTE III – *Coscinasterias tenuispina*

Capítulo 3. The effect of asexual reproduction on intra-specific genetic structure and divergence: the case of an amphi-Atlantic starfish. [*El efecto de la reproducción asexual en la estructura genética y en la divergencia intraespecíficas. El caso de una estrella anfiatlántica.*]

- Autores: <u>Alex Garcia-Cisneros</u>, Creu Palacín, Carlos Renato Rezende Ventura, Barbara Feital, Paulo Cesar Paiva, Rocío Pérez-Portela

Capítulo 4. Hope springs eternal in the starfish gonad: preserved potential for sexual reproduction in a single-clone population of fissiparous starfish [*La esperanza es eterna en las gónadas de una estrella de mar: el potencial para la reproducción sexual se mantiene en una población monoclonal de una estrella de mar fisípara*]

- Autores: <u>Alex Garcia-Cisneros</u>, Rocío Pérez-Portela, Owen S. Wangensteen, Marta Campos-Canet, Creu Palacín

Capítulo 5. Long telomeres are associated with clonality in wild populations of the fissiparous starfish *Coscinasterias tenuispina* [*Los telomeros largos están relacionados con la clonalidad en poblaciones naturales de la estrella de mar* Coscinasterias tenuispina]

- Autores: <u>Alex Garcia-Cisneros</u>, Rocío Pérez-Portela, Bethanie Carney Almroth, Sophie Degerman, Creu Palacín, Helen Nilsson Sköld

Informe de los directores

Las doctoras **Creu Palacín** y **Rocío Pérez Portela**, co-directoras de esta tesis, certifican que el candidato a doctor **Alex Garcia Cisneros** ha participado activamente en todos los trabajos de la tesis, tanto en el diseño, como en el análisis de muestras y datos, y también en la redacción de los artículos que finalmente forman parte de cada capítulo de la tesis.

A continuación se describen los diferentes capítulos y el trabajo realizado por cada autor. También se indica qué capítulos han sido publicados en revistas indexadas por el *Institute for Scientific Information (ISI)* y el factor de impacto listado en el *Journal of Citation Reports (JCR)* correspondiente a 2015.

1. Characterization of thirty-two microsatellite loci for three Atlanto-Mediterranean echinoderm species. <u>Alex Garcia-Cisneros</u>, Claudio Valero-Jiménez, Creu Palacín y Rocío Pérez-Portela

Revista: *Conservation Genetic Resources* (2013) – Publicado. (IF = 1.172)

Volumen 5, Tema 3, pag 749-753; DOI 10.1007/s12686-013-9897-5

Diseño del trabajo: AGC, CP, RPP

Análisis de las muestras: AGC, CVJ

Redacción: AGC, CP, RPP

2. Low genetic diversity and recent demographic expansion in the red starfish *Echinaster sepositus* (Retzius 1816). <u>Alex Garcia-Cisneros</u>, Creu Palacín, Yousra Ben Khadra, Rocío Pérez-Portela

Revista: *Scientific Reports* – En revisión (IF = 5.578)

Diseño del trabajo: AGC, CP, RPP

Análisis de las muestras: AGC, YBK

Redacción: AGC, CP, RPP

3. The effect of asexual reproduction on intra-specific genetic structure and divergence: the case of an amphi-Atlantic starfish. <u>Alex Garcia-Cisneros</u>, Creu Palacín, Carlos Renato Rezende Ventura, Barbara Feital, Paulo Cesar Paiva, Rocío Pérez-Portela

Revista: *Molecular Ecology* – En revisión. (IF = 6.494)

Diseño del trabajo: AGC, CP, CRRV, RPP

Análisis de las muestras: AGC, BF, PCP

Redacción: AGC, CP, RPP

4. Hope springs eternal in the starfish gonad: preserved potential for sexual reproduction in a single-clone population of fissiparous starfish. <u>Alex Garcia-Cisneros</u>, Rocío Pérez-Portela, Owen S. Wangensteen, Marta Campos-Canet, Creu Palacín

Revista: *Hydrobiologia* – En revisión. (IF = 2.275)

Diseño del trabajo: AGC, RPP, CP

Análisis de las muestras: AGC, OSW, MCC

Redacción: AGC, OSW, CP, RPP

5. Long telomeres are associated with clonality in wild populations of the fissiparous starfish *Coscinasterias tenuispina*. <u>Alex Garcia-Cisneros</u>, Rocío Pérez-Portela, Bethanie Carney Almroth, Sophie Degerman, Creu Palacín, Helen Nilsson Sköld

Revista: *Heredity* (2015) –Publicado. (IF = 3.805)

Volumen 115; Tema 5; pág 437-443; DOI 10.1038/hdy.2015.43

Diseño del trabajo: AGC, BCA, HNS

Análisis de las muestras: AGC, SD

Redacción: AGC, CP, RPP, HNS

Barcelona, a 8 de Abril de 2016

Firmado:

Dra. Creu Palacín Cabañas   Dra. Rocío Pérez Portela

PARTE I

HERRAMIENTAS MOLECULARES
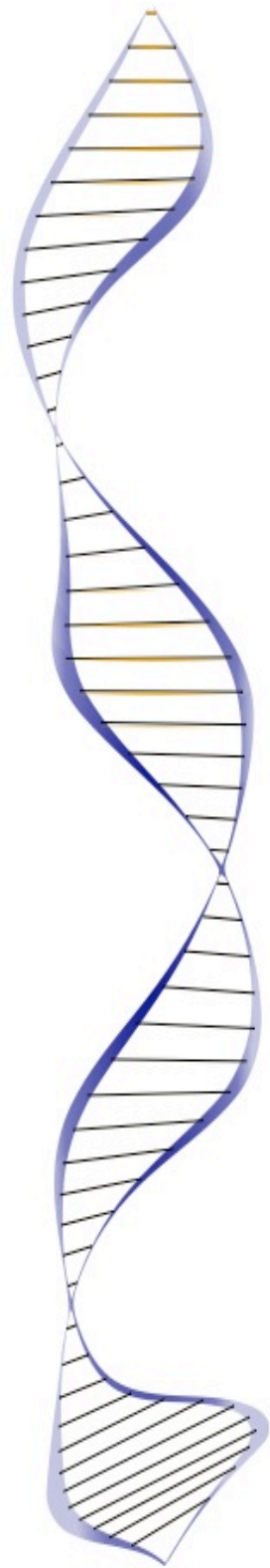
Capítulo 1

Characterization of thirty two microsatellite loci for three Atlanto-Mediterranean echinoderm species.

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Abstract

Thirty two microsatellites were optimized from 454 pyrosequencing libraries for three Atlanto-Mediterranean echinoderms: *Coscinasterias tenuispina*, *Echinaster sepositus* and *Arbacia lixula*. We observed different frequency of microsatellite types (di-, tri-, tetra- and pentanucleotide) throughout the genome of the species, but no significant differences were observed in allele richness among different microsatellite repeats. No loci showed linkage disequilibrium. Heterozygosity deficit and departure from Hardy–Weinberg equilibrium were observed for some loci, in two species, probably due to high levels of inbreeding. Heterozygosity excess observed in *C. tenuispina* could be explained by selection against homozygotes and/or outcrossing.

Keywords. Pyrosequencing, inbreeding, clonality, conservation, starfish, sea urchin.

Resumen

Treinta y dos microsatélites fueron optimizados a partir de bibliotecas generadas por pirosecuenciación 454 en tres equinodermos atlanto-mediterráneos: *Coscinasterias tenuispina*, *Echinaster sepositus* y *Arbacia lixula*. Se han observado diferentes frecuencias de tipos de microsatélite (di-, tri-, tetra- y pentanucleotidos) a lo largo del genoma de las especies, pero no se detectaron diferencias significativas en la riqueza de los diferentes alelos microsatélites. En ningún loci se observa desequilibrio de ligamiento. Dos de las especies mostraron un déficit de heterocigosidad y un desequilibrio Hardy-Weinberg para algunos loci, probablemente debido a altos niveles de endogamia. El exceso de heterocigosidad observada en *C. tenuispina* podría explicarse por la presencia de selección contra homocigotos y / o fecundación cruzada.

Palabras clave: Pirosecuenciación, endogamia, clonalidad, conservación, estrella de mar, erizo de mar.

During last century, Mediterranean Sea has suffered an extensive loss of biodiversity due to high anthropogenic pressures and environmental perturbations (Coll et al. 2010). Introduction of non-native species, increase in water temperature and extensive gaps in the distribution of natural populations due to urbanization, are among the most important environmental pressures (Thibaut et al. 2005, Lejeusne et al. 2009).

In this study we described new microsatellite loci for three of the most common Atlanto-Mediterranean echinoderms with important implications for conservation; the starfishes *Echinaster sepositus* and *Coscinasterias tenuispina*, and the sea urchin *Arbacia lixula*. *E. sepositus* is an emblematic species along the Atlanto-Mediterranean area but some populations at the North-Western Mediterranean have suffered a severe decline (Villamor 2010, and authors' pers. obs.). This species is now scarce in areas with high anthropogenic pressure and affluence of divers, and larger populations are only observed within marine protected areas. Due to the short-distance dispersal of its lecithotrophic larva, studies about populations' connectivity, inbreeding and genetic structure are crucial to design future management strategies for restoring their populations (Jones et al. 2007).

On the other hand, mitochondrial data suggested a recent colonization of the Mediterranean from the Atlantic Ocean by the thermophilous species *A. lixula* and *C. tenuispina* (Wangensteen et al. 2012 and authors' unpublished data), and whose densities may increase dramatically in the foreseeable future. Global warming might facilitate population blooms and thus turn these species into an ecological problem. Both species can modify sublittoral habitats because of their voracity generating barren grounds when populations reach high densities (Guidetti et al. 2003; Bonaviri et al. 2011). Populations' monitoring, including recruitment and connectivity studies between Atlantic sources and Mediterranean stocks based on microsatellites, is highly recommendable to evaluate the potential threat of these species for Mediterranean ecosystems.

We used 454 pyrosequencing to isolate novel microsatellite loci in *C. tenuispina*, *E. sepositus* and *A. lixula*. Genomic DNA was extracted using QIAamp® DNA Mini Kit (QIAGEN) to a final DNA concentration of 5 ng/µl and distributed in three physically separated lanes of a plate. Pyrosequencing was performed on a Roche Life Science 454 GS-FLX System at the Scientific-Technical Services of University of Barcelona. Sequences were trimmed to remove regions with a greater than 0.5 % chance of error per base using GENEIOUS version 5.5 (Drummond et al. 2010). Total number of sequences which passed quality filtering, number of microsatellites detected, and reads mode length were variable between species, and all details are summarized in Online Resource 1. Sequences were searched for perfect microsatellites (di-, tri-, tetra- and pentanucleotides) with at least eight repeats and enough priming regions with QDD1 v. 1.3 (Meglécz et al. 2010). Primers were designed with the software PRIMER 3 (Rozen and Skaletsky 1999).

Amplification success and polymorphism were tested in two populations per species: Costa Brava (42°29'N, 3°10'E) and Tenerife (28°25'N, 16°19'W) in *C. tenuispina*, Costa Brava (41°46'N, 3°05'E) and Marseille (43°16'N, 49°34'E) for *E. sepositus*, and Costa del Sol (36°34'N, 4°34'W) and Costa Brava (42°24'N, 3°07'E) in *A. lixula*. Total DNA was extracted from feet tube and amplified using the REDExtract-N-Amp Tissue PCR Kit (Sigma Aldrich). Forward primers were labelled with a fluorescent dye as shown in Table 1. PCR amplifications were performed as described in Valero-Jimenez et al. 2012. Allele length was estimated relative to the internal size standard 70-500 ROX (Bioventures) using the software Peak-Scanner (Applied Biosystems).

Dinucleotides were the most frequent microsatellites followed by tri, tetra and pentanucleotides throughout the genome of the species (see Online Resource 2). A total of thirteen, nine and ten polymorphic microsatellite were optimized for *C. tenuispina, E. sepositus* and *A. lixula*, respectively, including a selection of different microsatellite types (see Table 1). Linkage disequilibrium,

observed and expected heterozygosity, and deviation from Hardy–Weinberg equilibrium were calculated with ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010). Bonferroni corrections of the p-values for multiple tests were run.

No evidence of linkage disequilibrium was detected across all pairwise comparisons. Failed amplifications due to presence of null alleles were not detected for any loci. Nineteen markers showed Hardy–Weinberg disequilibrium after Bonferroni corrections. Heterozygosity deficit observed in two species may be explained by high levels of inbreeding, as demonstrated in other marine invertebrates (Pérez-Portela et al. 2008; Calderón et al. 2009). The heterozygosity excess observed in *C. tenuispina* may be explained by clonal reproduction, selection against homozygotes and/or outcrossing (Blanquer and Uriz 2010). After confirming normality and homoscedasticity of the dependent variable, we used a two-way ANOVA to test for differences in genetic diversity (measured as allelic richness) of different microsatellite types and species. Genetic diversity values were adjusted to population size with a rarefaction index calculated in CONTRIB V1.2 (Petit et al. 1998). Our results did not show differences in genetic diversity among di, tri, tetra and pentanucleotide repeats (F=0.233; p=0.872) but diversity was significantly different among species (F=35.69; p<0.0001) (see Online Resource 3). This result suggests that different microsatellite types are equally valid in terms of genetic diversity to assess population genetics in echinoderm species.

Specie	Locus (dye), GenBank accession number	F and R primer sequence	Repeat motif	T_a (ºC)	Size range (bp)	Population 1				Population 2		
						N	N_A	H_O/H_E	H-W	N	N_A	H_O/H_E
C. tenuispina	m.ten1 (6'FAM)	F: TCAAGGCTGTGTAGTACTCT	(ATT)*12	51 °C	171-174	22	2	0.045/0.045	1.0	16	2	0.812/0
	KC699497	R: TCAATCAAACTGTGTACCTT										
	m.ten6 (NED)	F: CATGAGAGCTTACAGAAAAG	(TAA)*7	51 °C	160-163	21	2	0.952/0.511	0.001*	16	2	0.812/0
	KC699498	R: CTTAGGTGTAATGAAGTGCT										
	m.ten13 (6'FAM)	F: GACAGAGTGCTTTCTTAATG	(ATAC)*12	51°C	360-364	19	1	0/0	-	15	2	0/0.3
	KC699499	R: AGTTCTGGAATAAACTACCC										
	m.ten14 (HEX)	F: CACTCTGAGCCTATAAGAGA	(TAA)*7	51 °C	137-138	22	2	1.0/0.512	0.001*	11	1	0/0
	KC699500	R: GTTAATTTCTCCCTACCT										
	m.ten19 (HEX)	F: CTGCTGGCTCCAGCTGCTAT	(GATT)*8	51 °C	133-150	22	1	0/0	-	12	2	0.583/0.
	KC699501	R: TCAACCAGGTCGTTGATCTTTGT										
	m.ten25 (HEX)	F: TAACTGTTGAATCCATCCT	(GTA)*10	51 °C	295-298	22	1	0/0	-	16	2	1.0/0.
	KC699502	R: CCTGTCATGATTATGTTTGT										
	m.ten24 (HEX)	F: CTCATAAGGGTGCTGTTT	(GT)*11	51 °C	365-367	22	1	0/0	-	16	2	0.437/0
	KC699503	R: ATGAATCATACGTGTGTGG										
	m.ten27 (6'FAM)	F: CTTCATAAGAGGTTAGTTGG	(AT)*9	53 °C	293-295	13	1	0/0	-	10	2	0.6/0.
	KC699504	R: TCCAAGTCATGGAATAACTA										
	m.ten30 (NED)	F: GGTACCAGTCGTCATAAATA	(AGTC)*17	51 °C	397-409	22	3	1.0/0.638	0.001*	16	2	0.812/0
	KC699505	R: AGGTCCACACACTACAGAT										

	m.ten31 (6' FAM)	F: GTGAGTGAAGCCAGAAACTT	(TGTT)*9	51 °C	298-302	18	1	0/0	-	16	2	1.0/0.5
	KC699506	R: ACATTTGGAATGTTTCCATC										
	m.ten32 (6'FAM)	F: ATGAGAGTGGATGACTGACA	(TAGA)*8	51 °C	245-249	19	2	0.947/0.512	0.002*	14	2	0.571/0
	KC699507	R: CCATAAGCTTAGCACTACAGG										
	m.ten33 (HEX)	F: CTGTTGAATCCATCCTTGTT	(GTA)*10	51 °C	290-296	19	2	0.789/0.490	0.012	16	4	1.0/0.
	KC699508	R: GCCCTGTCATGATTATGTTT										
	m.ten40 (6'FAM)	F: CCAGCTTGTTTCCATCCAAGGC	(AG)*11	51 °C	151-154	19	1	0/0	-	16	4	0.312/0
	KC699509	R: TCTGCACCTCGGGCGCATAGA										
E. sepositus	mES 2 (JOE)	F: CGTATTTTATGTGCAGTTG	(TTA)*9	51 °C	232-254	25	7	0.520/0.619	0.012	11	8	0.636/0
	KC699510	R: ATCATCCCATTAGAGGTTTA										
	mES 4 (6'FAM)	F: GCCAAAGATGCCATAAAT	(CAA)*6	51 °C	115-148	26	9	0.692/0.788	0.087	16	8	0.688/0
	KC699511	R: CTGTAGGCTAGCTGAGTTT										
	mES 11 (FAM)	F: GTTGTAGTGATTTCCTGATG	(TTA)*8	51 °C	128-256	21	3	0.143/0.138	1.000	8	3	0.250/0
	KC699512	R: CCGTGTTGAGAATATGTAA										
	mES 23 (6'FAM)	F: ATCATTGTTCTTCAGTTTCC	(TG)*10	51 °C	85-91	19	5	0.611/0.607	0.771	1	2	1.00/1
	KC699513	R: TTGTTAAATAGTCCCCAACT										
	mES 24 (HEX)	F: AGAGATCATTAACCCATTCA	(TTCA)*12	51 °C	87-195	26	10	0.115/0.838	0.000*	15	7	0.333/0
	KC699514	R: ACTAGTATGTATCCGTTGGC										
	mES 25 (HEX)	F: TAATTGATCCCATTCCTGTA	(TAAA)*10	51 °C	154-199	25	11	0.680/0.873	0.118	14	16	1.00/0.
	KC699515	R: TCACTGTATCCAGATTTCCT										

A. lixula	mES 29 (6'FAM)	F: ACTAGAATGTGGAGTGACAG	(AC)*12	51 °C	203-288	26	13	0.833/0.891	0.465	16	12	0.938/0
	KC699516	R: GTCGCTTAGGAAACATCT										
	mES 30 (HEX)	F: AAAGGTCTCTTTGAAGGTGTT	(CTG)*8	51 °C	262-290	26	8	0.269/0.767	0.000*	14	6	0.286/0
	KC699517	R: TTCAGGTAGTTGAAGAATTGC										
	mES 38 (HEX)	F: CCAGTTGACCCATCATAAAT	(GCA)*9	51 °C	256-317	25	9	0.320/0.796	0.000*	16	7	0.688/0
	KC699518	R: GTGATTATGTCCAAAGTGC										
	ALM 2 (6-FAM)	F: TGCTAAACGGCAACAATGAA	(AATC)*12	56 °C	283-355	23	12	0.739/0.756	0.5071	18	17	0,889/0
	KC699519	R: TGGTCGCTAATGGAGGTTTC										
	ALM 4 (6-FAM)	F: TGAGACAACGGGAAAGTCAA	(AATC)*14	56 °C	239-308	23	17	0.435/0.912	0.000*	18	18	0,778/0
	KC699520	R: CGATGGTCCTAGAGGTGACA										
	ALM 5 (6-FAM)	F: GTGGAATGGTGATGGAAAGG	(AGAT)*14	57 °C	120-228	23	16	0.696/0.903	0.000*	18	14	0.722/0
	KC699521	R: TCACGCCTGTTGAAATATCC										
	ALM 7 (HEX)	F: CATGGTTCATTTCTGCCTCA	(AATC)*11	56 °C	228-352	23	6	0.826/0.708	0.0835	18	13	0.500/0
	KC699522	R: GAATGGTTGACTTATTGGACGTT										
	ALM 8 (6-FAM)	F: CCATCCATTCATTCACTACTTCA	(AGGT)*11	57 °C	78-173	23	16	0.478/0.881	0.0906	18	14	0.444/0
	KC699523	R: ACAGATGGGTGGGTGGAG										
	ALM 9 (HEX)	F: TGTACGTACGTTGGCTGACGA	(AACT)*10	58 °C	221-275	23	11	0.261/0.857	0.000*	18	8	0.278/0
	KC699524	R: GCTCACATACAGCTCCCATGTT										
	ALM 11 (HEX)	F: CAGCTGAATCCGATGGTGTA	(AAATC)*9	57 °C	350-469	23	9	0.261/0.871	0.000*	18	8	0.222/0
	KC699525	R: TCACGTGCGAGATGTTCTTC										

ALM 14 (NED)	F: GCCTTATCATTAGGTGCAGGT	(AGT)*16	57 °C	181-259	23	18	0.609/0.911	0.000*	17	18	0,471/0
KC699526	R: CCGTCTAAGTGGAGAGCTATGG										
ALM 15 (HEX)	F: GAGGGCTTCATCCAACAATG	(ACT)*15	58 °C	75-125	23	14	0.478/0.797	0.000*	16	12	0,667/0
KC699527	R: TAATTGGCCGGCGTATATTG										
ALM 17 (NED)	F: GGATCCTACCATGAATTGTTACAT	(AC)*16	51 °C	177-356	23	13	0.799/0.911	0.259	18	11	0,625/0
KC699528	R: AATCAACCTGCTCCGTGAAT										

Table 1. Characteristics of 32 microsatellite markers for three echinoderm species. Ta annealing temperature, N number of individuals, N
alleles, Ho observed heterozygosity, HE expected heterozygosity and H-W p-value of the Hardy-Weinberg equilibrium test (*) sig
Bonferroni corrections.

Acknowledgments

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PARTE II
Echinaster sepositus

Capítulo 2

Low genetic diversity and recent demographic expansion in the red starfish *Echinaster sepositus* (Retzius 1816)

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Abstract

Understanding the phylogeography and genetic structure of populations, and the processes associated with patterns in them, is important in evaluating the vulnerability of marine species and developing strategies for their management. In this study, we explore how past climatic events and ongoing processes have shaped the genetic structure and diversity of the Atlanto-Mediterranean red starfish *Echinaster sepositus*. The species is relatively abundant in some areas of the Mediterranean Sea; but some populations have dramatically decreased over recent years due to direct extraction for the ornamental aquarium and souvenir industries. Analysis across most of the distribution range of the species based on the mitochondrial cytochrome c oxidase subunit I gene and eight microsatellite loci revealed very low intraspecific genetic diversity. The species presented a weak genetic structure within marine basins despite the a priori low dispersal potential of its lecithotrophic larvae. Our results also reveal a very recent demographic expansion across the distribution range of the species. The genetic data generated and presented here indicate that the species may be highly vulnerable, due to its low intraspecific genetic diversity.

Keywords: Starfish, phylogeography, genetic diversity, marine barriers.

Resumen

Entender la filogeografía y la estructura genética de las poblaciones, así como los procesos asociados a los diferentes patrones de distribución que se dan en las mismas, es importante para evaluar la vulnerabilidad de las especies marinas y desarrollar estrategias para su gestión. En el presente trabajo, se estudia cómo los procesos climáticos históricos y actuales han modulado la estructura y la diversidad genética de la estrella de mar roja *Echinaster sepositus* a lo largo de su distribución atlanto-mediterránea. Esta especie es relativamente abundante en algunas zonas del mar Mediterráneo, aunque algunas poblaciones han disminuido drásticamente en los últimos años debido a la extracción directa de ejemplares para acuarios o como recuerdos ornamentales. Los análisis del gen Citocromo c oxidasa subunidad I y ocho loci microsatélite en individuos de procedentes de todo el rango de distribución de la especie, han revelado una diversidad genética intraespecífica muy baja. Esta especie presenta una baja estructura genética dentro de cada una de las diferentes cuencas marinas, a pesar del hipotético bajo potencial de dispersión de sus larvas lecitotróficas. Los resultados de este estudio también muestran una expansión demográfica muy reciente en toda el área de distribución de la especie. Los datos genéticos generados y presentados indican que esta especie puede ser muy vulnerables debido a su baja diversidad genética.

Palabras clave: Estrella de mar, filogeografía, diversidad genetica, barreras marinas.

Introduction

In marine benthic species with limited mobility during adulthood, dispersal of the larvae plays an important role in the intraspecific distribution of genetic diversity, and both the genetic structure and connectivity of populations [1]. Traditionally, it has been though that planktotrophic larvae had a higher dispersal capability than lecithotrophic larvae [2–6]. Hence, species with lecithotrophic larvae that exhibit philopatric behaviour are expected to show populations that are genetically more structured at finer scales [5–10]. Nevertheless, during recent years, several studies have demonstrated that the time spent by larvae in the water column does not directly determine the genetic structure of populations [11,12]. Coastal water circulation, the availability of substrates, population size, fecundity and the stochasticity of the success of recruitment may determine the chaotic genetic structure found in many nearshore benthic species [13–16]. Additionally, other factors such as major oceanographic circulation as well as geographical straits and waterfronts are known to act as physical barriers that prevent propagule interchanges thereby limiting the connectivity between nearby areas [17–19].

Along the Atlanto-Mediterranean arch, the Almeria-Oran Front is considered the real boundary between the Mediterranean Sea and the Atlantic Ocean, acting as an important barrier to gene flow in a number of marine species [20–23]. The real influence of this marine transition from the genetic point of view still remains controversial due to its different effect and permeability on species displaying contrasting biological features [22,24–26]. The Mediterranean Sea itself possesses a complex system of oceanographic circulation [27], divided into two sub-basins separated by the Siculo-Tunisian Strait [20]. This sea has also suffered an intricate past history. The desiccation of the Mediterranean Sea, which reduced it to a series of hypersaline lakes during the so-called Messinian salinity crisis at the Mio-Pliocene transition (6–5.5 Mya), was followed by the refilling of the basin with Atlantic water [28,29]. More recently, the Quaternary climatic fluctuations that characterised the coastal fauna along the coast of northern Europe also had an important impact on the marine fauna of the south of Europe, including that of the Mediterranean Sea. During the cyclical glacial periods, which had a strong impact the northern European coast, the Mediterranean Sea and the southern European coast acted as separate marine refuges, when most of the north of Europe was covered by ice sheets [30]. These historical processes have determined the evolution of coastal species across the Atlanto-Mediterranean area [20,31–33]. The complexity of these historical, palaeo-geographical and ecological processes that have occurred in the Mediterranean explains the high biodiversity and rate of endemism in this small basin [34]. While the Mediterranean Sea is considered a hotspot of marine biodiversity, it is also one of world's most impacted seas [35]. It is exposed to considerable anthropogenic pressure from both short-term and long-term perturbations [36]. Mitigating further impact is hence a priority and to do this we need to understand the vulnerability of Mediterranean organisms. Molecular studies of the intraspecific distribution of genetic diversity can contribute to effective management and conservation strategies. Phylogeographic information and population genetic analysis assist us in exploring the most important evolutionary and contemporary factors that have shaped the current biodiversity and its geographical distribution. Therefore, molecular analysis provides data not only on inter-genetic and intra-genetic diversity and connectivity among populations, but also on the most important processes underlying the origin and maintenance of this diversity that should also be conserved where possible 37.

In this paper, we analyse one of the most emblematic starfish found in the Mediterranean Sea, and the first mentioned in science, by Aristotle 2,300 years ago in *Historia Animalium*: the red starfish *Echinaster sepositus* (Retzius 1783). The species is distributed across the Mediterranean and the temperate waters of the eastern Atlantic, from the south-eastern limit of the English

Channel to Cape Verde [38]. It inhabits shallow (from some 2 m) to deep waters, down as deep as 250 m, on sandy bottoms, rocky subtracts, and within seagrass systems [39]; and it has an affinity for coralline algae communities [40]. Although the species can be relatively abundant in some particular areas of the Mediterranean coast, during the last decade, some populations of *E. sepositus* in the north-western Mediterranean have dramatically decreased [40], probably at least partly as a result of direct extraction from nature by the ornamental aquarium and the souvenir industries. This activity is expanding rapidly in the absence of proper systems of the regulation and control of capture [41].

It has been reported that *E. sepositus* releases lecithotropic larvae that remain as free swimmers for no longer than 5-6 days [42,43]. Nevertheless, many features of the nature and behaviour of its larvae remain unclear. Whereas some authors [42] has reported floating eggs and larvae for this species, more recent laboratory experiments have showed that both the eggs and larvae sink to the bottom of tanks after spawning (Villamor, personal communication). In accordance with its short larval development, we may expect limited dispersal potential, low connectivity between distant areas and high genetic structure of populations; as observed for other asteroids with similar biology [5,44,45]. If this is indeed the case, these general biological characteristics will limit the potential for recovery of extinct populations via recruitment from other geographical sources. Nevertheless, in light of the most recent studies [14], information regarding the connectivity of a particular species should not be directly inferred from species displaying similar biology, because a number of biotic and abiotic factors are related to the effective dispersal of the larvae and intra-species connectivity.

For *E. sepositus*, very limited genetic information is available to date from which to infer the effective dispersal and levels of gene flow. The only genetic study with this species, based on one mitochondrial gene, included only a few populations from the coast of Tunisia [45]. Hence, the aim of this study is to obtain a more complete picture of the population genetic structure in *E. sepositus*, at both the evolutionary and geographical scales. We focus on the potential effect of major marine corridors, the Strait of Gibraltar and the Siculo-Tunisian Strait, on the genetic structure and connectivity; and also on the importance of geographical distance for the divergence of populations in a species with a short larval developmental stage. This represents a twofold contribution to marine biology: a) it increases knowledge of population genetics and phylogeography of benthic invertebrates across the Atlanto-Mediterranean transition, and between the two Mediterranean sub-basins, which is currently patchy and controversial; and b) it generates the knowledge required to understand the vulnerability of this species to exploitation, according to its genetic features. It is well known that the intraspecific genetic diversity of species plays a crucial role in the long-term survival of populations because it is the raw material on which natural selection acts [46]. Populations affected by human activity that results in habitat loss, pollution and overexploitation may experience a reduction in size. In turn, this may be translated to a reduction of genetic diversity due to the effects of bottlenecks, strong genetic drift and inbreeding depression, all of which can jeopardise population persistence [47]. These effects are particularly negative in species with low dispersal potential and low connectivity, because they limit the potential for recolonisation processes and gene interchanges with other sources.

To address our objectives, we use both mitochondrial and nuclear markers to understand past and present events shaping the geographical distribution of the intraspecific genetic diversity in *E. sepositus*. As a mitochondrial marker we chose the cytochrome c oxidase subunit I (COI) gene because of its high resolution in phylogeographic analysis [17,32,48] and potential comparison with a number of studies in echinoderms [22,24,26,45,49,50].

Results

Genetic diversity

From a total of 325 sequences (657 bp) of the COI fragment, we obtained 23 different haplotypes. Most individuals (92%) had one of three main haplotypes (H_1, H_2 and H_3), with only one mutation step between each other (see Figure 1). We detected 18 private haplotypes, and only two of them were separated by more than one mutation step from the three most common haplotypes. Nucleotide diversity (π) and haplotype richness (Hr) for the whole dataset were 0.0013 (± 1.03e-6 SD) and 0.619 (± 0.021 SD), respectively. Values of genetic diversity for COI were similar for the Atlantic and the Mediterranean basins, (Atlantic: Hr = 0.545, π = 0.00099; Mediterranean: Hr = 0.560, π = 0.00114), although within the Mediterranean basin, the eastern Mediterranean seemed to have lower values of diversity (Hr = 0.300, π = 0.00057). The population from Livorno, located in the western Mediterranean, presented only two haplotypes, and was the population with the fewest haplotypes and the lowest Hr (0.522).

The haplotype network obtained from COI (Figure 1) showed a very well-connected network, separated by only a few mutation steps, and no loops. However, 7 non-synonymous mutations were found in haplotypes that appeared infrequently. The three most frequent haplotypes appeared in most populations but displayed differences in geographical distribution. H_1, was
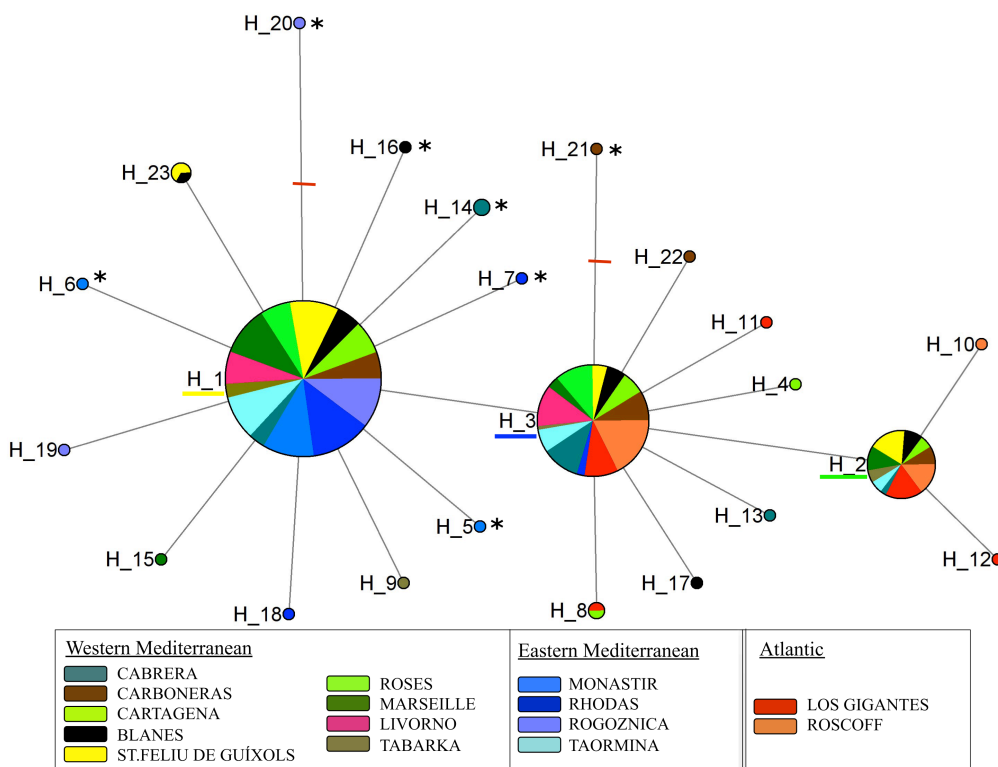

Figure 1. Haplotype network from COI sequences of *E. sepositus*. The area of the circles is proportional to the number of sampled individuals, and different colours represent different populations. Small red dashes represent mutation steps of more than one mutation. Asterisks (*) indicate non-synonymous mutations. The most common haplotypes, H_1, H_2 and H_3, are underlined using the colour pattern from Figure 2.

	Code	Coord.	N_{COI}	Nh	Hr	II	N_m	R_s	Allele number	Private Alleles	Ho	He	F
Cytochrome c oxidase I (COI)							**Microsatellites (8 loci)**						
<u>**Western Mediterranean**</u>													
CABRERA, Balearic Islands, Spain	CAB	39.152487, 2.946320	20	5	0.679 (±0.080)	0.00136 (±0.00025)	24	4.271	60	1	0.661	0.775	0.1
CARBONERAS, Andalusia, Spain	CAR	36.992822, -1.885877	23	6	0.700 (±0.061)	0.00154 (±0.00028)	24	4.039	57	0	0.657	0.734	0.1
CARTAGENA, Murcia, Spain	PAL	37.628559, -0.695339	22	5	0.645 (±0.085)	0.00133 (±0.00024)	23	3.830	52	1	0.605	0.705	0.1
BLANES, Catalonia, Spain	BLA	41.650626, 2.818985	20	6	0.742 (±0.074)	0.00166 (±0.00027)	8	3.822	38	0	0.531	0.739	0.28
St. FELIU, Catalonia, Spain	PSA	41.770913, 3.030400	30	4	0.598 (±0.082)	0.0014 (±0.00022)	26	4.082	57	0	0.493	0.735	0.3
ROSES, Catalonia, Spain	ROS	42.236866, 3.211675	22	3	0.567 (±0.051)	0.00093 (±0.00013)	24	3.959	56	2	0.561	0.733	0.2
MARSEILLE, Provence, France	MAR	43.276094, 5.335732	26	4	0.502 (±0.105)	0.0015 (±0.00027)	24	4.340	62	1	0.656	0.774	0.1
LIVORNO, Tuscany, Italy	LIV	43.472328, 10.332079	23	2	0.522 (±0.033)	0.00075 (±0.00005)	25	2.994	36	0	0.507	0.607	0.1
TABARKA, Jendouba, Tunisia	TAB	36.968067, 8.757856	9	4	0.694 (±0.147)	0.00169 (±0.00044)	10	3.991	43	0	0.587	0.734	0.2
TAORMINA, Sicily, Italy	TAO	37.845190, 15.296036	24	3	0.507 (±0.093)	0.00095 (±0.00021)	24	4.093	56	2	0.605	0.746	0.1
<u>**Eastern Mediterranean**</u>													
MONASTIR, Monastir, Tunisia	MON	35.787254, 10.838771	21	3	0.186 (±0.110)	0.0029 (±0.00018)	22	3.590	48	0	0.597	0.680	0.1
RHODAS, Dodecanese, Greece	RHO	36.446022, 28.228543	25	3	0.227 (±0.106)	0.0036 (±0.00017)	24	3.866	57	1	0.667	0.721	0.0
ROGOZNICA, Dalmatia, Croatia	CRO	43.522663, 15.95418	20	4	0.195 (±0.115)	0.00046 (±0.00029)	24	4.169	58	1	0.699	0.773	0.0
<u>**Atlantic Ocean**</u>													
LOS GIGANTES, Canary Islands, Spain	GIG	28.265344, -16.84775	18	5	0.667 (±0.083)	0.00132(±0.00025)	24	3.893	56	2	0.575	0.722	0.2
ROSCOFF, Brittany, France	RFF	48.753712, -3.972950	22	3	0.437 (±0.105)	0.00077(±0.00021)	24	4.184	65	4	0.567	0.738	0.2

	N_{COI}	Nh	Hr	π	R_s			Ho	He	F_{IS}
MEDITERRANEAN SEA	285	20	0.560 (±0.027)	0.00114(±0.00008)	4.189	94	9	0.602	0.727	0.1
WESTERN MEDITERRANEAN	195	15	0.634(±0.025)	0.0013(±0.00008)	4.188	86	7	0.586	0.728	0.2
EASTERN MEDITERRANEAN	90	8	0.300(±0.061)	0.00057(±0.00013)	4.065	78	2	0.654	0.725	0.1
ATLANTIC OCEAN	40	6	0.545(±0.069)	0.00099(±0.00017)	4.173	75	6	0.571	0.730	0.2
TOTAL	325	23	0.619(±0.021)	0.00129(±0.000)	4.224	100	-	0.598	0.727	0.1

Table 1. Populations of *E. sepositus*. Population name, code, coordinates, number of COI sequences (N_{COI}), number of haplotypes (Nh), rarefied diversity (Hr), nucleotide diversity (**π**), number of genotyped specimens with eight microsatellites (N_m), microsatellite rarefied allelic richness (R_s), m allele number, observed and expected heterozygosity (Ho and He), fixation index (F_{IS}) (***p-value < 0.05**) and p-values of the Hardy-Weinberg equilibriu values) for each location (**** p-value < 0.01**).

restricted to the Mediterranean basin, and H_3 was not found in the eastern Mediterranean populations, with the exception of Taormina (Figure 2).

The eight microsatellites showed between 6 and 32 different alleles per locus in the populations analysed. We observed differences in allelic richness among populations, but the population from Livorno displayed much lower values of allelic richness than any other population (Livorno R_s: 2.994; all the other populations R_s: from 3.882 to 4.340); a result consistent with the existence of only two mitochondrial haplotypes in this population (see Table 1). There was in general a significant deficiency of observed heterozygosity (H_o) in most populations, and populations from the north-western Mediterranean and the Atlantic showed significant F_{IS} values, which translated in Hardy Weinberg disequilibrium (Table 1; F_{IS} values).

Population structure and demography

The Bayesian clustering analysis performed with STRUCTURE did not revealed large differences between using only microsatellite loci or the combined microsatellite loci and COI dataset (see Figure 3). The resolution of the analysis seemed to be better when locations were implemented as priors, under the non-admixture model (Supplementary Figures S1 and S2). The most likely K values obtained from the microsatellite locus database were used to represent the results (Figure 3). For K=2, only the Mediterranean populations of Cartagena and Livorno appeared separated from all the other populations. When increasing K, the Atlantic population from the Canary Islands (Los Gigantes) and all the eastern Mediterranean populations grouped into different clusters (K=3 and K=4). For K=8, the main clusters detected for K=4 were maintain (Cartagena, Livorno, Los Gigantes, and eastern Mediterranean), and all the other Mediterranean populations and Roscoff from the Atlantic appeared as a mixture of different
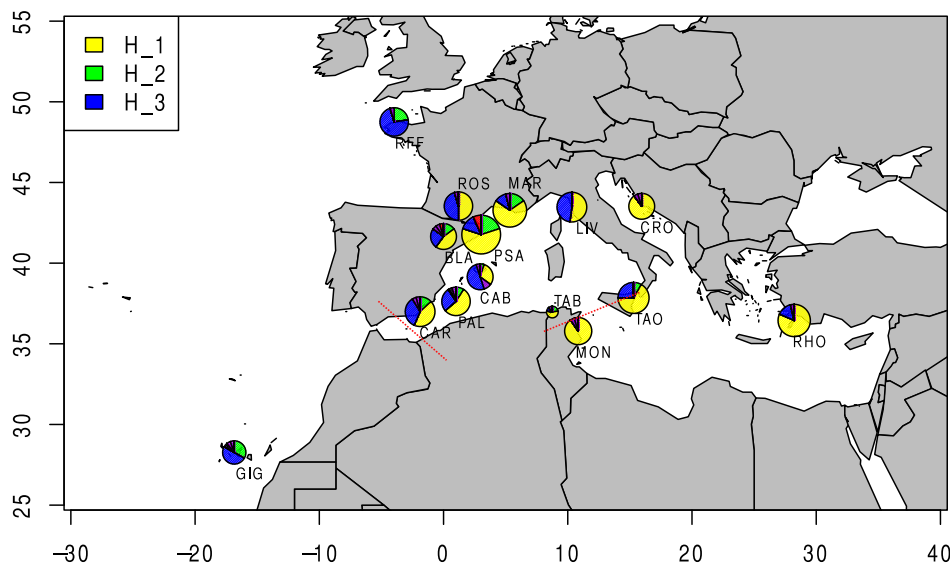

Figure 2. Map of the sampling locations in *E. sepositus*. Pie charts represent COI haplotype frequencies for each location, and size is proportional to the number of samples analysed. Private haplotypes are represented in purple. For location codes see Table 1. Red dotted lines represent the main marine barriers in the geographical area: the Almeria Oran Front (AOF) and the Siculo-Tunisian Strait. The map was drawn using Maps and Mapplots R packages and Inkscape 0.48 (Free Software Foundation, Inc.; Boston, USA).

clusters (See Figure 3A and 3B, and Supplementary Figure S2).

The results we obtained from the AMOVA for both COI and microsatellites supported the pattern obtained using STRUCTURE. The analysis revealed that most of the genetic diversity was retained within populations and individuals respectively (COI: >55%; microsatellites within individuals >80%). Significant differences were observed between groups (Atlantic, and eastern and western Mediterranean), and among populations within geographical groups for microsatellites, but not for COI. Taormina, a population on the distribution limit between the eastern and western Mediterranean, seemed to be more closely related to the western Mediterranean group; as observed by the increase in the variation between groups for the two groups (Table 2). The other populations on the limit of the Siculo-Tunisian Strait, Tabarka and Monastir, did not show more affinity for one specific area when moving between different sub-basins.
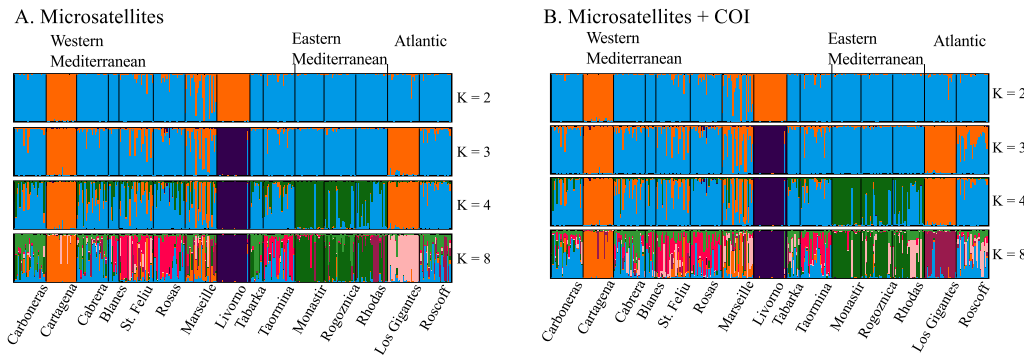

Figure 3. Bar plots of the Bayesian clustering analysis obtained using STRUCTURE for different K values. A. Analysis based on microsatellite loci. B. Analysis based on the combination of both mitochondrial sequences (COI) and microsatellite loci. Note that the same K values are presented, for comparison between databases.

Both Φ_{ST}-F_{ST} and Jost's D values showed significant genetic differences between populations from the Atlantic and Mediterranean basins, with only a few exceptions (Figures 4 and 5; Supplementary Tables S1 and S2). Most of the western Mediterranean populations did not show significant differences. We detected a significant positive correlation between Φ_{ST}-F_{ST} and Jost's D values (COI: r= 0.94, p= 0.001, and microsatellites: r= 0.93, p= 0.001, Supplementary Figure S3) indicating that both types of statistic detected similar genetic structure. Nevertheless, there was no significant correlation between pairwise differences between populations calculated from COI and microsatellites (correlation between Φ_{ST} and F_{ST}: r = 0.10 and between Jost's D: r = 0.13, Supplementary Figure S4). This is probably because the two types of genetic markers detect different signals of divergence between populations. In the case of the eastern Mediterranean populations, it did not show any significant differences in genetic structure according to COI; but most of them were significantly different according to the microsatellite data, which also revealed that Livorno and Cartagena were significantly different from all the other populations. With multidimensional scaling (MDS) analysis based on Φ_{ST} from COI, we observed a distribution of the populations related to geographical origin: on the right-hand side, were all the eastern Mediterranean locations; in the centre, the western Mediterranean; and on the left-hand side, the two Atlantic locations. Nevertheless this ordering was not maintained for the MDS based on F_{ST} from microsatellites, in which the Mediterranean populations of Livorno and Cartagena, and the Atlantic location of Los Gigantes, appeared clearly separated from all the other locations.

Source of variation	d.f.	SSD	% Variation	F-stat	F-value	p-value
Mitochondrial DNA						
Geographical regions Med vs Atl						
Within populations	309	105.531	55.57	Φ_{st}	0.442	0.000
Among populations	13	13.402	5.18	Φ_{sc}	0.085	0.000
Among groups	1	17.530	39.24	Φ_{ct}	0.3924	0.009
Geographical regions W Med vs E Med vs Atl						
Within populations	309	105.531	70.69	Φ_{st}	0.283	0.000
Among populations	12	4.93	1.82	Φ_{sc}	0.025	0.054
Among groups	2	15.95	27.78	Φ_{ct}	0.275	0.000
Taormina grouped with western Mediterranean						
Within populations	309	105.531	67.69	Φ_{st}	0.323	0.000
Among populations	12	4.859	0.58	Φ_{sc}	0.008	0.278
Among groups	2	24.562	31.73	Φ_{ct}	0.317	0.000
Microsatellite genotypes						
Geographical regions Med vs Atl						
Within individuals	282	688.5	80.1	Fit	0.199	
Within populations	269	934.537	16.19	Fis	0.175	0.001
Among populations	11	59.383	1.5	Fsc	0.015	0.001
Among groups	1	12.725	1.5	Fct	0.015	0.001
Geographical regions W Med vs E Med vs Atl						
Within individuals	282	689	80.6	Fit	0.194	
Within populations	269	935.729	17.1	Fis	0.175	0.001
Among populations	10	51.006	1.3	Fsc	0.013	0.001
Among groups	2	21.201	1	Fct	0.010	0.003
Taormina grouped with western Mediterranean						
Within individuals	282	689	80.5	Fit	0.195	
Within populations	269	935.724	17.1	Fis	0.175	0.001
Among populations	10	47.171	1	Fsc	0.010	0.001
Among groups	2	24.455	1.5	Fct	0.015	0.001

Table 2. Analysis of molecular variance (AMOVA) in *E. sepositus* for COI and microsatellite loci. Populations were grouped as follows: Med (Mediterranean) vs Atl (Atlantic) groups; within the Mediterranean basins with W Med (western Mediterranean) vs E Med (eastern Mediterranean); and with Taormina grouped with the western Mediterranean. For AMOVA with microsatellite loci, the Livorno and Cartagena populations were not considered.

Genetic differentiation between populations correlated with geographic distances when the whole dataset, including both the Atlantic and Mediterranean basins, was considered together (Table 3). However, the stratified isolation by distance (IBD) analysis did not reveal IBD within

the basins, indicating that the significant results were driven by regional divergence between basins [51].

The coalescence analysis using LAMARC based on COI sequences showed the largest effective population in the western Mediterranean sub-basin, considered as *theta* (θ) mean values, followed by the eastern Mediterranean and the Atlantic basin (Figure 6). The estimation of effective population size showed between 33,000 and 52,000 females in the western Mediterranean sub-basin; while fewer than 500 were estimated in the other two areas (Table 4). Demographic exponential growth was detected in the eastern Mediterranean and Atlantic populations (Supplementary Figure S5). However, the growth factor in the western Mediterranean area did not reach convergence for any of the replicates (Table 4, Supplementary Figure S5). The LAMARC results also indicated asymmetric migration between basins and sub-basins. The western Mediterranean sub-basins seemed to receive immigrants from the Atlantic and eastern Mediterranean basins; whereas migration in the opposite direction was not detected. Confidence intervals of the highest probability density (HPD) at 95% included the maximum value accepted by the program priors, indicating some limitations in the analysis. This was the case in the migration analysis from eastern to western Mediterranean, and in the demographic analyses of the eastern Mediterranean sub-basin and Atlantic basin.

Mantel tests	Standard		Stratified	
	r	p-value	r	p-value
COI	0.7552	0.0002*	0.7552	0.236
Microsatellites (all locations)	0.1202	0.1752	0.1202	0.263
Microsatellites (without LIV and CAR)	0.6179	0.0027*	0.6179	0.066

Table 3. Results of the standard and stratified Mantel tests. The coefficient of correlation (r) and p-values are presented for both types of analysis. * p-value < 0.05

	Growth	θ value	N_f
Atlantic Ocean	9,763 [3,035 − 15,000]	0.0092	194 − 304
Western Mediterranean	7,369 [200 − 14,191]	1.5740	33,274 − 52,081
Eastern Mediterranean	11,550 [5,997 − 15,000]	0.0114	241 − 377

Table 4. Demographic parameters of *E. sepositus* based on COI data. Mean population growth (Growth) and 95% HPD interval in brackets, *theta* (θ) values estimated from LAMARC, and effective population size of females (N_f) calculated from the *theta* values and substitution rates of 2.3%-3.6%.
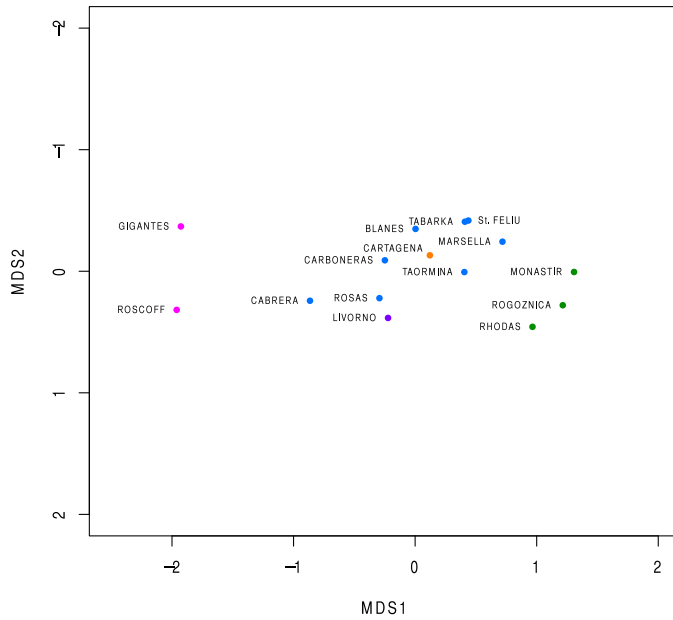
Figure 4. Multidimensional scaling (MDS) from the Jost's D pairwise matrix computed from COI sequences. Green, blue and fuchsia colours represent eastern Mediterranean, western Mediterranean and Atlantic localities respectively. Orange represents Cartagena and purple Livorno.
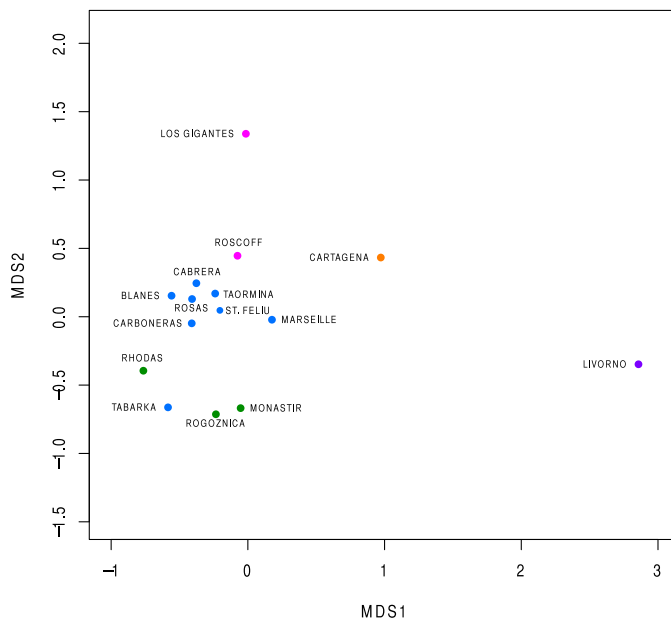

Figure 5. Multidimensional scaling (MDS) from pairwise Jost's D differences from microsatellite data. Green, blue and fuchsia colours represents Eastern Mediterranean, western Mediterranean and Atlantic localities respectively. Orange represents Cartagena and purple Livorno.

The unimodal mismatch distribution for the whole dataset revealed a peak close to the y-axis, which indicates recent expansion (Figure 7). Bayesian analysis using LAMARC with the COI data supports the expansion model in two of the three basins. Estimations of demographic expansion based on different substitution rates for starfish dated this event between 9,000 and 13,000 generations ago. Assuming one generation per year, this expansion happened between 7,000 and 11,000 years after the Last Glacial Maximum (LGM).

Other signals that could be interpreted as a result of a recent demographic expansion were also detected from microsatellite loci. The Wilcoxon test results showed a significant heterozygosis deficit related to the number of alleles in some populations; a common occurrence in populations in demographic expansion (Table 5). For the M-Ratio test, there were no values of M lower than the critical value, M_c, suggesting no population decline at any of the sites.

	Code	IAM	SMM	M	Mc
Mediterranean					
CABRERA, Balearic Islands, Spain	CAB	0.125	0.009*	0.76	0.65
CARBONERAS, Andalusia, Spain	CAR	0.037*	0.014*	0.83	0.65
CARTAGENA, Murcia, Spain	PAL	0.156	0.019*	0.73	0.60
BLANES, Catalonia, Spain	BLA	0.004*	0.002*	0.74	0.55
St. FELIU, Catalonia, Spain	PSA	0.004*	0.004*	0.84	0.66
ROSES, Catalonia, Spain	ROS	0.027*	0.004*	0.76	0.65
MARSEILLE, Provence, France	MAR	0.156	0.037*	0.74	0.65
LIVORNO, Tuscany, Italy	LIV	0.156	0.156	0.71	0.65
TABARKA, Jendouba, Tunisia	TAB	0.009*	0.006*	0.68	0.58
TAORMINA, Sicily, Italy	TAO	0.125	0.027*	0.71	0.65
MONASTIR, Monastir, Tunisia	MON	0.097	0.004*	0.79	0.64
RHODAS, Dodecanese, Greece	RHO	0.371	0.383	0.80	0.65
ROGOZNICA, Dalmatia, Croatia	CRO	0.273	0.027*	0.77	0.65
Atlantic					
LOS GIGANTES, Canary Islands, Spain	GIG	0.014*	0.004*	0.78	0.65
ROSCOFF, Brittany, France	RFF	0.006*	0.004*	0.78	0.65
ATLANTIC		0.97	0.01*	0.79	0.69
MEDITERRANEAN SEA		0.99	0.002*	0.81	0.74
WESTERN MEDITERRANEAN		0.99	0.004*	0.76	0.74
EASTERN MEDITERRANEAN		0.97	0.01*	0.82	0.70

Table 5. Demographic analysis of *E. sepositus* based on microsatellite loci. Results are based on the Infinite Allele Model (IAM) and Stepwise Mutation Model (SMM) using the Bottleneck software; and M values (M) and the critical value of M (*Mc*) from the M-ratio test.
* Significant when p < 0.05

Discussion

In this study, by combining both nuclear and mitochondrial markers, we demonstrate that the intraspecific genetic structure of *E. sepositus* is characterised by historical processes of divergence and a recent demographic expansion. This is combined with the disruptive effect of contemporary oceanographic barriers between the Atlantic and Mediterranean basins, and between the western Mediterranean and eastern Mediterranean sub-basins. This species presents low values of genetic diversity, which is important for the future conservation of their populations.

The genetic diversity in *E. sepositus* was much lower than that observed in other echinoderms living in the same distribution area with planktotrophic larvae [24,25,49,52–54]. There are no previous studies of echinoderms with lecitothrophic larvae for the same genetic markers and geographical area available for comparison, but the haplotype diversity found for COI data was comparable to some other benthic groups, such as some colonial ascidians and molluscs, with very limited dispersal potential [9,55]. Low allelic richness has been explained in other marine species as a result of high levels of inbreeding and/or population decline [56,57]. Nevertheless, for *E. sepositus*, we did not detect signs of current inbreeding for the whole distribution range, although we did for a few subpopulations, and there was no evidence of recent bottlenecks. Therefore, an inbreeding depression or recent population reduction cannot explain the low diversity observed in *E. sepositus*. However, low genetic diversity characterises coastal areas of recent re-colonisation after the LGM, dated some 20,000 years ago across the northern hemisphere, where the signal of a strong founder effect still persists when populations have not yet reached equilibrium [17,31,58,59]; this might be the case of *E. sepositus*. A dramatically reduction of *E. sepositus* populations along the European coast during the LGM might explain its low values of genetic diversity.

Due to the differences in nature and coalescence time between mitochondrial and nuclear
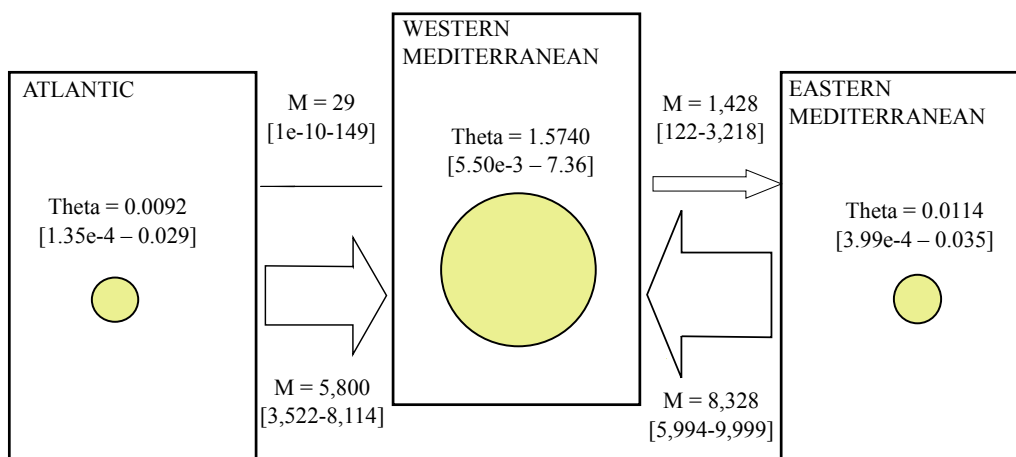

Figure 6. Values of *theta* and migration between basins and sub-basins. Yellow circles represent the value of *theta* (logarithmically transformed) per basin, and arrows represent the direction of migration. The thickness of the arrows is proportional to the migration value. Values in brackets are the 95% HPD confidence of *theta*.

markers [60], we need both types of markers to distinguish between past and contemporary processes involved in the phylogeographic pattern and genetic structure of the species. In *E. sepositus*, the COI data displayed genetic divergence in the structure of the population among the three oceanographic areas analysed: the Atlantic, western Mediterranean and eastern Mediterranean. This genetic divergence between the basins and sub-basins was also supported by results from microsatellite loci. We detected sharp phylogeographic discontinuities, according to the AMOVA and genetic pairwise comparisons between populations, with the absence of some ancestral haplotypes in the Atlantic basin and the easternmost section of the Mediterranean, and additionally the presence of private alleles within the three marine regions. These genetic divergences typically evolve in response to long-term extrinsic barriers to gene flow [61,62]. Oceanographic barriers, such as the well-known Almeria-Oran Front between the Atlantic and Mediterranean basins, and the Siculo-Tunisian Strait between the western and eastern Mediterranean, limit gene flow between different hydrographic basins in *E. sepositus*, as extensively documented for other marine benthic invertebrates [9,20,23,49,50,54,63]. However, although oceanographic breaks exist, they should be understood as transition breaks with a discontinuous barrier effect [64], and populations at the edges between biogeographical areas may be influenced by the oceanographic circulation between areas; as in the case of the populations at Taormina or Cartagena analysed in this study. The long-existing separation between Atlantic and Mediterranean haplotypes and a posterior recolonisation of Atlantic haplotypes to the Mediterranean basin would partially explain the higher haplotype diversity found in the western Mediterranean, as observed in other echinoderm species [54]. This hypothesis is supported by a clear unidirectional gene flow observed from the Atlantic to the western Mediterranean. A predominant superficial current across the Strait of Gibraltar from the Atlantic to the Mediterranean Sea [27] promotes directional gene flow by dispersion of larvae. This oceanographic circulation pattern has probably favoured past secondary contact between basins after periods of divergence during the Pleistocene glaciations, partially homogenising the genetic structure between populations [54]. Additionally, a very recent demographic expansion in *E. sepositus*, estimated between 9,000 and 13,600 generations ago, after the LGM has also marked the phylogeographic pattern in this species; a pattern observed in a number of marine invertebrates across the North Atlantic [17,20,31,65,66], even in other Echinaster species [67]. This recent demographic expansion probably favoured geographical redistribution of the most
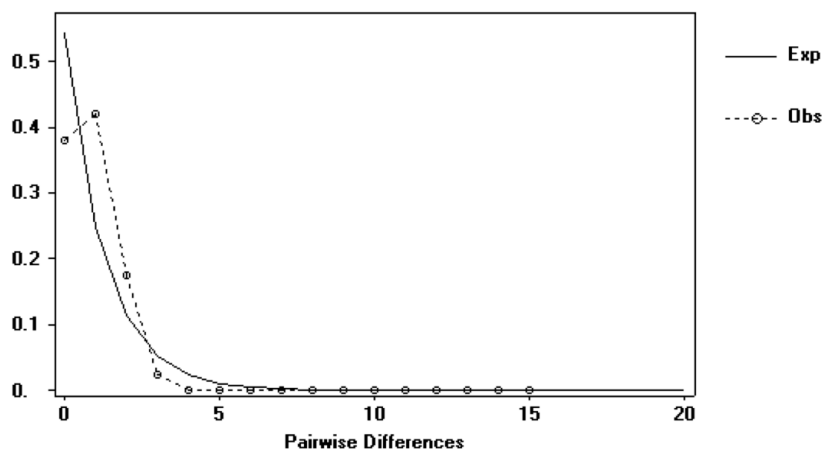

Figure 7. Mismatch distribution of *E. sepositus* for all samples. Observed (Obs) and expected (Exp) values of the distribution are represented as dashed and solid lines, respectively.
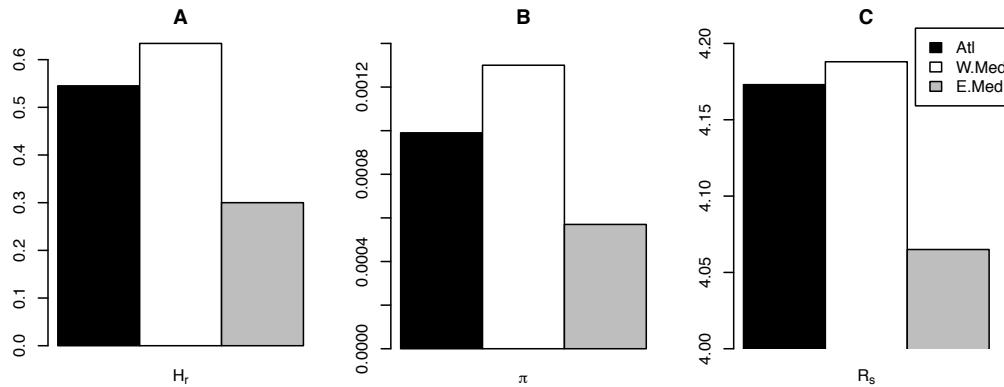
Figure 8. Bar plots representing diversity values from COI and microsatellites per basin: A) rarefied haplotype diversity, B) nucleotide diversity, and C) allelic richness. Black bars represent Atlantic populations (Atl), white bars western Mediterranean populations (W. Med), and grey bars eastern Mediterranean populations (E. Med).

frequent haplotypes, and secondary contact, as discussed above. The greater genetic diversity for both nuclear and mitochondrial markers (Figure 8), and the presence of the three ancestral haplotypes (H_1, H_2 and H_3) in the western Mediterranean basin, also points to this particular area as the central origin of the distribution of the species [59,68]. Nevertheless, other hypotheses cannot be disproved, since a higher genetic diversity in the western Mediterranean might also be a consequence of the larger effective population size, or the influence of genetic inflow and genetic admixture from the eastern Mediterranean and Atlantic sources.

However, our results based on the LAMARC Bayesian approach assumes a stable migration for a long period [69] and therefore some considerations must be taken into account. Results of the process of migration into the western Mediterranean could include an effect on the exponential expansion from this basin to the other basins. Additionally, in order to translate the N_f values into absolute population sizes, we note that N_f only counts mature females, and we have no information on the age of maturity of the species. Finally, results for the estimated number of individuals were within a range, and different markers in MCMC analysis already reported variation within a species [70]. Nevertheless, the larger population size could indicate better environmental conditions for this species along the western Mediterranean area, reinforcing the hypothesis of the origin of expansion in this basin.

The genetic structure of the populations in *E. sepositus* did not follow a pattern of IBD when the disruptive effect of marine fronts between basins was eliminated [51]. Aquarium experiments in *E. sepositus* showed that both gamete and larvae immediately sink to the bottom of the experimental tanks [71] after induced spawning, although floating eggs are also described elsewhere in *E. sepositus* [42,43]. Hence, although some experimental data and literature seem contradictory for *E. sepositus*, the release of both floating and non-floating eggs has been observed in other *Echinaster* species during the same reproductive event [72]. The existence of gamete and larvae with different behaviour and/or dispersal potential (floating vs non-floating) in *E. sepositus* might explain the low differentiation between populations due to high connectivity promoted by floating and long-dispersal gamete and larvae. Additionally, the absence of IBD could be related to the stochastic nature of larva connectivity in nearshore species due to intermittent and heterogeneous processes of marine circulation, which varies over time, habitat availability and other biological features, such as competition and predatory pressure [13]. These complex systems of physical and biological interactions can generate large variation on the recruitment process, complicating connectivity networks over time [13]. Hence, further studies of larval behaviour and the

ultrastructure in *E. sepositus* are needed to understand their relevance to the connectivity of populations and the potential evolutionary significance of divergent strategies of dispersal.

Interestingly, two Mediterranean populations, Livorno and Cartagena, displayed sharp differences in genetic structure based on microsatellites, and appeared as separated clusters. Those differences were less noticeable in mtDNA sequences, which may indicate that the divergence of these two populations is a relatively recent process. These two particular populations are located close to large marine harbours, and coastal areas affected by industry. In the case of Livorno, only 11 km from our sampling site there is a famous white beach produced by the industrial carbonate discharges of Rosignano Solvay. The load of heavy metals around the Livorno harbour has been demonstrated to have effect on the genetic diversity of other marine species [73,74]. The population at Cartagena is separated by just 15 km from an iron mine that deposited tones of sediments rich in heavy metals over several decades [75]. Although our experimental design does not allow us to assess the effects of pollutants on the genetic structure of *Echinaster*, those effects may be related in some way with the low diversity and divergent structure found at these two particular sites. The genetic structure of populations in polluted areas may be due to the existence of selective sweeps [76] or changes in the reproductive biology of the population with effects on the population structure [77]. Further studies considering both neutral and non-neutral markers such as single nucleotide polymorphisms (SNPs) could provide valuable information on potential adaptations to pollutants.

Population decline was not detected at any of the sites analysed, but the significant F_{IS} values, which translated into Hardy Weinberg disequilibrium, might be related with inbreeding in some populations. The general patterns of genetic structure in *E. sepositus*, together with the low values of genetic diversity detected and potential inbreeding, make this species highly vulnerable to overfishing and environmental perturbations at a small to medium geographical scale [66,78]. Uncontrolled and constant extraction of specimens from nature could dramatically reduce effective population size, and increase the risk of losing genetic diversity by genetic drift, which is more marked in small populations. This effect might be particularly important in *E. sepositus* due to the stochasticity of the recruitment process that does not assure gene flow, and recruitment of larvae from nearby sites, limiting the recovery process of populations.

The genetic data presented here for *E. sepositus* set the base for further studies to design policies of management for a sustainable exploitation of this iconic starfish. Any conservation strategy for this species should consider how genetic diversity is geographically distributed and the genetic divergence among the Atlantic, western Mediterranean and eastern Mediterranean basins.

Methods

Sampling

Tube feet samples from 332 specimens of *E. sepositus* were collected at 15 different locations: 9 in the western Mediterranean (Cabrera, Carboneras, Cartagena, Blanes, St. Feliu de Guíxols, Roses, Marseille, Livorno, Tabarka), four in the eastern Mediterranean (Taormina, Monastir, Rhodas, Rogoznica) and two in the Atlantic Ocean (Roscoff, Los Gigantes) (Figure 2). The specimens were collected by scuba diving at depths of between five and 30 meters in most locations, and by trawling, at 100 metres, at the Mediterranean location of Blanes. Between 9 and 30 individuals per population were processed, depending on the abundance of the species at each location (See Table 1). We used a minimally invasive system for tissue collection, and animals were rapidly released back to the same place where they were collected. The tube feet were preserved in absolute ethanol and kept at -20ºC until processing.

Genotyping and Sequencing

DNA was extracted from all tube feet samples using a REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, www.sigmaaldrich.com), following the protocol proposed by the manufacturer.

Microsatellites genotyping: We used eight specific microsatellite loci (mES_2, mES_4, mES_23, mES_24, mES_25, mES_29, mES_30, mES_38) already optimised for this species [79]. All the microsatellites were separately amplified by PCR with the exception of mES_4 and mES_30, which were amplified together in multiplex. Forward primers were labelled with a fluorescent dye [79]. All PCR amplifications were performed in 10 μl total volume, using 5 μl of the REDEXtract-N-Amp Tissue PCR Kit (Sigma-Aldrich), 5 pmol of each primer, 3.7 μl of ultrapure water, and 0.5 μl of DNA extraction. Thermal cycling was performed in a Bio-Rad S1000 dual thermal cycler (BioRad, www.bio-rad.com) with a first step of 95°C for 60 sec, followed by 35 cycles of 95°C for 20 sec, 50°C for 20 sec and 72°C for 2 minutes, and a final extension of 5 minutes. Amplification products were purified and analysed on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, www.appliedbiosystems.com) at the Scientific-Technical Services of the University of Barcelona. Allele length was estimated relative to the internal GENESCAN 400HD ROX size standard (Applied Biosystems) using Peak-Scanner software. Alleles were scored using the MsatAllel 1.0 package [80] for R 3.1.

Sequencing of mitochondrial DNA: A fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) was amplified with the primers F210-CO1 (5'-GTAATGCCAATTATGATTGG-3') and COA (5'-AGTATAAGCGTCTGGGTAGTC-3') 81. PCR reactions were performed in a total volume of 20 μl, using 8 μl of the REDEXtract-N-Amp Tissue PCR Kit (Sigma-Aldrich), 10 pmol of each primer, 8.4 μl of ultrapure water, and 1 μl of DNA extraction. Thermal cycling was performed in a dual thermal cycler as explained before with 96°C for 90 sec; followed by 35 cycles of 96°C for 20 sec, 48°C for 80 sec and 72°C for 90 sec, and a final extension of 5 minutes. Amplifications were visualised in agarose gels, and purification and sequencing of the PCR products were performed in a 3730xl DNA Sequencer at the Macrogen services (www.macrogen.com) with the same primers as were used for amplification of the fragment. Sequences were edited and trimmed with MEGA v. 5.2 software [82] and aligned with CLUSTALW. Sequences with singletons were re-amplified and sequenced again. The sequences obtained have been deposited in GenBank (accession numbers pending, www.genbank.com).

Genetic diversity, structure and demography

The genetic descriptors: haplotype number (Nh), nucleotide diversity (π) and haplotype diversity of COI, were calculated using the software DNASP v. 5.0 [83]. Haplotype richness (Hr) was calculated after rarefaction to size 8, which was the size of the smallest population analysed, with CONTRIB v. 1.02 [84] in order to compare populations with different sample sizes. A network of COI sequences was reconstructed with Network v. 4.6 (Fluxus Technology, www.fluxus-engineering.com/sharenet.htm) using an infinite site model. Haplotype frequencies in each population were plotted in geographic coordinates using the packages Maps and Mapplots in R [85,86].

Allele number per microsatellite locus, allele richness, observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated using hierfstat package in R [87]. The fixation index F_{IS} was also calculated for each population, and confidence intervals at the 0.05 level were tested after 100 bootstrapping resampling operations over loci with the same package [83]. The Hardy-Weinberg equilibrium per population and its significance were tested after 1,001 permutations in GenoDive [88].

In order to infer major homogeneous genetic units (K) across the distribution range of the species, the software STRUCTURE v. 2.3 [89] was used for microsatellite data, and for the combination of microsatellite data and COI sequences. This software detects major genetic groupings based on a Bayesian clustering approach. The software was initially run with the whole dataset, a non-admixture model, and with a K value ranging from 1 to 15, using the sampling site as a prior, due the low F_{ST} values obtained for this species (see Results section)[90]. However, because the admixture model gives more flexibility to the analysis, it was repeated with an admixture model using the location as prior [91]. For sequences of COI, the second allele was treated as missing data, following the software manual for haploid loci. Twenty independent replicates of 10,000 MCMC each were run, and a 10,000 burn-in period applied following the optimised parameters for STRUCTURE software [92]. The most likely value of 'real' clusters was identified by comparing the rate of change in the likelihood of K (L(K)). The optimal K value per run was determined and graphically visualised using the ad hoc statistic ΔK [92] with the software Structure Harvester [93] following the Evanno method [92]. The results of the 20 runs from all K values were summarised and graphically illustrated using the pipeline of CLUMPAK [94]. In order to optimise the comparisons between the different STRUCTURE runs, we represented the results from the same number of K for each, using the best ΔK from the microsatellite analysis (Supplementary Figure S1).

Hierarchical analysis of the molecular variance (AMOVA) was performed with Arlequin v. 3.5 [95] in order to detect populations grouping for COI and microsatellite loci. Populations were *a priori* grouped within basins and sub-basins, which are considered different ecoregions [96], to test the potential effect of the Strait of Gibraltar and the Siculo-Tunisian Strait. AMOVA analysis was performed separately for COI and microsatellite loci, due to the different signal provided by the markers (see Results section). Populations were grouped as follows: Atlantic (Los Gigantes, Roscoff), eastern Mediterranean (Rogoznica, Rhodes, Monastir and Taormina) and western Mediterranean (Tabarka, Livorno, Marseille, Blanes, Roses, St. Feliu, Cartagena, Cabrera and Carboneras). Additionally, populations on the limit of distribution between the eastern and western Mediterranean (Taormina, Monastir and Tabarka) were interchanged between groups for AMOVA analysis, due to the difficulties in assigning these populations to a particular area. AMOVA analysis based on microsatellites was run both including and excluding the populations at Livorno and Cartagena, because of the large genetic differentiation of these two populations due to unknown processes (see a full explanation in Results and Discussion).

Pairwise differences in genetic structure between locations were separately explored for COI and microsatellites by two different approaches; the Φ_{ST} for COI and F_{ST} for microsatellites, and the Jost's D coefficients of dissimilarity [97] for both COI and microsatellite loci. The Φ_{ST} and F_{ST} values were computed in Arlequin v. 3.5 [95], and the Jost's D values in SPADE [98] for the COI, and in DEMEtics R package [99] for microsatellites. In all cases, p-values or confidence intervals were calculated after 1,000 permutations, and p-values were adjusted for multiple comparisons using Benjamini-Hockhberg corrections [100]. In order to explore the potential different signal of divergence obtained from COI and microsatellites, we calculated the Pearson correlation between the Φ_{ST} and F_{ST} values. If mitochondrial and nuclear markers show similar trends in genetic divergence between populations, then the Φ_{ST} (from COI) and F_{ST} (from microsatellites) should be highly correlated. Additionally, the correlations between the Φ_{ST} and F_{ST} with the Jost's D for COI and microsatellites, respectively, were also calculated to explore whether the two types of analysis yield similar results. Dissimilarity genetic matrixes based on Jost's D coefficients were graphically represented using a non metric MDS for both kinds of markers with the Vegan R package [101].

Genetic differentiation due to geographical distance (IBD) was calculated using the Mantel test procedure in the Vegan package [101]. We separately correlated the Φ_{ST} and F_{ST} dissimilarity matrixes for COI and microsatellites, respectively, and a matrix of geographical distances.

Geographical distances were considered as the shortest linear distance in kilometres by sea. The IBD analysis was performed with all populations with a stratification method to consider the divergence associated with the "basin" factor: Mediterranean and Atlantic basins [51]. Analysis was performed including and excluding Livorno and Cartagena from the microsatellite dataset. The significance of the tests was evaluated by 1,000 permutations of individuals between populations and basins.

Migration between geographical areas, effective population size (expressed as *theta*), and demographic events, such as population growth or decline, were estimated with a Bayesian approach as implemented in LAMARC v. 2.1.9 [102] from COI data. We ran this analysis for the Atlantic basin and the two Mediterranean sub-basins separately. The best evolutionary method for our dataset was inferred with jModelTest 2 [103] and then implemented in LAMARC. An initial run was performed with default parameters as priors, followed by a series of runs adjusting these priors in order to find the optimal parameters for our data. Once optimal priors were obtained from the initial runs, they were implemented in a final run. The final run was based on three different 1,000,000 MCMC replicates each, and a burn-in period of 100,000. For each of the three replicates, four simultaneous heating searches were performed with relative temperatures of 1, 3, 7 and 11. Priors for the final analysis are presented in Supplementary Table S3. The Effective Sample Size (ESS) was visualised in Tracer v. 1.6 [104] to confirm a large enough number of independent simulations (over 250), and the Gelman and Rubin diagnosis with the *coda* R package [105] was applied to test convergence of multiple MCMC runs [106] (Supplementary Table S4). Migration rates (Mt) were expressed as the number of migrants per generation $Mt = m/\mu$, where m is the migration rate per generation, μ the substitution rate, and θ the *theta* value. The *theta* value is defined by $\theta = 2N_f\mu$, where N_f is the effective population size for mitochondrial markers; we calculated N_f using a substitution rate of between 2.3% and 3.6%/million generations applied in other asteroids [17,32,33,48,107]. Hence, *theta* values (θ) and growth rates (G) are defined from the evolution of different *theta* values: $\theta_t = \theta_1^{-Gt}$. The high computational requirements of LAMARC in both time and memory, which cannot be parallelised in a super-computer cluster, did not allow us to analyse microsatellites with this software; thus, other methods had to be applied for microsatellite loci.

Demographic changes based on COI sequences were also inferred with a mismatch distribution [108] in DNAsp. Sudden expansions are typically represented by a unimodal distribution in the mismatch distribution; while multimodal distributions represent populations at equilibrium. We used the formula $\tau = 2ut$ [108] to approximate the time in generations (*t*) of the demographic changes from the coalescent methods. We used the same lineage substitution rate as before, between 2.3% and 3.6%/million generations; due to a lack of information on the generation time of *E. sepositus*, we assumed 1 year per generation, as applied to different echinoderms[17,32,33,48,107].

For microsatellite loci, recent population size changes were explored using two different approaches: the software Bottleneck v. 1.2 [109], and critical_M for the M-ratio test [110]. We ran Bottleneck in order to detect excess or deficiency of heterozygosity in our populations using an infinite allele model (IAM), and a stepwise mutation model (SMM). The significance of the tests was determined using the Wilcoxon signed rank test after 1,000 replicates. The heterozygosity excess method exploits the fact that allele diversity reduces faster than heterozygosity during a bottleneck, because rare alleles are lost rapidly and have little effect on heterozygosity, thus producing a transient excess in heterozygosity relative to that expected in a population of constant size [109,111]. Nevertheless, Bottleneck is more successful at detecting population expansions than population declines [112]. The M-ratio test, which is the ratio between the number of alleles and the range in allele size, is based on the fact that during population decline, the microsatellite allele size range decreases more slowly than the number of alleles because only the less frequent alleles are lost due to genetic drift. Hence the M-ratio test calculates the M

value, a ratio between the number of alleles and the range in allele size, and its significance, the critical M value (M_c), was calculated after 10,000 simulations. We then expect population decline when M_c is greater than the mean M value [110].

Acknowledgments

Contributions

AGC, CP and RPP designed the study and experiments. AGC, YBK, CP and RPP participated in sample collection at the different locations. AGC and YBK carried out laboratory analysis. AGC performed bioinformatic analysis; while CP and RPP reviewed that analysis. AGC wrote the first draft of the manuscript, while RPP and CP made major contributions to the writing. All the authors have reviewed the final version of the manuscript.

Competing Financial interests

The authors declare no competing financial interests.

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PARTE III
Coscinasterias tenuispina
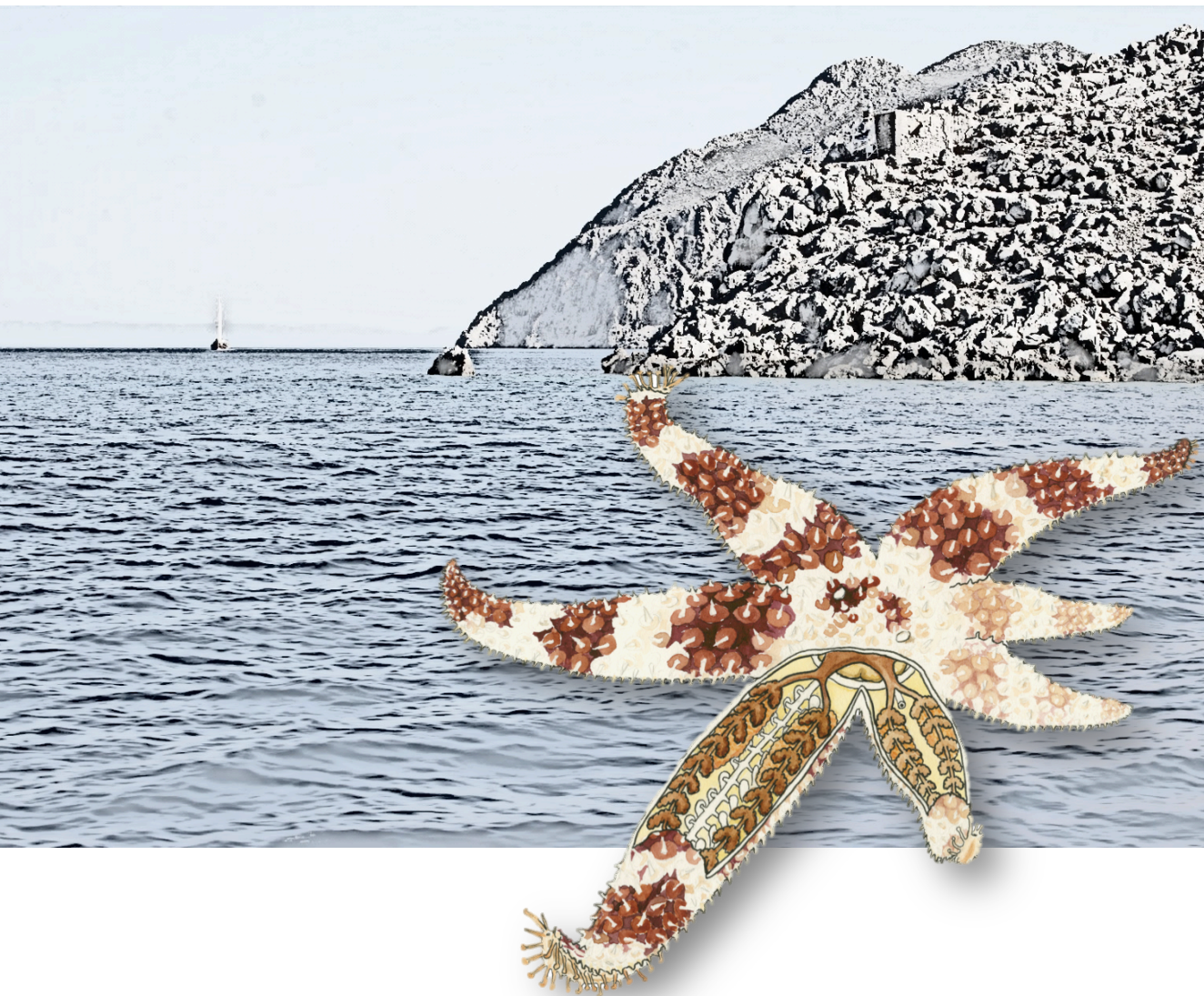
Capítulo 3

The effect of asexual reproduction on intraspecific genetic structure and divergence: the case of an amphi-Atlantic starfish

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Abstract

Intraspecific genetic diversity and divergence have a large influence on the adaption and evolutionary potential of species. Different reproductive strategies influence this intraspecific structure considerably. The widely distributed starfish, *Coscinasterias tenuispina,* combines sexual reproduction with asexual reproduction via fission. Here we analyse the phylogeography of this starfish to reveal historical and contemporary processes driving its intraspecific genetic divergence. We further consider whether asexual reproduction is the most important method of propagation throughout the distribution limits of this species. Our study included 326 individuals from 16 populations, covering most of the species' distribution range. A total of 12 nuclear microsatellite loci and sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene were analysed. COI and microsatellites were clustered in two isolated lineages: one found along the south-western Atlantic and the other along the north-eastern Atlantic and Mediterranean Sea. This suggests the existence of two different evolutionary units. The south-western Atlantic lineage probably originated from the eastern Atlantic and the mid-Atlantic barrier. Marine barriers would be responsible for population clustering: the Cabo Frio upwelling in the south-western Atlantic; the Siculo Tunisian strait that divides the two Mediterranean basins; and the Almeria-Oran Front that limits the entrance of migrants from the Atlantic to the Mediterranean.

The presence of identical genotypes and low values of genetic diversity were detected at all the locations with the most extreme monoclonal populations at the distribution limits of the species, where annual mean temperatures and minimum values were lower.

Keywords: phylogeography, genetic diversity, distribution limits, clonality, marine barriers, starfish.

Resumen

La diversidad genética y la divergencia intraespecífica tienen una gran influencia en la adaptación y el potencial evolutivo de las especies. Diferentes estrategias reproductivas influyen considerablemente en esta estructura. La estrella de mar *Coscinasterias tenuispina*, combina la reproducción sexual con la reproducción asexual a través de procesos de fisión. En este trabajo se analiza la filogeografía de esta estrella de mar para revelar los procesos históricos y contemporáneos de su divergencia genética intraespecífica. Además, se explora si la reproducción asexual es la predominante dentro de los límites de distribución de esta especie. Se han trabajado con un total de 326 individuos de 16 poblaciones, que cubren la mayor parte del área de distribución de la especie. Se analizaron un total de 12 loci microsatélite y secuencias del gen mitocondrial citocromo c oxidasa subunidad I (COI). El COI y los microsatélites han mostrado dos linajes aislados: uno a lo largo del sudoeste del Atlántico y el otro a lo largo del Atlántico noreste y del Mar Mediterráneo. Esto sugiere la existencia de dos unidades evolutivas diferentes. El linaje del Atlántico sudoeste probablemente se originó a partir del Atlántico oriental y la barrera del Atlántico medio. Las barreras marinas serían las responsables de los grupos de poblaciones: el afloramiento de Cabo Frío en el sudoeste del Atlántico, el estrecho Siculo-Tunecino que divide las dos cuencas del Mediterráneo, y el Frente Almería-Orán, que limita la migración desde el Atlántico al Mediterráneo.

Se detectó la presencia de genotipos idénticos y de valores bajos de diversidad genética en todos las zonas muestreadas, con poblaciones monoclonales en los límites de distribución de la especie, donde las temperaturas medias y los valores mínimos anuales son más bajos.

Palabras clave: Filogeografia, diversidad genética, limites de distribución, barreras marinas, clonalidad, estrella de mar.

Introduction

Sexual reproduction, an ancient capacity in multicellular eukaryotes, is a complex process that involves a large number of different gene families (Cavalier-Smith 2002; Otto & Lenormand 2002). Additionally, independent evolutionary strategies of asexual or clonal reproduction, which are in fact synonymous (de Meeûs *et al.* 2007), have also appeared and spread within the metazoan tree (Schwander & Crespi 2009). Whereas some asexual propagation is related to colonial growth (e.g. in corals, bryozoans, and colonial ascidians), in other animal groups, clonal reproduction produces new generations that are genetically identical to the corresponding parental generation, with the occasional appearance of somatic mutations (de Meeûs *et al.* 2007). Obligate asexual reproduction in metazoans is notably rare and in general, animal taxa display complex life cycles combining sexual and asexual phases (Otto & Lenormand 2002; de Meeûs *et al.* 2007).

Theoretical works have evaluated many advantages and disadvantages of "sex" (Ayala 1998; Otto & Lenormand 2002; de Meeûs *et al.* 2007; Otto 2008, 2009; Barton 2009), and asexual reproduction certainly provides some important advantages over sexual propagation. Clonality offers exemption from the "two-fold cost of sex" that arises from the need for two different genders for sexual reproduction (Maynard Smith 1976; Hurst & Peck 1996). Moreover, that well-adapted individuals are expected to double their genetic input into the next generation because asexual reproduction maintains the co-adapted complexes of genes together (Becks & Agrawal 2012). Ecologically, one of the major advantages of clonal reproduction is the potential for colonising new areas: rapid spreading and establishing of new populations (Achituv & Sher 1991). In this way populations can be founded from just one or very few well-adapted individuals (Mladenov & Emson 1984; Mergeay *et al.* 2006; Barbuti *et al.* 2012; Pérez-Portela *et al.* 2012; Kronauer *et al.* 2012), even when unfavourable biotic or abiotic conditions prevent the complete sexual cycle of the species (Honnay & Bossuyt 2005). Thus, it has been observed that clonal reproduction is more frequent at the distribution limits of species (Silvertown 2008), where physical and biological conditions are harsher, and when the specific limits for survival and sexual reproductive success are reached (Mladenov 1996; Silvertown 2008). Nevertheless, asexual reproduction misses out on the advantages of sexual reproduction. The lack of genetic diversity in the next generations and the absence of recombination promote the accumulation of deleterious mutations in the genome in an irreversible way; the so-called Müllers ratchet principle (Muller 1964; Kondrashov 1994; Hurst & Peck 1996). Additionally, the popular hypothesis of the Red Queen postulates that organisms must constantly adapt and evolve with a new genetic pool provided by sexual reproduction on which natural selection acts, to face abiotic changes and co-evolve with parasites, hosts and competitors (Bell 1982; Bell & Smith 1987; Hamilton 1990; Judson & Normark 1996). Thus, some authors have recently highlighted that sexual organisms may have major advantages for long-term evolutionary persistence (Agrawal 2006; Becks & Agrawal 2012); whereas asexual organisms experience higher short-term success, because the lack of genetic diversity may compromise the long-term survival of their populations (Barton 2009).

The particular life cycle of species and the prevalence of one type of reproduction over the other (sexual versus asexual) have a great influence on its demography, phylogeography and genetic diversity (Kyle & Boulding 2000; Sherman *et al.* 2008; Uthicke *et al.* 2009; Fernández *et al.* 2014). As the rate of clonation affects the genetic structure of populations, it also has consequences on their evolutionary potential and their capacity to respond to environmental shifts (de Meeûs *et al.* 2007). However, the genetic consequences of asexuality vary depending on the complexity of the life cycle of the species. Inferring the genetic structure of populations is a good indicator of their biology. The existence of repeated identical multilocus genotypes (MLGs) is a recognisable sign of clonal reproduction. However, other genetic signs can be assessed when clonal

reproduction is prevalent in populations. Clonality tends to modify the expected distribution of genotypes by producing an excess of heterozygotes, which drives populations to a deviation from the Hardy-Weinberg equilibrium (Balloux *et al.* 2003; de Meeûs *et al.* 2007). The proportion of clonal reproduction also has a strong effect on the differentiation of populations and effective population size (Balloux *et al.* 2003). When reproduction tends toward strict asexuality, population dissimilarity is drastically reduced; while the effective population size (a parameter summarising the amount of genetic drift) approaches infinity because genetic diversity cannot be lost within clonal lineages. Under strict clonality, there is also more statistical linkage disequilibrium because loci within the whole genome are linked (Balloux *et al.* 2003).

It is common for marine invertebrates to display complex biological cycles that combine sexual and asexual phases (Emson & Wilkie 1980). This potentially allows them to maximise their survival and expansion because they receive benefits from both mechanisms of reproduction. Within the phylum Echinodermata, a marine group characterised by its high regenerative capacity (Carnevali 2006), there are species with the capacity for asexual division at both the adult and larval stages (Emson & Wilkie 1980; Bosch *et al.* 1989; Lawrence & Herrera 2000); although the number of species that reproduce asexually during adulthood is small (Emson & Wilkie 1980). This capacity was first described for echinoderms in the starfish *Coscinasterias tenuispina* (Lamarck 1816) by Streenstup (1856; *apud* Emson & Wilkie 1980). Clonality has been reported in only three classes of Echinodermata: Holothuriodea, and more frequently in Ophiuroidea and Asteroidea (Emson & Wilkie 1980). In echinoderms, rates of fission are modulated by both biological and environmental conditions, depending on the species (Crump & Barker 1985; Uthicke *et al.* 1999; Sköld *et al.* 2002; Rubilar *et al.* 2005; Sterling & Shuster 2011).

The four species of the starfish genus *Coscinasterias* can reproduce both sexually and asexually by fission (Emson & Wilkie 1980). In sexual reproduction, *Coscinasterias* species release a planktonic larva that lives in the water column for several weeks (Barker 1978; Shibata *et al.* 2011). In contrast, asexual reproduction, which is very common in the genus, seems to produce a strong sex bias in the populations which can even be composed of only one gender, as observed in *C. muricata* in New Zealand (Crump & Barker 1985; Sköld *et al.* 2002), *C. acutispina* in Japan (Seto *et al.* 2000) and some populations of *C. tenuispina* in Brazil (Alves *et al.* 2002). Some authors have even suggested that species of the genus have populations strictly maintained by asexual reproduction (Barker 2013). This hypothesis is at least partially supported by some genetic studies that detected only a few genotypes in some populations (Sköld *et al.* 2003; Ventura *et al.* 2004; Perrin *et al.* 2004; Haramoto *et al.* 2006; Pazoto *et al.* 2010). However, it worth mentioning that some of those studies used low-resolution genetic markers that might underestimate the real number of identical genotypes (clones), thereby masking the standing genetic diversity.

In this study, we focus on *C. tenuispina* (Lamarck, 1816); an amphi-Atlantic species found around the Macaronesian Islands and Iberian Peninsula, throughout the whole of the Mediterranean Sea, off Bermuda, in the Gulf of Mexico and south of Brazil (Hansson 2001), although it is apparently absent from equatorial areas. The combination of a larva with a large dispersal potential and clonal reproduction provides *C. tenuispina* with considerable biological plasticity, and the potential to be an efficient and rapid coloniser (Mladenov & Emson 1984). Some authors originally suggested human translocation as the most likely cause of the broad distribution of *C. tenuispina* (Fisher 1928; Clark 1946; Hyman 1955), although that hypothesis was later rejected by Waters & Roy (2003) based on molecular evidence. Due to its wide and unusual distribution, *C. tenuispina* is currently exposed to a large variation of environmental conditions, biogeographic breaks and oceanographic barriers. Barriers such as the mid-Atlantic break (Luiz *et al.* 2012; Lessios *et al.* 2012), and the Almeria-Oran Front, which divides the Atlantic and the Mediterranean basins, have an important effect on the connectivity of populations of other echinoderms (Baus *et al.* 2005; Calderón *et al.* 2008; Pérez-Portela *et al.*

2010 for Atlantic-Mediterranean echinoderms; Wangensteen *et al.* 2012 for an amphi-Atlantic echinoderm). The scarce phylogenetic information available on *C. tenuispina*, based on only a few sequences of mitochondrial DNA fragments, suggested the existence of three major lineages, separating Brazil, Bermuda and other North Atlantic populations (Waters & Roy 2013); but the impact of clonal reproduction on the intraspecific genetic diversity was ignored.

One of the critical features in the resilience and long-term persistence of species is the standing genetic variation that allows them enough plasticity to adapt to environmental changes (Hoffmann & Sgro 2011). Nevertheless, Garcia-Cisneros *et al.* (2015) demonstrated how *C. tenuispina* has adaptive mechanisms to circumvent some negative effects of asexual reproduction and the low genotype diversity in its populations. Those authors pointed out the existence of systems for telomere elongation during fission; a mechanism related to the absence of senescence and genetic defects during prolonged periods of asexuality (Garcia-Cisneros *et al.* 2015). Those findings opened up important questions regarding the molecular mechanisms underlying the plasticity and colonisation successes of asexual species.

Despite being a very common and biologically interesting species, little is known of the reproduction systems across the distribution range of *C. tenuispina* or the effect of major biogeographical breaks and clonality on its intraspecific genetic structure. All of these are fundamental features when it comes to understanding the evolutionary potential of the species and the ongoing genetic processes at work. To determine levels of genetic variation, and how that variation is partitioned within and between populations, here we provide genetic data with the following main objectives.

(i) To explore past and contemporary factors promoting evolutionary divergence and diversity. Since larval stages are widely recognised as an important vector for natural dispersal of benthic marine invertebrates, levels of genetic connectivity may be greatly affected by the absence of sexual reproduction in some populations, as well as the different effects of genetic drift on the standing genetic diversity. To date, studies of echinoderms across the eastern and western Atlantic, and the Mediterranean Sea, have mostly been based on strictly sexual species; little is known of the potential effect of oceanographic barriers on non-colonial organisms with complex life cycles.

(ii) To infer the different prevalence of sexual and asexual reproduction across the distribution range of the species and to explore whether clonality is the most important system of reproduction at the distribution limits of the species, where environmental conditions may be suboptimal and make completing the sexual cycle difficult.

To achieve these objectives we combined databases of mitochondrial sequences and nuclear markers to obtain accurate information on the most important factors driving the intraspecific divergence of this non-colonial species.

Material and Methods

Sampling sites

A total of 326 individuals of *C. tenuispina* were sampled from 16 locations (see details in Figure 1). The sampling sites were located across the whole distribution range of the species as follows: six Mediterranean locations (eastern and western Mediterranean), one from the Cantabrian Sea, four from the Canary Islands, one from the Gulf of Mexico (Florida), and four from Brazil. This sampling scheme includes distinct geographical areas with divergent oceanographic conditions, including the Mediterranean Sea, north-eastern Atlantic, and southern and north-western Atlantic (see details in Table 1 and Figure 1). Most of the samples were collected at a depth of

between 0 and 5 metres by snorkelling, and only individuals from Tenerife (Canary Islands, north-eastern Atlantic) and Greece (eastern Mediterranean) were collected by scuba diving; between 10 and 25 m deep.

Tube feet were removed with forceps from each individual collected in the field and the starfish were immediately returned to the sea. The tube feet were then preserved in absolute ethanol, and stored at -20ºC once in the laboratory.

Genotyping and sequencing

Total DNA of the preserved samples was extracted using the REDExtract-N-Amp™ Tissue PCR kits, and following the manufacturer's protocol (Sigma-Aldrich, www.sigmaaldrich.com).

DNA sequencing: The mitochondrial gene cytochrome oxidase *c* subunit I (COI) was amplified with the primers F210-COI (5'-GTAATGCCAATTATGATTGG-3') and CoA-R (5'-AGTATAAGCGTCTGGGTAGTC-3') (Palumbi *et al.* 1991). PCR was carried out in a final volume of 20 µl of PCR reaction, including 8 µl of REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich), 10 pmol of each primer, 8 µl ultrapure water, and 1 µl of DNA template, and following the standard protocol described by the manufacturer. PCR was performed in a Bio-Rad S1000 dual thermal cycler (BioRad, www.bio-rad.com). Purification of the PCR products, and sequencing of the fragments with the same primers were performed at the Macrogen services (www.macrogen.com).

Additionally, DNA from ten individuals collected from Llançà that presented heteroplasmy for the COI (see Figure 2 and Results section) was re-extracted, re-amplified and re-sequenced in order to double-check for potential contamination. Additionally, to rule out heteroplasmy of the COI due to the presence of a pseudogene in the nuclear DNA, the putative mitochondrial control region, which is described as a more variable fragment than the COI in starfish (Waters & Roy 2004), was also amplified and sequenced for those 10 individuals, with the following

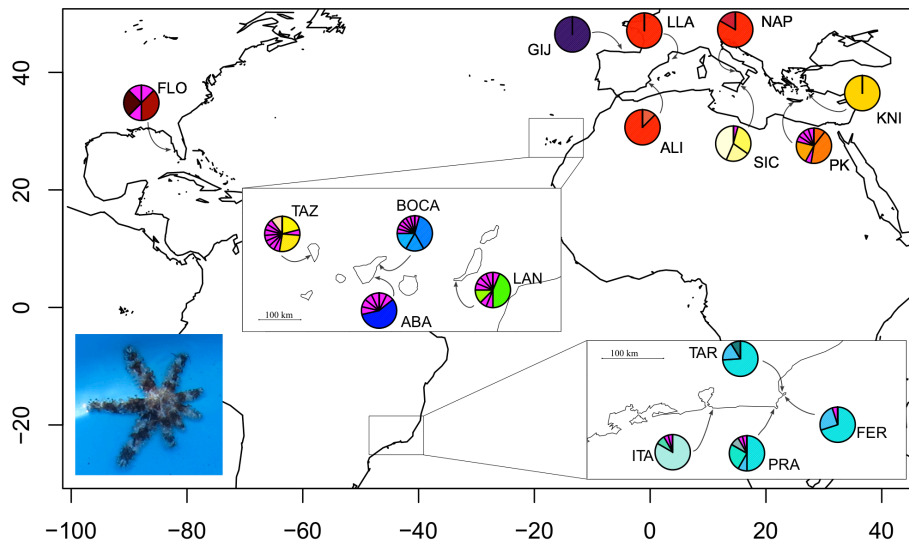**Figure 1.** Map of the *C. tenuispina* sampling locations. Frequency of MLGs based on microsatellite data is represented as pie charts per location. Each different colour represents a different clonal MLG, except violet which represents single MLGs. Bottom left: *C. tenuispina*.
See Table 1 for full names of locations. Note that pie chart size is the same for all locations and does not represent the number of individuals.

primers: E12Sa (5' ACACATCGCCCGTCACTCTC 3') and E16Sb (5' GACGAGAAGACCCTATCGAGC 3') (Waters & Roy 2004), and the same protocol used for the COI. If heteroplasmy appeared for two mitochondrial fragments, the presence of a COI pseudogene in the nuclear DNA could be ruled out.

DNA sequences were edited with MEGA v 5.2 (Tamura *et al.* 2011), and aligned with CLUSTALW implemented in the same software. All the different haplotypes obtained in this study were deposited in GenBank (accession numbers "*pending*" for manuscript acceptance).

Microsatellite genotyping: Twelve polymorphic microsatellites previously reported for the species (m.ten1, m.ten6, m.ten13, m.ten14, m.ten19, m.ten25, m.ten24, m.ten27, m.ten30, m.ten31, m.ten32 and m.ten40, in Garcia-Cisneros *et al.* 2013) were amplified. The forward primers in that work were labelled with a fluorescent dye and microsatellites amplified in multiplex, with two microsatellites per reaction, with the exception of loci m.ten13 and m.ten27 which were individually amplified. PCR was carried out in 10 μl of final volume, including 5 μl of REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich), 3 pmol of each primer, 3 μl of ultrapure water and 1 μl of DNA template. The PCR included an initial step of 96ºC for 60 sec, followed by 35 cycles of 95ºC for 20 sec, 49ºC for 20 sec and 72ºC for 80 sec, and a final extension of 72ºC for 5 minutes in a Bio-Rad S1000 dual thermal cycler. The amplification products were analysed on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems) at the Scientific-Technical Services of the University of Barcelona. Allele length was estimated relative to the internal GENESCAN 400HD ROX size standard (Applied Biosystems) using the software Peak-Scanner. Allele scoring was graphically performed using the R package MsatAllele (Alberto 2009).

Genetic diversity and clonality

COI: Haplotype richness (Hr) was calculated using Contrib (Petit *et al.* 1998) with a rarefaction size of 12, smaller than the smallest population with at least two haplotypes, to make comparison among populations with different sampling sizes possible. Nucleotide diversity (Π) was calculated with the DNAsp v 5 software (Librado & Rozas 2009) per location and geographical area. Sequences were also collapsed into haplotypes using that same software.

Microsatellites: For general characterisation of the populations, all individuals were used to calculate the observed and expected heterozygosity (H_o and H_e, respectively), allelic richness (Ar) by rarefacting to the smallest sample size (n = 7), number of alleles, fixation index F_{IS} and Hardy-Weingberg equilibrium (HWE). These genetic descriptors were estimated using GenoDive (Meirmans & Van Tienderen 2004) per location and geographical area.

Measurements of genetic diversity and clonal richness from microsatellite loci were also used to infer the prevalence of asexual reproduction in *C. tenuispina*. Because previous studies in other clonal species used different genetic statistics, here we summarised several descriptors for comparison with those studies (Arnaud-Haond *et al.* 2007). The descriptors of genotype number and diversity were: (i) number of MLGs or multilocus lineages (MLLs), (ii) clonal richness (P_d), and (iii) corrected clonal richness (R); this last factor depends on the sample size. We also calculated clonal heterogeneity and evenness: (i) Nei's (1987) genetic diversity (corrected for sampling size) (div) similar to the well-known Simpson's index in ecology; (ii) the Shannon-Wienner index (corrected for sampling size) (Chao & Shen 2003) (shc); and (iii) the clonal evenness index (eve), which is an indicator of how equally each genotype is represented.

The different MLGs were determined using a distance matrix based on an infinite allele model (IAM) for the whole dataset, location and geographical area. Missing data were not considered when performing the distance matrix and therefore only the available scores were used for identification of MLGs. Additionally, MLGsim v 2.0 (Stenberg *et al.* 2003) was used to calculate

the probability of finding the same MLG due to a sexual event (P_{sex}), based on the observed allele frequencies, and the sample size of the dataset. We ran 1,000 simulations with random mating to obtain the significance values of P_{sex}. For the significance values of P_{sex}, GenoDive found a total of 69 different MLGs (see Results section), whereas MLGsim found 77 MLGs because missing data were considered as different alleles.

MLLs, defined as individuals with different MLGs derived from the same sexual event (Arnaud-Haond *et al.* 2007), were detected using the same method as above for MLGs, but limiting the threshold of allele differences to a maximum of 1 per individual. A threshold of only 1 mutation was used because only a few alleles per microsatellite locus appeared per geographical area, and a higher threshold would have reduced the probability of discriminating between two individuals stemming from distinct reproductive events.

The prevalence of fission over sexual reproduction in echinoderms is modulated by environmental factors (Alves *et al.* 2002; Rubilar *et al.* 2005; Haramoto *et al.* 2007); moreover, seawater temperature and salinity are important factors for larval development in temperate species. Therefore, to infer the potential relationship between these oceanographic variables and clonality, pairwise correlations between environmental variables and different genetic descriptors that assess genetic diversity and clonality (as described above) were performed (see details in Supplementary Table S1). The environmental variables considered were: minimum, maximum, mean, and range (difference between the maximum and minimum) of seawater temperate during the year; and the mean salinity value for each site. These variables were obtained from the Bio-ORACLE dataset (Tyberghein *et al.* 2012) using the R packages raster (Robert & van Etten 2012) and rgdal (Keitt & Bivand 2011) (details in Supplementary material Table S2). Benjamini-Yecutieli corrections for multiple tests were applied for the *p*-values (Benjamini & Yekutieli 2001; Narum 2006).

Phylogeny, phylogeography and genetic structure

To understand the evolutionary relationships between haplotypes, and the divergence of lineages in *C. tenuispina*, a phylogenetic tree of the COI haplotypes obtained in this study was reconstructed together with only the different sequences of the same species available from Genbank. Different haplotypes from other species of *Coscinasterias* (Acc numbers AF485006 – AF485016; AF485018 – AF485025; AF485028 – AF485035; AF485040; AF485041; and AF485043 – AF485045) and one distant genus were included as outgroups (*Pisaster ochraeus*; Acc number DQ021906). A maximum-likelihood phylogenetic tree was produced using RAxML v 8 (Stamatakis 2014) implemented in CIPRES Gateway (Miller *et al.* 2010). Different partitions between the first two codon positions and the third codon position were applied on a GTR+G model. The program estimates different parameters for each partition and implements them separately. Pairwise divergence between haplotypes was calculated using a Kimura (1980) 2-parameter evolution model; that used for the same species by Waters & Roy (2003). We applied molecular calibrations from other asteroids for COI, 2.3% – 3.6% (Hart *et al.* 1997; Wares & Cunningham 2001).

The evolutionary relationships and geographical distribution of genetic variants were explored by phylogeographic analysis based on both mitochondrial and nuclear data. A network of haplotypes from the COI sequences was built with a median joining algorithm implemented in Network v 4.6 software (Fluxus Technology, htttp://fluxus-engineering.com/sharenet.htm), with default parameters (epsilon = 0, weight of characters = 10, and rate of transversions - transitions = 1-1). A posterior maximum parsimony option was applied to simplify the vector links. Additionally, a minimum-spawning network was reconstructed based on a distance matrix of the microsatellite data using the HapStar software (Teacher & Griffiths 2011). The genetic

distance matrix was calculated from an infinite allele model in Genodive as explained before, and using all the MLGs.

To infer the genetic structure of the species, Bayesian clustering analysis was performed with the STRUCTURE v 2.3 software (Pritchard *et al.* 2000). Hierarchical analysis was applied to detect the most homogeneous clusters (*k*) from the microsatellite data for *C. tenuispina* without geographical priors. We performed an initial analysis with the whole dataset; and afterwards, different analyses for the different geographical areas and clusters obtained from the previous analyses. In further analyses, the four Brazilian populations and all the other populations (North Atlantic and Mediterranean populations) were analysed separately. A final analysis was performed including only one of the previously obtained clusters, which included the populations from the eastern Mediterranean, Canary Islands and Florida. All the runs were computed under the admixture model with allele frequencies correlated between populations. Twenty independent replicates and 10,000 MCMC (Markov chain Monte Carlo) iterations were performed per run, with a 10,000-run burn-in period, following the optimised parameters found in Evanno *et al.* (2005). The prior number of *k* for each analysis was from 1 to the number of populations plus one. The *ad hoc* statistic ΔK (Evanno *et al.* 2005) was used to infer the most likely number of clusters. The 20 independent runs obtained for the best number of clusters (*k*) were then averaged using the clumpak server (http://clumpak.tau.ac.il) and represented in the same server.

Analysis of the molecular variance (AMOVA) was performed to test the spatial division of the genetic diversity grouping of populations within the major geographical areas and according to the clusters found with STRUCTURE (see Results section). AMOVA was performed in Arlequin v 3.5 (Excoffier *et al.* 2005; Excoffier & Lischer 2010). Populations for AMOVA were grouped as follows: Brazil (four populations), Canary Islands (four populations), eastern Mediterranean (three populations) and western Mediterranean (three populations). AMOVA tested for subdivisions among geographical areas, among populations within geographical areas, and among individuals within populations. We used 1,000 permutations to measure significant genetic differences from zero (p-values). The Gijón and Florida populations were not considered in this analysis since only one population from each of these two geographical areas was available.

In order to assess detailed differences in genetic structure between pairwise populations, we performed the F_{ST} estimator from allele frequencies (Weir & Cockerham 1984) and Jost's D (Jost 2008). Both types of marker, COI and microsatellites, were used separately to measure dissimilarity between populations using the Arlequin software for F_{ST}, and both SPADE (Chao & Shen 2010) and DEMEtics R package (Gerlach *et al.* 2010) for Jost's D. The genetic distances matrixes between pairwise populations were then graphically represented using a multidimensional scaling method (MDS). Genetic distance methods such as F_{ST} may be greatly affected by deviations from the HWE (Waples 2014) in nuclear markers, and therefore potential differences in the genetic structure inferred from mitochondrial and the microsatellite markers were also inspected by assessing the correlation between F_{ST} of COI and microsatellite loci of the populations.

Connectivity and genetic drift are two evolutionary processes shaping the neutral spatial structure of species. Genetic drift is highly related to the contemporary effective population size (Ne). In order to estimate Ne of populations, we used a linkage disequilibrium method implemented in NeEstimator v 2 (Do *et al.* 2014).

Connectivity in *C. tenuispina* was only studied between and within two large geographical areas: the Canary Islands and the eastern Mediterranean (see Results of STRUCTURE, last run). They were the only two areas containing individuals with "admixed" genotypes from different clusters. Other populations were not included because previous analyses demonstrated genetic

Locality	Geographical Area	Code	Latitude	Longitude	$N_{ind.COI}$	Hr (sd)	Π (sd)	$N_{ind.Micros}$	$N_{alleles}$	Ar	H_o	H_e
						Mitochondrial marker (COI)			Nuclear marker (Microsatellite			
Alicante, Murcia, Spain	Western Mediterranean	ALI	-0.379	38.434	27	0.000(0.000)	0.000(0.000)	24	17	1.368	0.344	0.177
Llançà, Catalonia, Spain	Western Mediterranean	LLA	3.162	42.385	28	0.000(0.000)* heteroplasmy	0.000(0.000)* heteroplasmy	25	16	1.332	0.333	0.167
Napoli, Campania, Italy	Western Mediterranean	NAP	14.088	40.790	14	0.000(0.000)	0.000(0.000)	18	18	1.422	0.333	0.179
Taormina, Sicily, Italy	Eastern Mediterranean	SIC	37.850	15.299	27	0.000(0.000)	0.000(0.000)	23	24	1.687	0.247	0.275
Knidos, Datça, Turkey	Eastern Mediterranean	KNI	36.688	27.370	27	0.000(0.000)	0.000(0.000)	15	14	1.500	0.167	0.083
Plakias, Crete, Greece	Eastern Mediterranean	PK	35.191	24.389	28	0.254(0.095)	0.00037(0.00014)	19	24	1.661	0.368	0.260
Abades, Tenerife, Spain	Canary Islands	ABA	28.134	-16.446	13	0.154 (0.126)	0.00023(0.00018)	14	26	1.836	0.435	0.347
Bocacangrejo, Tenerife, Spain	Canary Islands	BOCA	28.406	-16.314	20	0.195(0.115)	0.0017(0.00073)	24	25	1.880	0.569	0.391
Playa Blanca, Lanzarote,	Canary Islands	LAN	28.857	-13.802	16	0.125(0.106)	0.00092(0.00078)	16	27	1.845	0.396	0.326

Spain

Tazacorte, La Palma, Spain	Canary Islands	TAZ	28.644	-17.946	19	0.205(0.119)	0.00092(0.00068)	19	24	1.823	0.263	0.355
Gijón, Asturias, Spain	Cantabric Sea	GIJ	43.550	-5.640	29	0.000(0.000)	0.000(0.000)	30	14	1.166	0.167	0.083
St. Petersburg, Florida, USA	Florida, Golf of Mexico	FLO	28.466	-83.043	4	0.000(0.000)	0.000(0.000)	8	25	1.897	0.556	0.351
Ferradura, Rio de Janeiro, Brasil	South-western Atlantic, Brazil	FER	-22.772	-41.885	17	0.118(0.101)	0.00017(0.00015)	20	21	1.556	0.421	0.254
Itaipú, Rio de Janeiro, Brasil	South-western Atlantic Brazil	ITA	-22.977	-43.031	16	0.125(0.106)	0.00018(0.00016)	24	16	1.293	0.243	0.138
Prainha, Rio de Janeiro, Brasil	South-western Atlantic Brazil	PRA	-22.975	-42.017	22	0.506(0.050)	0.00074(0.00007)	24	21	1.694	0.441	0.297
Tartaruga, Rio de Janeiro, Brasil	South-western Atlantic Brazil	TAR	-22.755	-41.902	13	0.000(0.000)	0.000(0.000)	23	18	1.483	0.409	0.233
-	Mediterranean Sea	-	-	-	151	0.345(0.042)	0.00052(0.00038)	124	30	2.497	0.305	0.281

					N_ind.COI	Hr (sd)	Π (sd)	N_ind.Micros	N_alleles	Ar	H_o	H_e
-	Western Mediterranean	-	-	-	69	0.459(0.024)* heteroplasmy	0.00072(0.00004)* heteroplasmy	67	19	1.354	0.337	0.175
-	Eastern Mediterranean	-	-	-	82	0.094(0.043)	0.00014(0.00006)	57	28	1.289	0.261	0.310
-	Canary Islands	-	-	-	68	0.169(0.061)	0.00084(0.00035)	70	27	2.193	0.432	0.421
-	South-western Atlantic, Brazil	-	-	-	68	0.556(0.052)	0.00128(0.00013)	94	27	2.163	0.373	0.277
-	TOTAL	-	-	-	320	0.645 (0.027)	0.00452(0.00023)	326	41	3.411		

Table 1. Genetic descriptors of *C. tenuispina* for COI and microsatellites: location where samples were collected (Location), geographical area (Area), popu... (Code), geographical coordinates (Latitude and Longitude), number of individuals analysed ($N_{ind.COI}$ for COI and $N_{ind.Micros}$ for microsatellites), Hr and Π for... standard deviation in brackets (sd) and (*) indicates that heteroplasmy is present in the population, number of alleles ($N_{alleles}$), allelic richness (Ar), observe... expected (H_e) heterozygosis, fixation index (F_{IS}) for microsatellites and (**) indicates significant deviation from HWE with p-value < 0.01.

isolation. A genetic assignment method based on microsatellites was performed to assign or exclude reference populations as possible origins of individuals on the basis of MLGs. Genetic assignment methods allow us to infer where individuals were born, providing estimates of real-time dispersal through the detection of migrants. They are useful in addressing relationships when genetic differentiation at the population level is not high. The assignment test was performed using GenoDive (Meirmans & Van Tienderen 2004) following the method of Paetkau *et al.* (2004), with 10,000 permutations for resampling and a significance threshold set at 0.002.

Isolation and oceanographic barriers

The potential effect of isolation by geographical distance (IBD) was tested for using a classic Mantel test and a stratified Mantel test according to geographical areas, implemented in the R vegan package (Oksanen *et al.* 2013). The Mantel test procedure explores the correlation between matrixes of geographical distance and genetic dissimilarity, and the stratified Mantel avoids the IBD bias from major population structure (Landguth *et al.* 2010; Meirmans 2012). Geographical distances were estimated as the minimum linear distance across the sea between populations (logarithmically transformed), and genetic dissimilarities extracted from the F_{ST} estimators of COI and microsatellites.

Nevertheless, since oceanographic factors other than geographical distance have been demonstrated to prevent gene flow even between nearby populations, the software BARRIER v. 2.2 (Manni *et al.* 2004) was used to identify major barriers to genetic admixture across the whole distribution range of *C. tenuispina*. BARRIER detects zones of abrupt changes in genetic structure. The genetic descriptors used for this analysis were the F_{ST} and Jost's D matrixes, based on microsatellite loci; and five of the most important barriers were represented based on Voronoï tessellation (Voronoi 1908) and Delaunay triangulation (Brassel & Reif 1979), as implemented in the software.

Results

Genetic diversity and clonality

A total of 683 bp of the COI fragment was sequenced from the samples of 320 individuals. Only 13 haplotypes were found for the whole dataset, with 19 variable sites. The values of genetic diversity for the COI were low in terms of the number of haplotypes (between 1 and 3 per population), Hr (0.000-0.503) and Π (0.000-0.00092) (see Table 1). Large differences in Hr and Π were detected between populations, with most populations dominated by only one haplotype with a high frequency (see Figure 2). Several populations, such as in Florida, Gijón (Cantabrian Sea), Tartaruga (Brazil), and most of those from the Mediterranean with the exception of Plakias, showed only one COI haplotype. All the specimens from Llançà (north-western Mediterranean) presented heteroplasmy for the COI, with the presence of two of the most common haplotypes (H_3 and H_8) in the same specimens. The former finding was confirmed by double sequencing from independent DNA extractions; while additional sequencing of the mitochondrial control region confirmed the existence of two different haplotypes for both genes within individuals. The latter result supported the idea of there being two different mitochondrial haplotypes rather than a pseudogene of the COI in the nuclear DNA. Hence, for some genetic analyses including the Llançà samples, we considered the two haplotypes separately (Figure 3, H_3 and H_8).
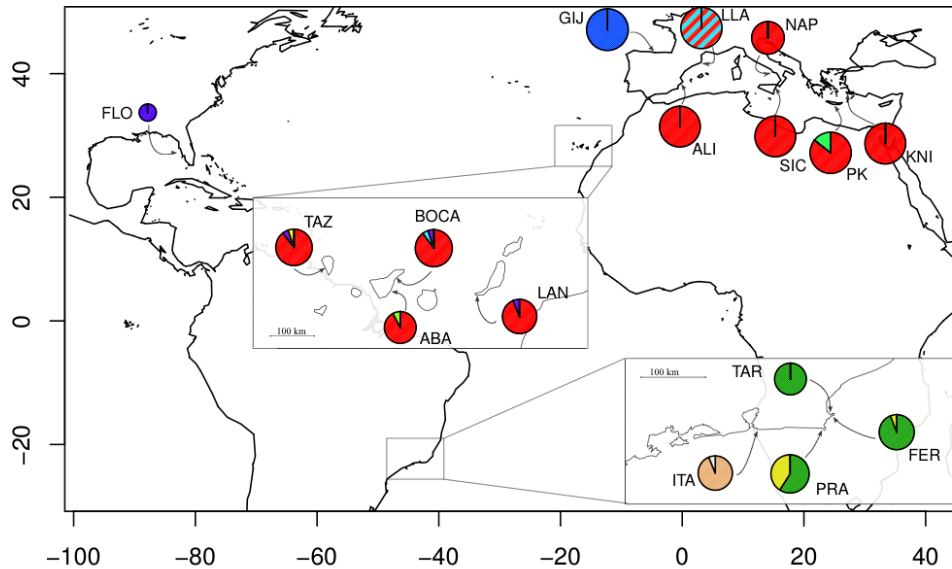
Figure 2. Map of the *C. tenuispina* sampling locations. Pie charts represent frequencies of the COI haplotypes at each location, and their size is proportional to the number of individuals analysed. Different colours represent different haplotypes.
See Table 1 for full location names. Note that the dual colour in Llançà represents a heteroplasmy detected in all individuals from this location.

For microsatellites, a total of 326 individuals were successfully genotyped. Genetic diversity descriptors for each locus are presented as Supplementary material (Supplementary Table S3). The heterozygosity observed (H_o) was higher than expected under conditions of equilibrium in all populations, which translated into negative values of the fixation index (F_{IS}), with the exception of those from Sicily and Tazacoste (see Table 1), and deviation from HWE in all populations, except Sicily. The excess of observed heterozygotes in *C. tenuispina* is probably a result of a prevalence of clonal reproduction.

A reduced number of alleles per locus (between 2 and 5) and MLGs were detected across all the populations. A total of 69 different MLGs were found for the whole dataset (Figure 1 and Table 2) with different prevalence of clonality (clones defined as individuals sharing the same MLG) between populations and geographical areas (Figure 1, Table 2). All clonal MLGs had low probabilities of being a product of sexual events, as demonstrated by the significant values of P_{sex} (p-value < 0.001): 2.34E-09 to 4.00E-15. The high prevalence of clones was also in concordance with low genotype diversity (div: 0 - 0.901), and a high clonal evenness value (eve: 0.355 - 1) in some populations (Table 2). The populations from the Canary Islands and Florida retained the highest number of alleles, allele richness and genotype diversity (measured using different descriptors). The Canary Island population also concentrated the highest number of MLGs and MLLs, with 36 MLGs in 73 specimens, and clonal richness (P_d=0.507; R= 0.500, See Table 2). This number of MLGs was larger than that from all the other 12 populations together (with 33 MLGs from 253 individuals) (see details in Table 2). These results suggest that in the Canary Islands the prevalence of asexual reproduction is lower than in all the other geographical areas. The highest values of clonal reproduction were inferred for the geographical areas of: Cantabrian Sea (Gijón), western Mediterranean and Brazil, according to the clonal richness (P_d = 0.033, 0.045 and 0.111; and R = 0.00, 0.030 and 0.100 for Gijón, western Mediterranean and Brazil, respectively). Extreme cases of clonality were observed in the monoclonal

Population	size	Clonal richness				Genotype diversity		
		MLG (private)	MLL (private)	Pd	R	div	eve	shc
Alicante	24	2 (0)	1 (0)	0.083	0.043	0.228	0.64	0.168
Llançà	25	1 (0)	1 (0)	0.040	0	0	1	0
Napoli	18	2 (0)	2 (0)	0.111	0.059	0.294	0.692	0.201
Knidos	15	1 (0)	1 (0)	0.067	0	0	1	0
Plakias	19	8 (5)	4 (2)	0.421	0.389	0.795	0.507	0.916
Sicily	23	4 (1)	4 (1)	0.174	0.136	0.7	0.756	0.549
Abades	14	7 (6)	7 (6)	0.500	0.462	0.692	0.4	0.929
Bocacangrejo	24	10 (7)	7 (5)	0.417	0.391	0.826	0.48	1
Lanzarote	16	9 (7)	6 (4)	0.563	0.533	0.817	0.474	1.09
Tazacoste	19	11 (8)	9 (7)	0.579	0.556	0.901	0.619	1.189
Gijón	30	1 (0)	1 (0)	0.033	0	0	1	0
Florida	8	5 (3)	3 (2)	0.625	0.571	0.857	0.8	0.879
Ferradura	20	3 (1)	3 (1)	0.150	0.105	0.468	0.601	0.368
Itaipú	24	4 (2)	2 (0)	0.167	0.130	0.308	0.355	0.368
Prainha	24	6 (2)	4 (0)	0.250	0.217	0.699	0.505	0.681
Tartaruga	23	3 (0)	2 (0)	0.130	0.091	0.435	0.571	0.336
Mediterranean Sea	124	16 (6)	11 (0)	0.129	0.122	0.731	0.227	0.864
Western Mediterranean	67	3 (0)	2 (0)	0.045	0.030	0.167	0.400	0.164
Eastern Mediterranean	57	13 (6)	9 (3)	0.228	0.214	0.865	0.511	1
Canary Islands	73	37 (28)	28 (22)	0.507	0.500	0.956	0.475	1.608
Brazil, South-western Atlantic	91	10 (3)	5 (0)	0.111	0.100	0.713	0.34	0.726
TOTAL	326	69 (40)	50 (27)					

Table 2. Intra-population diversity in *C. tenuispina*. Clonality descriptors were separated into clonal richness and genotype diversity. First, the number of analysed per population (size). Clonal descriptors: number of different genotypes (MLGs) with single MLGs in brackets; number of genotypes grouped in line with the number of MLLs that only occurred once in brackets (lineages that had at least 2 MLGs are in bold); clonal ratio (Pd) and corrected clonal ratio (R) diversity: Nei's (1987) genotype diversity (div), clonal evenness value (eve) and Shannon corrected index (shc).
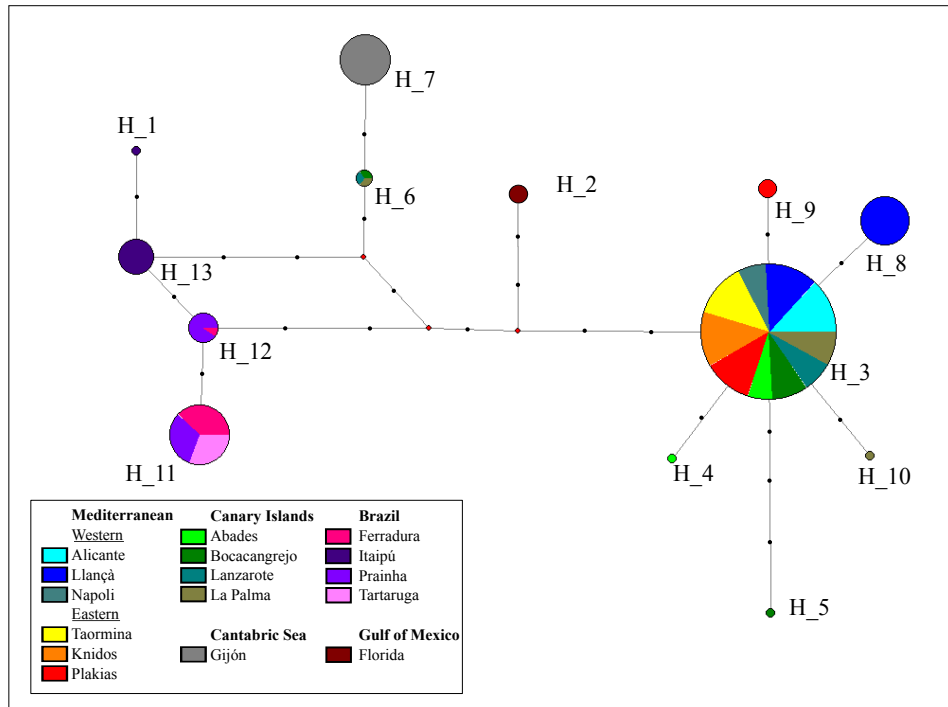
Figure 3. Network of all COI haplotypes found in *C. tenuispina*. Pie charts represent different haplotypes and colours, the different locations sampled. The size of the pie charts is proportional to the number of individuals with the same haplotype. Dots on the lines identify mutational steps between haplotypes. Note that blues are reserved for the western Mediterranean; warm colours for the eastern Mediterranean; greens for the Canary Islands; and pink and purples for the Brazilian locations.

populations of Gijón, and two Mediterranean populations, Llançà and Knidos, although in other Mediterranean population, such as Alicante, only one MLL was also found (Table 2).

Significant correlations were detected between the mean or minimum seawater temperature and some genetic descriptors. Values of evenness were negatively correlated with minimum temperature after Benjamini-Yekutieli corrections (R^2_{eve}= -0.72, p< 0.05) and they also correlated significantly with annual mean temperature (R^2_{eve} = -0.61, p< 0.05); while Nei's diversity correlated positively with this latter variable, but only when non-corrected p-values were considered. Salinity did not correlate significantly with any of the genetic variables (See Supplementary Table S1).

Phylogeny, phylogeography and genetic structure

The phylogenetic tree constructed from the COI sequences was not supported strongly by the bootstrap values, but revealed haplotype clustering into two main groups or clades. The Brazilian sequences formed a clade (the south-western Atlantic clade) with 75% of bootstrap support. Most of the north-eastern and western Atlantic and the Mediterranean samples clustered together; although this clade was not well supported (<50%). Two haplotypes (from Gijón and Lanzarote) did not group together with either of these two clades (see Figure S1 in Supplementary material).

The COI network, which includes information on the haplotype frequencies for each population, showed that all the populations from the Mediterranean Sea and the Canary Islands shared the most frequent haplotype (H_3), which was also the only haplotype found in four Mediterranean populations (Alicante, Napoli, Taormina and Knidos) (Figure 3). The Brazilian populations showed four different haplotypes on the opposite side of the network; greatly separated from the common H_3, by up to 7 mutations: a K2P divergence of 0.88%-1.18% with H_3. Neither did these populations share any haplotypes with other geographical areas; there was 0.44%-1.48% K2P divergence between Brazil and all the other haplotypes. Three of the Brazilian populations shared the second most common haplotype (H_11), whereas the population of Itaipú did not have any haplotype in common with the other Brazilian populations. The Gijón population (Cantabrian Sea, north-eastern Atlantic) appeared closely related via one haplotype found at three locations in the Canary Islands, but distant from the other North Atlantic haplotypes and those from Brazil. The only haplotype from Florida also seemed to be separated from other geographical areas, with a K2P divergence of 0.58% - 1.03%.

The MLG network based on microsatellites showed similar results to the COI, but it allowed more detailed analysis of the relationships between genotypes or clones (Figure 4). The minimum spanning network revealed that the MLGs from the Canary Islands and the Mediterranean Sea appeared spread over the central area of the network and related among themselves. MLGs from the western Mediterranean remained in a small cluster, but closely related to MLGs from the Canary Islands rather than the eastern Mediterranean genotypes. As observed from the COI network, MLGs from the south-western Atlantic (Brazil) and Florida were separated from those of other geographical areas, appearing on opposite sides of the network. The Gijón population (Cantabrian Sea, north-eastern Atlantic) was composed of only one MLG, but it was closely related to a group of MLGs from the Canary Islands (as observed
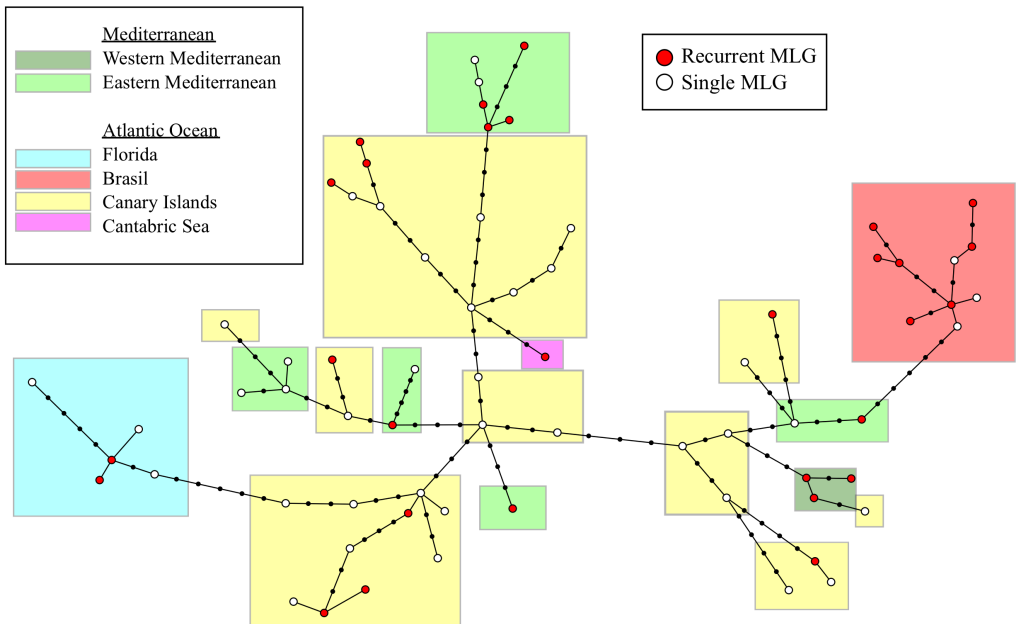

Figure 4. Minimum spanning network of *C. tenuispina* from microsatellite allelic distances. Black dots represent mutation steps, and white and red circles represent different MLGs. Red circles show recurrent and therefore clonal MLG, while white circles are single MLGs found in only one individual. Squares indicate MLGs from the same geographical area.
Note that the size of the circles does not represent the number of individuals.

from the COI haplotypes).

Hierarchical analyses using STRUCTURE identified two main homogeneous clusters from microsatellite loci in the first run, with a clear increase in Δ K (Evanno *et al.* 2005) for *k*=2. The clusters corresponded to the south-western Atlantic (Brazilian populations; orange in Figure 5-A) and the North Atlantic and Mediterranean (blue in Figure 5-A); coherent with the results obtained from the COI phylogenetic tree. Separate analyses of these two main clusters demonstrated more detailed structure within the major geographical areas. Within the south-western Atlantic area of Brazil, three different clusters were detected (optimal *k*=3) (Figure 5-B). The Itaipú population showed a very homogeneous structure (pale orange in Figure 5-B), different from the other Brazilian populations which presented a more heterogeneous structure. The North Atlantic + Mediterranean cluster (Figure 5-C) also had an optimal *k*=3, which corresponded to: Gijón (Cantabrian Sea; dark purple in Figure 5-C); western Mediterranean + the eastern Mediterranean population from Knidos (purple in Figure 5-C); and Florida + Canary Islands + most eastern Mediterranean populations (light blue in Figure 5-C). The third run with the last group of populations (the light blue cluster) showed an optimal *k*=4, separating Florida (in light green) and the eastern Mediterranean populations of Plakias and Knidos (in
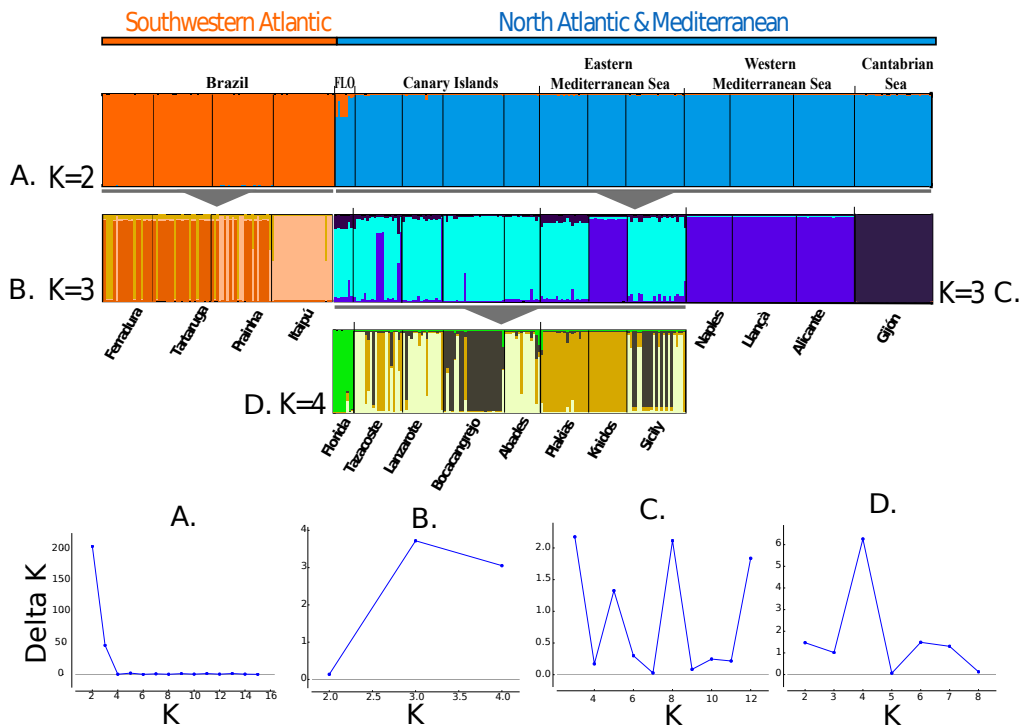

Figure 5. Bar plots from the Bayesian clustering analysis with STRUCTURE and (below) graphs of ΔK values (on y-axes) for each K (on x-axes) for the four different datasets. **A)** First analysis performed with the whole dataset (best clustering when k = 2) that separates south-western Atlantic (Brazil) and all the other locations from the North Atlantic & Mediterranean area. **B)** Analysis of the south-western Atlantic Brazilian populations (best k = 3) that separates Itaipú into a different cluster, with some admixture with the other three populations. **C)** Analysis of North Atlantic & Mediterranean populations (k = 3), clustering: Florida, the Canary Islands and most of the eastern Mediterranean populations; western Mediterranean; and Gijón. **D)** Analysis of the Canary Islands, eastern Mediterranean and Florida (best k = 4), revealing admixture between Canary Islands and Sicily; with Florida separated from the eastern Atlantic and Mediterranean Sea.

dark yellow) from the Canary Islands and Sicily (Figure 5-D). All the Bayesian analyses of *C. tenuispina* demonstrated that individuals of the species belonged to a specific cluster with a very high probability (80%-100%), with no "mixed genotypes" from different genetic clusters observed. Only a few individuals from the Canary Islands seemed to be a genetic mixture from different clusters (see Figure 5-D).

The AMOVA results, based on both COI and microsatellites, provided more statistical support for the geographical grouping observed in the Bayesian clustering (Table 3). For both type of markers, genetic differentiation among geographical groups (percentage of variation: 40.83% and p-value=0.002 for microsatellites; and 35.5% and p-value=0.001 for COI), and populations within geographical groups (26.2% and p-value=0.000 for microsatellites; and 14.7% and p-value=0.000 for COI) was large and significantly different from zero. For the COI, significant differences were also detected within populations (percentage of variation 32.9% and p-value=0.000) (see details in Table 3).

Source of variation	d.f	Sum of squares	Fixation index	Variance components	% of variation	p-value
COI						
Among groups	3	35.557	0.408	0.124	40.83	0.002
Among populations within groups	10	17.824	0.443	0.080	26.23	0.000
Within populations	301	30.178	0.671	0.100	32.94	0.000
Total	314	83.559		0.304		
Microsatellites						
Among groups	4	609.50	0.358	1.037	35.8	0.001
Among populations within groups	11	192.36	0.226	0.420	14.5	0.001
Within populations	310	240.31	-0.454	-0.653	-22.5	1.000
Within Individuals	326	682.50	0.278	2.092	72.2	--
Total	326	1,724.67		2.896		

Table 3. AMOVA analyses of COI and microsatellites. Groups of populations: western Mediterranean, eastern Mediterranean, Canary Islands and Brazil.

Tables with the original values of the pairwise genetic distances are available as Supplementary material (Table S4 and Table S5); while the results of the MDS performed from pairwise matrixes are presented as Figure 6 and Supplementary Figure S2. For both types of markers, the populations from the areas of Brazil, Gijón and Florida were the most distant ones (see Figure 6 and Tables S4 and S5). There was a significant correlation between the F_{ST} values from the COI and microsatellites, supporting the evidence of congruence in genetic structure from different markers (correlation= 0.638, p<0.001, see Supplementary Figure S3). Moreover, this confirms that, in this case, microsatellites give robust results, despite the violation of the HWE. However, microsatellites seemed to display more significant pairwise differences between populations (Tables S4 and S5). Populations from the Canary Islands and the Mediterranean
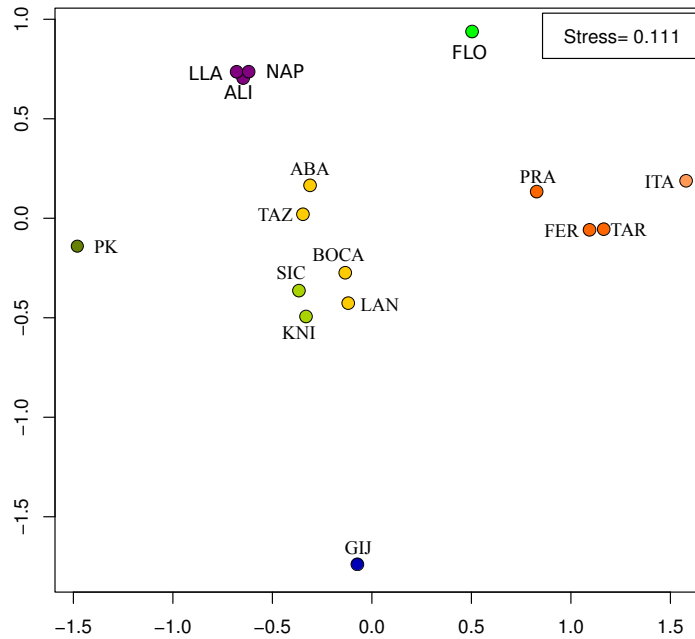
Figure 6. Multidimensional scaling (MDS) based on the F_{ST} pairwise differences from microsatellite data with the first two dimensions plotted. Populations are presented by their codes. Colours follow a similar pattern represented in structure figure with K=3 and K=4.

Sea, with the exception of the Llançà population, did not show significant differences in genetic structure for the COI, because of the prevalence of the common haplotype H_3 (Figure 3). Nevertheless, significant differences were observed between most comparisons using microsatellite data, when comparing both populations from different geographical areas and also populations within geographical areas, with the exception of Alicante, Napoli and Llançà due to the prevalence of only one MLG (see Table S5 and Figure 6). Within the area of Brazil, the populations of Itaipú and Tartaruga displayed large differences in genetic structure, despite their geographical proximity (see Figure 6, and Tables S4 and S5).

Estimations of *Ne* showed that for monoclonal populations (those dominated by only one MLG), such as those from Knidos (eastern Mediterranean), the Cantabrian Sea (Gijón), and two Brazilian populations (Itaipú and Tartaruga), this value tends to infinite. For populations with infinite *Ne,* the effect of genetic drift over time on the genetic structure is estimated to be zero. For all the other populations, estimations of *Ne* were very small (from 0.5 to 1.9), indicating that these populations are susceptible to changes in their genetic structure due to a strong effect of genetic drift.

The connectivity between geographical areas of *C. tenuispina* was limited. Previous analyses indicated the absence of common clusters in Brazil, Gijón, Florida, and the western Mediterranean (see STRUCTURE results). Meanwhile, connectivity measured within and between the Canary Islands and the eastern Mediterranean demonstrated that gene flow was only effective between populations from these islands (See Figure 7). The assignment test detected only one migrant from the Mediterranean population of Sicily (eastern Mediterranean) to Bocacangrejo (Canary Islands), and three migrants from Tazacoste (Canary Islands) to Plakias (eastern Mediterranean) (see Figure 7). Although still low, gene flow seemed to be higher from the Atlantic to the Mediterranean basin than in the opposite direction. Among populations

in the Canary Islands, 17 migrants could be identified, ranging from three to five per population (see Figure 7).

Isolation and Oceanographic barriers

The Mantel test procedure detected IBD signals with a significant correlation between genetic differentiation (F_{ST}) and geographic distances for COI and microsatellites (r = 0.668 and 0.676, respectively; p-value < 0.001) (Table 4). However, no significant IBD was detected using a stratified Mantel test (Table 4), indicating that the significance in IBD was due to major population structure generated by oceanographic breaks such as those reported below.

Different oceanographic barriers could be identified as abrupt changes in genetic structure (Figure 8). The two most abrupt changes separated the south-western Atlantic (Brazil), and the north-eastern Atlantic and the Mediterranean Sea, followed in importance by a barrier that separated the two Mediterranean sub-basins (western and eastern). Other minor though significant breaks were between: a) the Cantabrian Sea and the Canary Islands, and b) Florida and the other areas. Finally, there was a weaker break separating the Itaipú population from the more northern Brazilian populations (Figure 8).

Isolation by distance	
<u>Microsatellites</u>	Correlation (r) (p-value)
Mantel Test	0.668 (<0.001)*
Stratified Mantel Test	0.668 (0.599)
<u>COI</u>	
Mantel Test	0.676 (<0.001)*
Stratified Mantel Test	0.676 (0.499)

Table 4. IBD results. Values of the correlation (r) of the Mantel and stratified Mantel tests from microsatellites and COI, using F_{ST} matrixes and a distance matrix. Associated p-values; * when p-value < 0.01).

Discussion

Studies exploring phylogeographic patterns of solitary benthic invertebrates with asexual reproduction are scarce (see some examples in Johnson & Threlfau 1987; Uthicke *et al.* 1999; Sköld *et al.* 2003; Ventura *et al.* 2004; Perrin *et al.* 2004; Haramoto *et al.* 2006). The lack of empirical studies that evaluate the effects of asexual reproduction on the intraspecific divergence and connectivity of marine invertebrates, limits our understanding of the evolutionary potential of species with complex life cycles, and prevents us from developing appropriate theoretical concepts related to the population genetics of asexual species.

Phylogeography and divergence in *Coscinasterias*

The phylogeography of *C. tenuispina* was characterised by the divergence of populations into two main groups, in concordance with the disjunctive geographical distribution across the equator. Both mitochondrial and nuclear markers detected clustering of populations from the south-

western Atlantic (Brazil), north-eastern Atlantic and Mediterranean Sea. Although the global phylogeny of the genus *Coscinasterias* had previously been reported to exhibit three groups of sequences (Waters & Roy 2003), the shallow phylogeny of the COI was considered evidence of recent long-distance dispersal; during the Pleistocene glaciations (Waters & Roy 2003). The data presented here reflect the existence of only two major groups; while neither common nuclear ancestral alleles nor evidence of recent migration was observed between the two main clusters. Our estimations of divergence from COI (0.44%-1.48% K2P; 191 – 122 Ky ago) are similar to those of Waters & Roy (2003), and therefore the hypothesis of expansion during an interglacial period of the Pleistocene and posterior divergence by vicariance is coherent with our data. Despite the low value of divergence for the COI between the Brazilian and North Atlantic sequences, their disjoint distribution and the absence of both gene flow and ancestral nuclear alleles provide robust arguments in favour of the view expressed by other authors that they should be considered as different taxonomic units (Clark & Downey 1992). Independently of the taxonomic consideration of these two major clusters, as species or subspecies, they need to be treated as independent evolutionary units.

Interestingly, although the COI did not clarify the relationship between the Brazilian sequences and those from the northern hemisphere due to the low support in our phylogenetic reconstruction, the nuclear markers pointed to the eastern Mediterranean area as the origin of the Brazilian cluster in the minimum spanning network. More thorough sampling from the coast of western Africa would be necessary to fully understand this relationship, since the origin of the Brazilian cluster could be prior to the colonisation of the Mediterranean Sea (see full explanation below) in accordance with the COI divergence. Therefore, despite this particular result, we hypothesise an origin of the *C. tenuispina* complex in the eastern Atlantic (around the Canary Islands), where this starfish retains the highest values of genetic diversity and number of genotypes. From there, larvae could have crossed the mid-Atlantic barrier (MAB) during some Pleistocene expansion event, 191 – 122 Ky ago, to establish themselves off the coast of Brazil. The disruptive effect of the Amazon-Orinoco Plume (AOP) (Rocha 2003; Luiz *et al.* 2012) was detected by the BARRIER software. The AOP is a strong barrier that promotes abrupt changes in genetic structure, isolation and divergence in *C. tenuispina*; resulting in a genetic cluster that has remained in the southern hemisphere with no connection with the north-eastern Atlantic and Mediterranean evolutionary unit. The AOP, a freshwater and sediment plume, produces
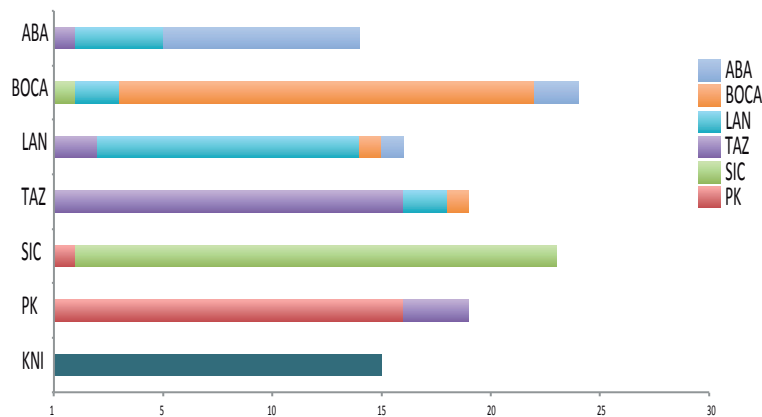
Figure 7. Results of the assignment test between the Canary Islands and the eastern Mediterranean. Each bar represents the number of individuals (on x-axes) sampled in each population (on y-axes) and colour represents the origin of each individual. Populations have a colour assigned, as in the legend. Individuals that have different colours from the colour of the population where they were sampled, represent probable migrants and the population they came from.

changes in the physical and chemical properties of inshore waters; factors that prevent larval dispersal and have promoted a number of speciation processes along the coast of Brazil (see Rocha 2003 for a review and reference herein). Only multi-habitat species with a capacity to survive in different ecotypes are capable of connecting across the AOP (Luiz *et al.* 2012). The fact that in Brazil, *C. tenuispina* is not found north of Nova Viçosa (south of Bahia State, -17º54', -39º07'), much further south than the beginning of the AOP, supports the hypothesis of habitat limitations for the species.

Within the Brazilian cluster, other oceanographic barriers were also detected. There was no evidence of connectivity between the Itaipú and the other three Brazilian populations located only ~100 km apart; a pattern that could not be explained by IBD, according to the non-significant stratified Mantel test. These two areas appeared to be grouped in different clusters from the STRUCTURE analysis, displayed high and significant values of F_{ST} (0.185-0.311; p< 0.05), and did not share any COI haplotypes. The upwelling of Cabo Frio that divides two large biogeographic areas in Brazil (Spalding *et al.* 2007; Coelho-Souza *et al.* 2012), also represents an ecological barrier for marine species. It prevents gene flow even in broadcast spawners (Maggioni *et al.* 2003; Wangensteen *et al.* 2012); an effect that may be even stronger than that caused by major marine currents (Waters & Roy 2004). Additionally, the presence of only a few "admixed" genotypes in Brazil and reproductive information on the species from some Brazilian locations (Alves *et al.* 2002) confirm that the Brazilian populations are mainly maintained by asexual reproduction, with the absence of a larval dispersal phase. This likely accentuates the disruptive effect of the Cabo Frio upwelling detected by BARRIER analysis.

In the north-eastern Atlantic, the Canary Islands seem to be located within the geographic distribution of *C. tenuispina*'s. We have different data that support this hypothesis. First, the Canary Islands retained the highest values of genetic diversity and number of genotypes; which is a characteristic sign of central distribution (Ehrich *et al.* 2008; Besnard *et al.* 2013b; a; Planas *et*
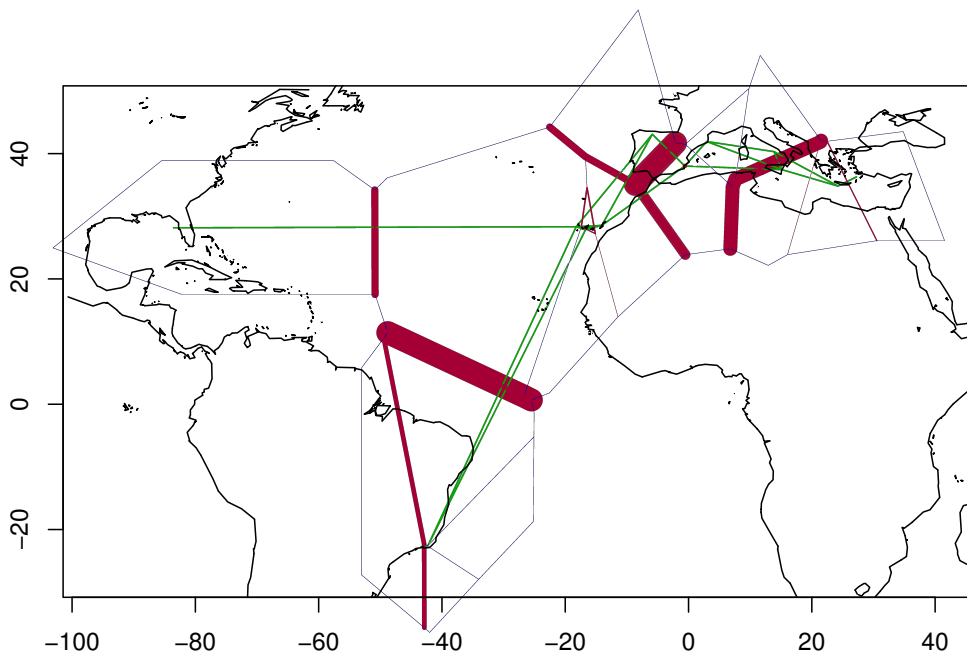

Figure 8. Graphical representation of a Voronoï tessellation (in blue) and a Delaunay triangulation (in green) of sampled populations, as computed using BARRIER. Detected oceanographic barriers are represented in red, and the width of the barrier (line) is related to its breaking effect.

al. 2014). Additionally, the presence of some "admixed" genotypes in most locations on these islands is also indicative of the mixture of genotypes and recombination during sexual reproduction. This was not in general observed elsewhere in the north Atlantic and Mediterranean cluster, according to the results obtained from STRUCTURE. This hypothesis is also supported by the smaller values of mean body size recorded in areas other than to the Canary Islands, such as some Mediterranean locations (Garcia-Cisneros *et al.* 2015; and author's unpublished data), as is fission more frequent in small starfish (Emson & Wilkie 1980) such as those found in the Mediterranean.

C. tenuispina could also have expanded to other areas of the north-western Atlantic, including Florida, even more recently than to Brazil, according to the genetic distances, mitochondrial network and microsatellite clustering data. The hypothesis of independent colonisations from east to west across the MAB indicates that although the MAB is a deep ocean boundary, it can be relatively permeable to species with vectors for long pelagic dispersal (Luiz *et al.* 2012). Therefore, the relationship between Florida and the north-eastern Atlantic for *C. tenuispina* seems to be based on occasional gene flow via sporadic migration of larvae across the North Atlantic Ocean. As in other species with a distribution range that includes both sides of the Atlantic, *C. tenuispina* presented a large divergence between its Florida and eastern Atlantic populations, based on the presence of distant haplotypes, significant F_{ST} between populations across the MAB, and being separated in a different genetic cluster in the STRUCTURE and minimum spanning network (see other examples of echinoderms in Lessios *et al.* 2001, 2012; Wangensteen *et al.* 2012).

The genetic structure within the north-eastern Atlantic & Mediterranean cluster, was characterised by: a) the presence of three major clusters and b) the absence of an exclusively mitochondrial lineage in the Mediterranean. The three major clusters correspond to the Cantabrian Sea population, the western Mediterranean, and the Canary Islands with the eastern Mediterranean; none of which followed a pattern of IBD as demonstrated by the non-significant values of the stratified Mantel test. The absence of an endemic Mediterranean lineage contrasts with what is generally observed in Atlanto-Mediterranean echinoderms (Duran *et al.* 2004; Calderón *et al.* 2008; Pérez-Portela *et al.* 2010; Boissin *et al.* 2011; Borrero-Pérez *et al.* 2011), but follows the pattern detected in the sea urchin *Arbacia lixula*. The pattern observed in *A. lixula* was attributed to recent colonisation of the Mediterranean Sea from eastern Atlantic sources during the last interglacial period (Wangensteen *et al.* 2012). This last and long interglacial period was marked by minimum seawater temperatures higher than 19ºC in winter, which favoured the entrance and establishment of temperate-tropical Atlantic species in the Mediterranean Sea. This could be the case of *C. tenuispina* and could also explain the colonisation of the Cantabrian Sea. In the case of *A. lixula*, this hypothesis was supported by a recent demographic expansion detected from the COI (Wangensteen *et al.* 2012). However, due to the small number of haplotypes found in *C. tenuispina*, related to the prevalence of asexual reproduction, exploring demographic expansions cannot be used as a straightforward approach to test this hypothesis. Instead the much lower values of div and the presence of several monoclonal populations can be taken as a sign of recent colonisation (Arnaud-Haond *et al.* 2007).

In contrast, the Canary Islands and the Mediterranean Sea revealed little difference in COI, due to both areas sharing the most frequent haplotype. However, microsatellite loci gave better resolution of the genetic structure in these two areas, and these markers clearly showed significant differentiation between most population pairs within and between the two major clusters found: the Canary Islands with the eastern Mediterranean; and the western Mediterranean. The closer relationship between the Canary Islands and the eastern Mediterranean is surprising. Even from the assignment analysis, we could detected some evidence of gene flow between these two areas, with three migrants of the last generation from

the Canary Islands (TAZ) in Plakias (eastern Mediterranean), and one migrant from Sicily (eastern Mediterranean) in Bocacangrejo (Canary Islands). Nevertheless, although the Canary Islands and the eastern Mediterranean seemed to have some genetic connectivity, it is not enough to homogenise the genetic structure within and between basins on both sides of the Strait of Gibraltar (or the Alboran Front), and the Siculo-Tunisian Strait: abrupt changes in genetic structure were detected. The high levels of clonality and the prevalence of one clone in the western Mediterranean basin probably causes the large differentiation of this area compared to the Canary Islands and the eastern Mediterranean. The absence of divergence between populations within the western Mediterranean basin contrasts with the other two neighbouring basins. Its homogeneity is not necessarily due to an extended gene flow between sites. The presence of only one clone, or two MLLs, increases the effective population size almost to infinite, thereby reducing the loss of genetic diversity due to stochastic events, and the probability of genetic divergence among populations due to genetic drift (Delmotte *et al.* 2002; Balloux *et al.* 2003). In other populations, genetic drift probably plays an important role in divergence due to the very low values of Ne. Our most outstanding finding was the presence of the same genotype along the western Mediterranean; a fact that cannot be directly attributed to a stepping-stone system of spreading of this clone, since populations are discrete and patchily distributed across this area. The hypothesis of translocation of starfish, or part of them, through human activity (Fisher 1928; Clark 1946; Hyman 1955) by ships or recreational boats, was initially ruled out by Waters and Roy (2003) for long distances. Nevertheless, occasional fouling over short distances, as demonstrated in other echinoderms (Sponer & Roy 2002), could explain the presence of a single clone along the western Mediterranean. However, other natural processes, such as dispersion during storms and rough weather, cannot be completely ruled out either. Although other species of starfish perform seasonal migrations (Pabst & Vicentini 1978; Kashenko 2003; Gallagher *et al.* 2008), a factor that could eventually favour gene flow and dispersal, this behaviour has never been observed in *C. tenuispina*. One question that remains is how migrations occur between the Canary Islands and the eastern Mediterranean, while the western Mediterranean remains isolated from those two basins. Further sampling along the African coast of the Mediterranean Sea may clarify this particular point, since gene flow may happen along the south coast of the Mediterranean; an area that was not explored in this work.

Clonality in *C. tenuispina*

Our results indicate some important features of the predominance of an asexual or a sexual reproductive strategy in this starfish across different geographical areas. Monoclonal populations or populations dominated by only one genotype of MLLs were observed in the Cantabrian Sea, the western Mediterranean basin, and also in one population from the eastern Mediterranean, indicating that these populations are exclusively maintained by asexual reproduction. Although some genetic variation was measured in the western Mediterranean due to somatic mutations, mutations that spread in the population are through fission. Somatic mutations were detected in the form of heteroplasmy in COI in the Llançà population, and MLLs separated by only one mutation (allele) were found in the other two western Mediterranean populations (Alicante and Napoli).

Fission, although common in the genus *Coscinasterias*, only becomes the exclusive mode of reproduction under sub-optimal or stressful environmental conditions, as is the case with other organisms (Barker & Xu 1991; Mladenov 1996; Lawrence & Herrera 2000; Sköld *et al.* 2002; Silvertown 2008), or when some of the essential steps of the sexual cycle cannot be completed (Honnay & Bossuyt 2005). Therefore, monoclonality across the distribution limits of *C. tenuispina* may be related to the existence of "sub-optimal conditions" for sexual reproduction, or may also be related to recent colonisation. The Cantabrian Sea and two of the three western Mediterranean populations (Llançà and Napoli) are on the northernmost distribution of *C.*

tenuispina, and the monoclonal population at Knidos on the easternmost. The significant negative correlation found between evenness, which measure genetic homogeneity, and seawater temperature, seemed to highlight this variable as one of the environmental conditions determining the levels of clonality. The lowest values of genetic diversity, represented by monoclonal populations, were found at two of the locations displaying the lowest minimum and mean values of seawater temperature at the distribution edges of the species, Gijón (Cantabrian Sea) and Llançà (north-western Mediterranean). This result is coherent with the relationship found between fission rates and temperature in *C. acutispina* (Haramoto *et al.* 2007; Seto *et al.* 2013). It can be explained by unfavourable conditions preventing maturation of the gonads and larva survival may be permanent at the edge of the range of the species (Eckert 2002). However, seawater temperature can only partially explain the general pattern of clonality found in our study, because populations were monoclonal, even at locations where temperatures are significantly higher, along the western Mediterranean, and in the Knidos population (eastern Mediterranean). Therefore, other factors that were not analysed in this study cannot be completely ruled out. For instance, the presence of parasites infecting the gonads or decreasing individual fitness can sometimes prevent completion of the biological cycle (Sköld *et al.* 2003; Haramoto *et al.* 2007; Seto *et al.* 2013); but have never been observed in *C. tenuispina*. These high rates of clonality probably respond to a combination of historical, environmental and biological factors. Therefore, before reaching further conclusions as to the potential causes of this general pattern of clonality across most of the Mediterranean Sea, more information on the biological cycle, presence of new recruits, and sex ratios of the populations are required for this species.

As theoretically expected, populations of *C. tenuispina* tending towards clonality had a significant heterozygosity excess, which translates into deviations from HWE and negative F_{IS} values (Balloux *et al.* 2003; Stoeckel & Masson 2014). This process has been explained by the accumulation of somatic mutations, and therefore new alleles, which increase heterozygosity in clonal lineages. However, some authors defend the idea that heterozygosity can be considered as an evolutionary characteristic that retains diversity within individuals, and therefore potentially increase fitness (Marriage & Orive 2012). Although the results of this study do not shed light on this particular debate, whether this hypothesis is true or not, the species *C. tenuispina* would experience powerful mechanisms to maintain fitness that would include both retention of genetic diversity and telomere elongation during fission (Garcia-Cisneros *et al.* 2015). Both mechanisms would have considerable evolutionary impact in the long and short term, avoiding many of the most negative effects of the absence of sexual reproduction and providing this species with a large coloniser potential (Lavergne & Molofsky 2007).

Conclusions

The phylogeographic pattern of *C. tenuispina* shows that even its very low values of genetic diversity do not seem to affect the fitness of the populations, and the starfish has been capable of spreading across warm and temperate areas of the Atlantic Ocean and across the entire Mediterranean Sea, with different environmental conditions. Its biological plasticity, and the existence of mechanisms for preventing senescence by telomere elongation in asexual populations, may play a major role in the colonisation potential and success of this species. As in other marine invertebrates, major marine barriers limit genetic interchange, although more accentuated in some areas where the populations are mostly asexual and cannot generate larvae for dispersal. Nevertheless, under the foreseeable scenario of global warming (Hoegh-Guldberg & Bruno 2010), the temperature might not be an obstacle to the development of sexual reproduction and viable larvae for dispersal and colonisation of new marine areas, thereby expanding the current distribution of the species. Future studies considering thousands of independent nuclear markers, such as single nucleotide polymorphisms (SNPs), could provide a better understanding of the most important polymorphisms associated with local adaptation,

and which have facilitated the expansion of this species across the warm and temperate Atlantic and Mediterranean areas.

Acknowledgements

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Data accesibility

DNA sequences: Genbank accessions of all sequence haplotypes (accession numbers pending of manuscript acceptance).

Sampling locations and microsatellite genotypes: Dryad doi (pending of manuscript acceptance).

Capítulo 4

Hope springs eternal in the starfish gonad: preserved potential for sexual reproduction in a single-clone population of fissiparous starfish

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Abstract

Among echinoderms, asexual reproduction by fission occurs in few species. This strategy is considered as a response to stressful conditions and usually alternates with sexual reproduction events, being the populations that only reproduce asexually extremely rare. The occurrence of a single-clone population of the starfish *Coscinasterias tenuispina* at Llançà (NW Mediterranean) allowed us to study intra-clonal variation of the reproductive cycle during a two-year study. The few developed gonads (all male) were found in winter months, coinciding with the minimum photoperiod (ρ=-0.82; p<0.001) and lowest temperatures (ρ=-0.75; p<0.001), only in best-fed individuals, indicating that food availability influences individual ability for gonad development. Fissiparity happened throughout all the sampled period, but its rate increased with warm temperatures (ρ=0.68; p<0.0001). In contrast to what has been reported in other species, no correlation between fission rates and population density was found. The population was maintained over time by asexual reproduction and remained monoclonal. Though sexual reproduction has probably not occurred in this all-male population for a long time, the ability to yearly produce mature gonads is retained by some individuals, indicating that potential to reproduce sexually may be preserved, even in the case of strictly asexual populations.

Keywords: sea star, echinoderm, reproductive cycle, asexual reproduction, fission, photoperiod.

Resumen

Entre los equinodermos, la reproducción asexual por fisión se produce en algunas especies. Esta estrategia se considera una respuesta a condiciones de estrés y, en general, se alterna con eventos de reproducción sexual, siendo las poblaciones que sólo se reproducen asexualmente extremadamente raras. La aparición de una población de la estrella de mar *Coscinasterias tenuispina* en Llançà (NW Mediterráneo) formada por un solo clon, ha permitido estudiar la variación intra-clonal del ciclo reproductivo durante un periodo de dos años. Las pocas gónadas desarrolladas (únicamente del sexo masculino) fueron encontradas en los meses de invierno, coincidiendo con el fotoperíodo más corto (ρ = -0,82; p < 0,001) y las temperaturas más bajas (ρ = -0,75; p < 0,001), y sólo se hallaron en los individuos mejor alimentados, lo que parece indicar que la disponibilidad de alimentos influye en la capacidad individual para desarrollar gónadas. A lo largo de todo el período de muestreo fue constante la fisiparidad, pero su tasa aumentó con las temperaturas cálidas (ρ = 0,68; p < 0,0001). En contraste con lo que se ha reportado para otras especies, no se encontró ninguna correlación entre las tasas de fisión y la densidad de la población. La población se ha mantenido a lo largo del tiempo por reproducción asexual y además, ha permanecido monoclonal durante todo el período de estudio. Aunque probablemente en esta población de machos la reproducción sexual hace tiempo que no ha sucedido, la capacidad de producir anualmente gónadas maduras se ha mantenido en algunos individuos, lo que indica que el potencial de reproducción sexual se conserva, incluso en el caso de poblaciones estrictamente asexuales.

Palabras clave: Estrella de mar, equinodermo, ciclo reproductivo, reproducción asexual, fisión, fotoperíodo.

Introduction

Echinoderms have evolved a number of deviant reproductive strategies (Lawrence & Herrera, 2000) of which fissiparity, frequently associated with stressful conditions (Mladenov 1996), has been considered as an extreme response (Achituv & Sher, 1991). Even though regeneration abilities are widespread in echinoderms, only about 1.3 % of echinoderm species are able to reproduce by fission during adulthood, being Ophiuroidea, followed by Asteroidea, the classes with most species showing this capacity (Emson & Wilkie, 1980; Lawrence & Herrera, 2000). Asexual reproduction in echinoderms is characterized by fragmentation or fission in free-living individuals, thus yielding mobile ramets of considerable dispersal capacity, whereas most other benthic clonal organisms (e.g. tunicates, bryozoans or corals) are colonial and composed of immobile ramets (Jackson & Coates, 1986). In echinoderms, like in most marine invertebrates, sexual and asexual phases usually alternate within the same population. However, some populations may be obligately fissiparous, having lost the ability to develop gonads (e.g. Mladenov et al. 1986). Despite being based in asexual reproduction as a main strategy, populations are usually composed of different genetic individuals (genets).

Coscinasterias tenuispina (Lamarck 1816) is a starfish species found in the Mediterranean Sea and temperate and subtropical Atlantic rocky shores. Like all representatives of the genus, *C. tenuispina* is heterogonic (i.e. able to reproduce both sexually and asexually, Crozier, 1921; Alves et al., 2002). Detailed studies described the reproductive cycle of congeners *C. muricata* from New Zealand (Crump & Barker, 1985; Georgiades et al., 2006; Barker, 2013), and *C. acutispina* from Japan (Seto et al. 2000). Though these studies noted some intraspecific differences between localities in the biological cycles and fission behavior (Seto et al., 2000; Alves et al., 2002; Sköld et al., 2002), clonality was found to be widespread in most studied populations (Crump & Barker, 1985; Sköld et al., 2003; Haramoto et al., 2007; Barker, 2013). Sex biased ratios are frequently reported in asexual echinoderms, with presence of only one gender (most frequently males) in some localities (Achituv & Sher, 1991; Chao & Tsai, 1995; Uthicke et al., 1999; McGovern, 2002; Rubilar et al., 2005; Barker & Scheibling, 2007), a fact regularly found in genus *Coscinasterias* (Crump & Barker, 1985; Seto et al., 2000; Alves et al., 2002; Sköld et al., 2002). Asexual reproduction is often related to some morphological treats in starfish, such as individuals having usually more than five arms (Mladenov et al., 1986) of different lengths. Crozier (1921) also noted the occurrence of multiple madreporites in fissiparous starfish, and proposed an association between the number of madreporites and the prevalence of asexual reproduction.

Environmental conditions are usually highly correlated with the biological cycles of echinoderms. Thus, environmental changes modulate the reproductive potential of species and are responsible of large density variations within populations (Uthicke et al., 2009). Seawater temperature, photoperiod, tidal cycles and food availability influenced differently the prevalence of sexual and asexual reproduction within genus *Coscinasterias* (Barker, 2013), as in other starfish genera (Rubilar et al., 2005). Body size and fed condition have been found to modulate clonal starfish fission (Emson & Wilkie, 1980; Crump & Barker, 1985; Sköld et al., 2003; Haramoto et al., 2007; Rubilar et al., 2011; Seto et al., 2013), being fission more frequent in small individuals (Crump & Barker, 1985; Barker & Scheibling, 2007). Other factors, such as population density, also modulate fission in echinoderms, being asexual reproduction more prevalent where population densities are lower (McGovern, 2003; Sterling & Shuster, 2011). Sexual reproduction is regulated by external factors as well. Gonad output is often correlated with seawater temperature in tropical and temperate echinoderm species, such as *Coscinasterias*, whereas other factors, such as photoperiod, regulate reproductive timing (Wangensteen et al., 2013b). The gonadal cycle of *C. tenuispina* has been studied only in two Western Atlantic localities, in Brazil (Alves et al., 2002) and Bermuda (Crozier, 1921), where it develops mature

gonads in winter. Surprisingly, the reproductive cycle of this species is almost completely unknown in Northeastern Atlantic or the Mediterranean Sea, though Tartarin (1953) studied fission and regeneration processes using Mediterranean individuals of *C. tenuispina*.

In a recent study, Garcia-Cisneros et al. (2015) revealed striking differences in the genetic structure of populations of *C. tenuispina* from Canary Islands (East Atlantic) and Northwestern Mediterranean. Canarian populations are partially clonal and composed of several genotypes, whereas only one genotype was found in the Northwestern Mediterranean population, thus suggesting that this population could be strictly monoclonal. The relative importance of sexual and asexual reproduction may then be related to the different environmental conditions in these localities. Furthermore, the species demonstrated the existence of enhanced mechanisms for telomere elongation to avoid senescence in Mediterranean populations (Garcia-Cisneros et al., 2015), suggesting a potential for long-term stability when fission is the main reproductive strategy.

In the present work, we empirically studied the reproductive traits and population structure of a putative strictly monoclonal wild population of *C. tenuispina* in the Northwestern Mediterranean. Our aims were: a) to confirm that the population (studied in Garcia-Cisneros et al., 2015) remained monoclonal over time, thus assessing its temporal stability and the absence of recruitment of new genets, b) to test whether both reproductive strategies, and specifically the potential for sexual reproduction, are preserved over time in such monoclonal population, and c) to study the temporal patterns and the individual variability of both sexual and asexual behaviors, population density and size structure dynamics, in order to infer which environmental or internal factors may modulate both reproductive strategies.

Materials and methods

Study site

The main sampling was carried out near Llançà, in Punta de la Figuera (Cap Ras, NE Spain, 42.385 N, 3.162 E) (Fig. 1), between January 2012 and April 2014. It is a sheltered bay opening towards South, with mixed sandy and rocky bottoms, covered by abundant boulders of different sizes near the coastal line. The boulders were covered by diverse turf and erect algal species. The sampling site was divided in three areas (Fig. 1), where the first two areas were intended to analyze every month the density and size structure of the starfish along the year, and a third one to characterize the size structure of the population. Individuals of *Coscinasterias tenuispina* were found mainly under boulders, coexisting with other metazoan species, of which the most common were small porcelain *(Porcellana platycheles, Pisidia longicornis)*, hermit *(Diogenes sp.)* and brachiuran crabs *(Xantus sp., Pachygrapsus marmoratus)*, different genera of polychetes, gastropods *(Gibbula sp., Haliotis sp.)*, polyplacophorans *(Chiton sp.)*, ophiuroids *(Ophiothrix sp.)*, small individuals of the echinoid *Paracentrotus lividus*, sponges, actiniaria *(Anemonia sp.)* and colonial ascidians.

For comparison purposes, in August 2013, November 2013, and February 2014, a total of 33 specimens of *C. tenuispina* were collected from La Palma, Canary Islands (Eastern Atlantic Ocean, 28.688 N, 17.759 W). These individuals were used to compare individual sizes in localities with different temperature patterns. Water temperature in Canary Islands is less variable and approximately ranges from 18° to 24° C, whereas in the Mediterranean locality here analyzed water temperature is colder in winter and warmer in summer, with an approximate range of variation from 12° to 27° C (www.meteoestartit.cat, www.puertos.es).
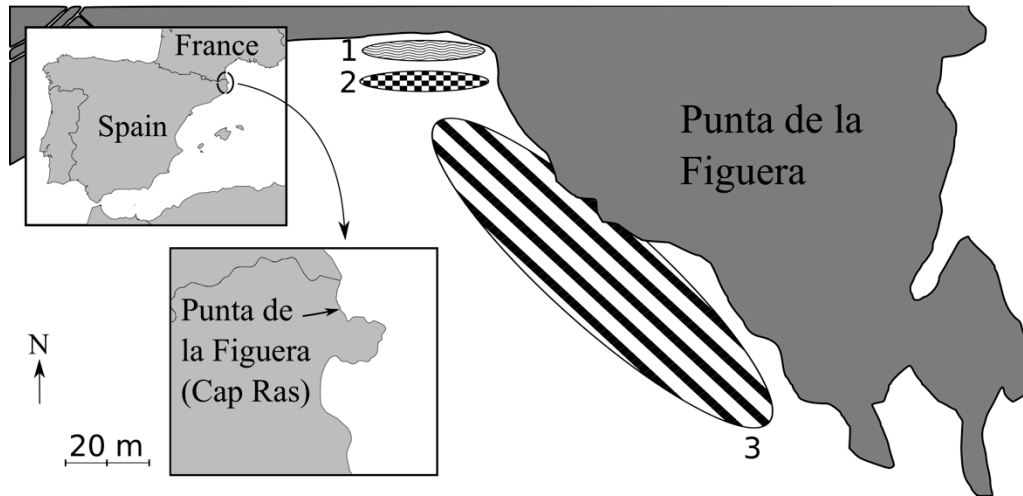
Figure 1. Map of the sampling locality near Punta de la Figuera (Cap Ras, Llançà, Spain), showing the Inshore (1, wave lines) and Offshore (2, checkered) areas where density measures were taken, and the additional area (3, diagonal lines) sampled for size distribution, fissiparity and gonadal cycle studies.

During the study, some relatively intense weather events occurred at the Mediterranean location, which should be considered as potentially able of affecting the studied biological cycle: two cold snaps in February 2012 and December 2013, two heat waves in August 2012 and November 2013 and two hard Levanter (easterly wind) episodes at the end of September 2012 and February 2013. Precipitations in this Mediterranean locality are scarce and their effect on coastal ecosystems is usually negligible. However, monthly rainfalls of over 100 mm m^{-2} month^{-1} were recorded in May 2012 (101 mm), October 2012 (157 mm), March 2013 (160 mm) and November 2013 (159 mm).

Clonality assesment

To confirm the number of genotypes (clones) found in the study site, tissue from 30 individuals was collected a few months before the monitoring study (June 2011), and 30 individuals after the study was completed (July 2014). DNA was obtained from tube feet. Tissue was collected with forceps, and kept in absolute ethanol until analysed in the molecular laboratory. All individuals were returned back to the water immediately after tissue sampling. Twelve microsatellite markers specifically designed for *C. tenuispina* were amplified (m.ten1, m.ten6, m.ten13, m.ten14, m.ten19, m.ten24, m.ten25, m.ten27, m.ten30, m.ten31, m.ten32 and m.ten40), and used for genotyping as specified in Garcia-Cisneros et al., (2013). Briefly, DNA extractions and microsatellite amplifications were carried out using a REDExtract-N-AmpTM PCR ReadyMixTM kit (Sigma-Aldrich, www.sigmaaldrich.com). PCR were performed following the Sigma-Aldrich protocol, with 10 pmol for each primer in 20 μl of PCR reaction. All amplifications were run using a protocol that included an initial step of 95 ºC for 60 s; 35 cycles of 95 ºC for 30 s, 49 ºC for 20 s, and 72 ºC for 80 s; and a final step of 72 ºC for 5 min. Amplicon sizes were analyzed in an Applied Biosystems 3730xl capillary electrophoresis system (Applied Biosystems, www.appliedbiosystems.com). Allele sizes were measured with Peakscanner v.3.2 (Applied Biosystems), and scored in MsatAllele (Alberto, 2009). Identical multilocus genotypes were identified as clones in the software Genodive (Meirmans & Van Tienderen, 2004).

Population density

Starfish density measures were made during 28 consecutive months (January 2012- April 2014) at two different areas (Fig. 1). The Inshore area (1) was closer to the shore, and $0 - 0.5$ m deep; and Offshore area (2), further from the shore, and 0.5 - 1 m deep. Density measures were carried out using a 1 m² PVC quadrat and 10 random replicates per area, avoiding the repetition of plots and sites with rocks outside the water. We carefully tumbled all rocky boulders inside the quadrat and the plot was thoroughly revised by at least two different divers, as the starfish were usually cryptic.

Size structure and asexual reproduction

In order to characterize the size population structure along the year, between 50 and 73 individuals randomly collected were measured every month, including individuals sampled in the Inshore and Offshore areas, and additional area 3 (Fig. 1). We performed size structure analyses grouping individuals of the three areas together to obtain an adequate sampling size, but also separately for the Inshore and Offshore areas.

Each individual was photographed on a scaled tablet for body size measurements, and arms were counted manually given the difficulty for recognizing smallest arms (1 - 2 mm long) in a picture (Fig. 2). Every individual was returned to the same place of capture, immediately after processing. The lengths of all arms were measured afterwards in the laboratory, from the scaled pictures using ImageJ 1.48k software (Abramoff et al., 2004).

To compare differences in size class distribution of the NW Mediterranean population (Cap Ras, Llançà) with those from the Atlantic Ocean, we also measured 33 randomly collected specimens of *C. tenuispina* from La Palma, Canary Islands, as a reference. All samples from Canary Islands were measured following the same procedure of body size measurement explained before.

For the study of asexual reproduction, a regeneration index (RI) was calculated as the ratio
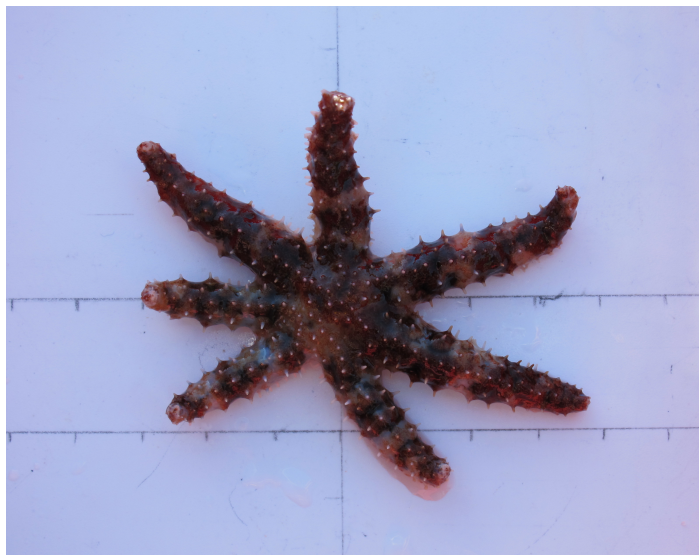

Figure 2. Picture of a scaled tablet with an individual of *C. tenuispina*.

between the longest and the shortest arm (Achituv, 1969; Achituv & Sher, 1991) for 50 individuals sampled in the Inshore, Offshore and Additional areas every month. Individuals were classified afterwards into three categories previously defined according to Alves et al., (2002) and Chao and Tsai (1995): a) recently split if RI was ≤ 25 %; b) regenerating individuals if 25 % < RI < 80 % and c) intact individuals when RI ≥ 80 %. Because some authors (Crozier, 1921) proposed an association between number of madreporites and fissiparity, the number of madreporites from each individual was also counted and recorded in the field.

Sexual reproduction

Every month, between April 2012 and April 2014, 10 individuals were collected from the Additional area 3. Samples were extracted from this area for histology analyses, because a periodic extraction from the Inshore and Offshore areas could have potentially affected posterior measurements of density and population's size structure. Furthermore, all 33 individuals from Canary Islands were also processed for histology analyses.

Individuals collected were then anesthetized in 37% $MgCl_2$ and seawater 1:1 in order to avoid the arms split by stress. Afterwards, individuals were stored in 4% formaldehyde until dissection. Given the remarkable asymmetry of *C. tenuispina* arms, gonads (when present) and pyloric caeca were weighted separately for every arm. Gonad index (GI) and pyloric caeca index (PCI) were calculated as weight of the organ / whole body weight x 100, following similar studies (Giese, 1966; Alves et al., 2002; Rubilar et al., 2005, 2011; Pastor-de-Ward et al., 2006; Haramoto et al., 2007).

All gonads found, even when they came from the same individual, were embedded in paraffin for histology, cut in 5 μm sections using a Microm HM325 Microtome, and stained in haematoxylin–eosin. Gender and developmental stage were determined (Delavault, 1961; Walker, 1980) under the optical microscope.

Temperature and photoperiod

Monthly mean values for sea surface temperature (SST) were obtained from the nearby L'Estartit Meteorological Station (http://www.meteoestartit.cat). Daily *in situ* measures of underwater temperature using electronic thermometers during the sampling days showed negligible differences with temperature recordings at L'Estartit. Photoperiod data were obtained from the Spanish Ministry of Development (http://www.fomento.gob.es).

Data analyses

Non-parametric statistic procedures were used for all analyses of population density, sexual and asexual cycles, and effect of temperature and photoperiod on these cycles, since data did not match normality and homoscedasticity assumptions. All analyses were performed in R (R Core Team, 2014).

Differences between mean density values in the Inshore and Offshore areas were assessed using a paired-samples Wilcoxon test. Temporal differences in population density were analyzed separately for both areas with Kruskal-Wallis tests using sampling date as factor and a Wilcoxon test was applied to detect differences between the first and the second semester of each year, in order to detect patterns in different seasons. The effect of density on asexual reproduction was assessed by Spearman correlation coefficients, comparing the frequencies of recently-divided

individuals and the densities in the Inshore and Offshore areas (separately or grouped), from the same month and also from the precedent month.

Kruskal-Wallis tests were also used to analyze monthly differences in individual size, GI, PCI and number of madreporites (using sampling date as a factor) and for comparing the number of madreporites of individuals in different regeneration stages (using regeneration stage as a factor). When significant, all Kruskal-Wallis tests were followed by pairwise Wilcoxon tests to detect differences between months or regeneration stages. Values of significance (*p*-values) were then corrected by Benjamini–Hochberg (1995) FDR corrections.

Spearman correlation coefficients between PCI, GI, temperature and photoperiod were calculated to assess the relationship among gonad maturation, the accumulation of reserves in the pyloric caeca, and the effect of environmental conditions. The correlation between the ratio of recently divided individuals and PCI was performed with a moving average of three months in order to filter fluctuation noise. Correlation between recently divided individuals and temperatures recorded during the previous month were also performed.

Results

Clonality assessment

All 60 individuals of *Coscinasterias tenuispina* genotyped using 12 microsatellite loci, before (2011) and after the study period (2014), shared exactly the same genotype, and coincident with the 17 individuals sampled in 2011 reported in Garcia-Cisneros et al., (2015) from the same locality, but collected at the most southeastern point of area 3 (Fig. 1). Thus, the studied population was genetically stable during the time of this study, and composed by a single genetic clone in absence of recruitment of new genotypes.

Population density

Population density of *C. tenuispina* fluctuated remarkably along the study period (Fig. 3), showing values between 0.0 and 3.8 individuals m^{-2} in the Inshore area, and between 0.0 and 1.8
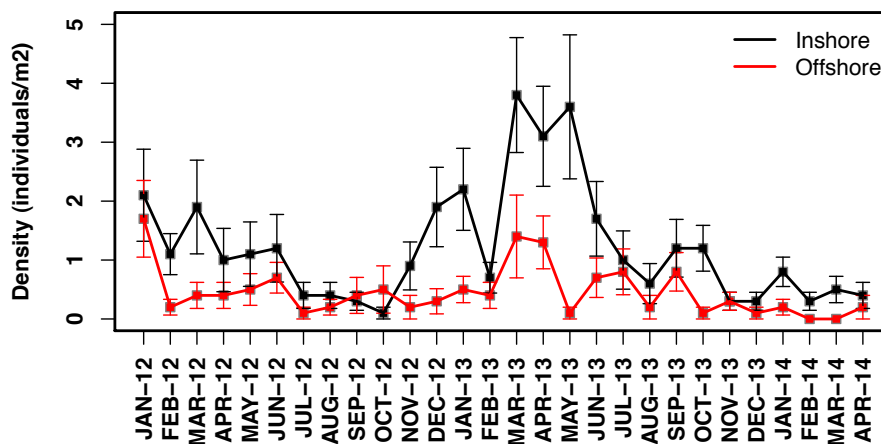
Figure 3. Monthly density values of *C. tenuispina* at two different areas throughout more than two years of sampling. Inshore area (1) is represented in black and Offshore area (2) in red. Vertical bars represent the standard error.

individuals m^{-2} in the Offshore area. The density values measured in the Inshore area were significantly higher than in the Offshore area (Wilcoxon's paired U = 366; p = 2 ·10^{-5}).

In the Inshore area, the temporal trend showed two consecutive population density peak periods roughly coinciding with some months of the first semester of each year (between January and June of 2012 and between December of 2012 and June of 2013), followed by a density drop in July (pairwise Wilcoxon's tests, p < 0.05) with the exception of February 2013 when a low density value was measured (Fig. 3). However, this trend was not found during the sampled months of 2014. In the Offshore area, the population density seemed to follow the same trend (remarkably higher values in January 2012 and March-April 2013), though the smaller densities in this area make the trend scarcely discernible. Following strong Levanter winds (end of September 2012 and February 2013), decreases of density in the shallower Inshore area were observed, while the Offshore area was not apparently affected. Increased precipitations, cold snaps or heat waves did not have any apparent effect in the measured population densities.

No correlations were found between measured densities and ratio of recently-divided individuals, neither Inshore nor Offshore, with Spearman ρ < 0.2 and p > 0.05 in all correlation comparisons.

Size structure and asexual reproduction

For analyzing the size structure, a total of 1,554 individuals were photographed and measured. The median number of arms was 7 in most sampled months (except January 2012, May 2012 with 6 and October 2013 with 8) and the maximum number of arms was 11 (in only one individual). Body size measurements confirmed that no newly arrived settlers were detected during the sampling, since only three individuals had the longest arm smaller than 10 mm. The largest individual from Llançà measured 57.6 mm for its longest arm, whereas the average size for the longest arm of all individuals of this population was only 26 mm. The size structure of
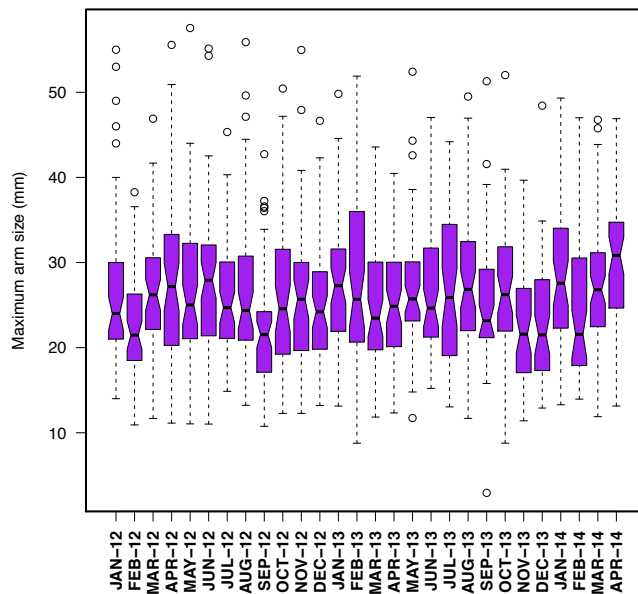
Figure 4. Monthly size structure (longer arm) of *C. tenuispina* population along the study. Box edges represent the first and third quartiles and whiskers extend to the most extreme data points. Outliers are represented by open circles. Notches represent median values.

Figure 5. Monthly percentages of individuals of *C. tenuispina* in each regeneration state, based on the regeneration index (RI) obtained from the whole population sampled. a) Recently split when RI was ≤ 25 % (in blue); b) regenerating individuals, 25 % < RI < 80 % (in red), and c) intact individuals, RI ≥ 80 % (in green).

the population stayed remarkably homogeneous along the sampling period, and only departed significantly from the main trend (being a little higher) at the end of the study (April 2014, Fig. 4 and Fig. S1). Thus, no clear seasonal trend in size structure was found.

The size class distribution comparison between the NW Mediterranean population (Llançà) and the Atlantic population (La Palma) showed large differences in body size frequencies (see Fig. S2). Specimens from the NW Mediterranean were much smaller, being 20-25 mm the most frequent size class, whereas in La Palma 45-50 mm was the most frequent size. The largest individuals from the Atlantic were over 120 mm for their longest arm.

The fissiparity cycle was explored based on the 1,554 individuals measured. All regeneration stages (recently split, regenerating and intact individuals) were found for every month sampled (Fig. 5). Intact individuals were scarce (0 − 5 %) throughout the sampling period, and most individuals were in regeneration stage or recently divided, indicating continuous fissiparity activity along the year. Recently split individuals were more abundant during late summer and early autumn months (September-November). The fission frequency increased with temperature, as indicated by a remarkable correlation found between the percentage of recently divided individuals and the average temperature during the previous month (Spearman ρ= 0.68; p < 0.0001; Fig. 6). PCI values were negatively correlated with the ratio of recently divided individuals when moving average of three months was considered (Spearman ρ= -0.46; p = 0.028; Fig. 7).

Recently divided individuals were more frequently found in the Inshore area; however, the difference with the Offshore was not significant (Fig. S3).

All individuals had between 1 and 4 madreporites. Slight differences were found between months. Overall, recently divided individuals were found to have less madreporites than those in regeneration (Fig. S4, Wilcoxon test: p < 0.001).
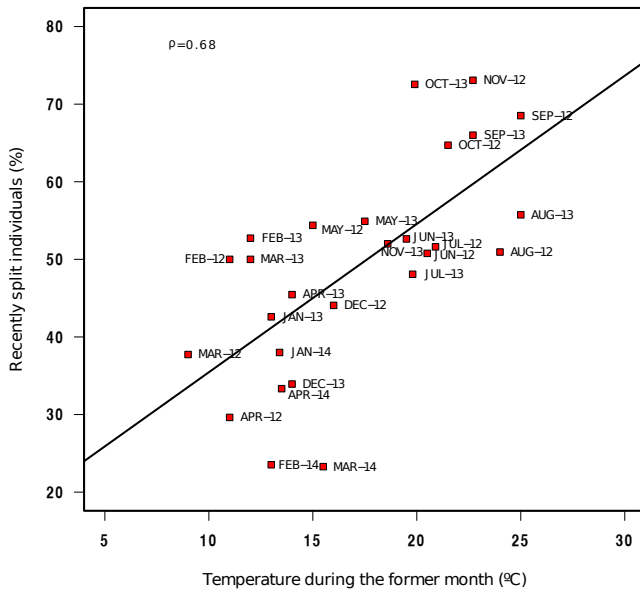
Figure 6. Correlation between the percentage of recently-divided individuals and the average water temperature during the previous month.
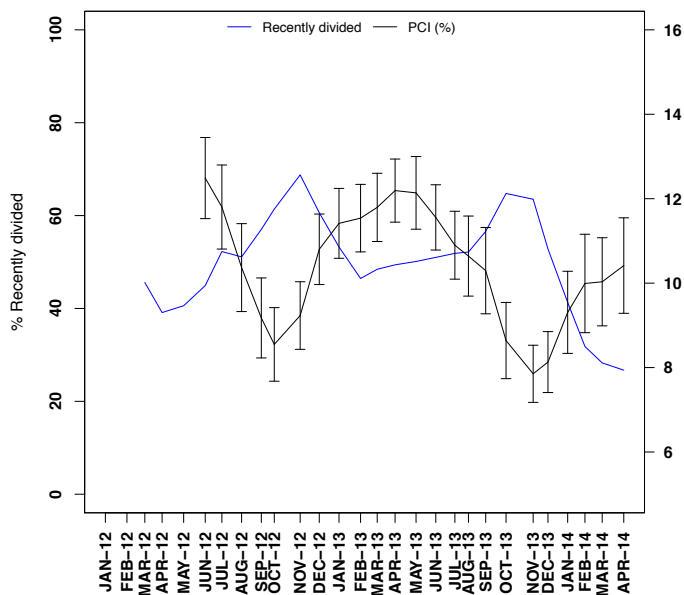
Figure 7. Gonad index (GI, in black) and pyloric caeca index (PCI, in red) for the ten dissected individuals of *C. tenuispina* per month, from April 2012 to April 2014. Note that each index has different scales, where GI scale range from 0 to 0.6 % on the left side, and the PCI scale range from 5 to 14 % on the right side of the graph. Vertical bars represent the standard error.
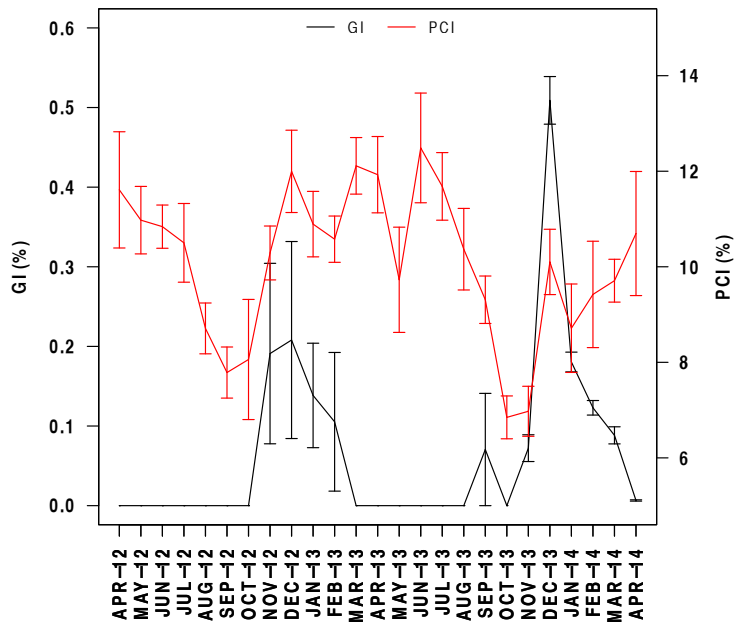
Figure 8. Plot of the pyloric caeca index (PCI), separately for individuals with developed gonads (in red) and individuals without gonads (in black). Vertical bars represent the standard error.

Sexual reproduction

A total of 237 individuals from Llançà were collected and dissected, from April 2012 to April 2014. From the total specimens dissected, only 55 had gonads (23 %). In most of these individuals with gonads, they were present in just one arm. When present, gonads were remarkably small, accounting only for 0.1 – 0.5 % of the total weight of the individual.

The histology of these gonads revealed that all of them were testes, and therefore males. Three different maturation stages were observed: growing, pre-mature and mature (Fig. S5). However, the first stage was found in just one gonad from September 2013. Gonads were more developed during winter months (November – February, Fig. 8), thus showing a negative correlation of GI with photoperiod (ρ = -0.82, p < 0.001) (Fig. 9) and temperature (ρ = -0.75; p < 0.001), and only present in arms larger than 12 mm. In contrast, one female and 5 males were observed from the Canary Islands.

Analysis of the PCI revealed that pyloric caeca represented the 7-12 % of the animal weight (Fig. 8). Arms smaller than 5 mm rarely had pyloric caeca. The temporal trend showed remarkable drops in the average PCI values of the population in summer, during the months of highest asexual reproduction activity and before gonad development. However, an inverse relationship between GI and PCI were not found. Moreover, a significant trend was found with individuals having gonads displaying higher PCI values than those without gonads from the same month (Fig. 10).
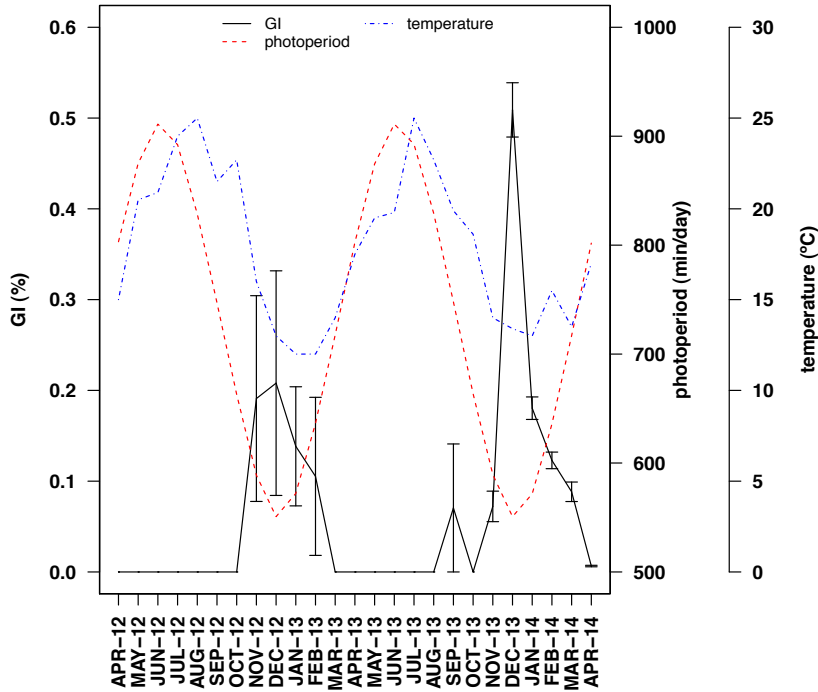
Figure 9. Plot showing the correlation of gonad index (GI, black line), photoperiod (red dashed line) and temperature (blue dashed line). Vertical bars represent the standard error.
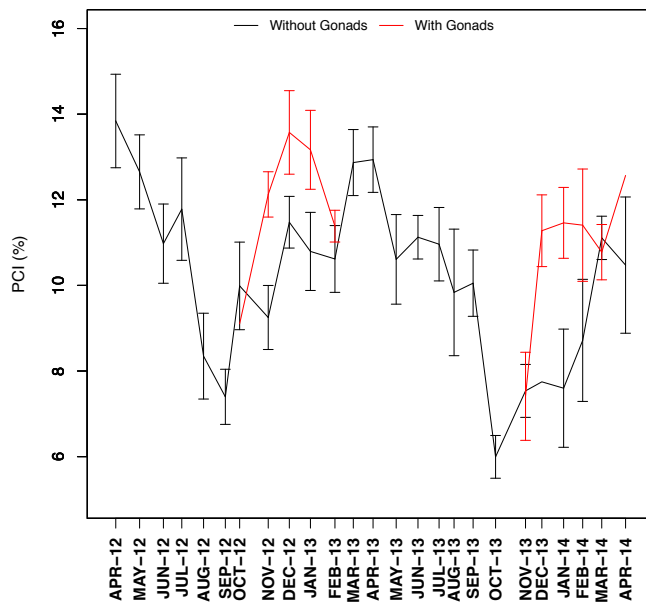
Figure 10. Plot representing differences between the GI from individuals with gonads (in red) and individuals without gonads (in black). Vertical bars represent the standard error.

Discussion

To the best of our knowledge, this is the first work studying the reproductive cycle of a single-clonal population of a Metazoan in natural conditions. Although *Coscinasterias tenuispina* is able to reproduce both sexually and asexually in warm waters, such as in the Canary Islands (Garcia-Cisneros et al., 2015), some populations in the colder waters of Northwestern Mediterranean are maintained over time by asexual fissiparity, as evidenced by all analyzed individuals before and after this work sharing exactly the same genotype. With 77 random individuals genotyped along three years, we can confirm that the entire population studied at Llançà remained strictly monoclonal throughout the whole sampling period. The number of asexual generations since the original colonizer settled is unknown, but the presence of enhanced mechanisms to avoid senescence in this population suggests the possibility of many asexual generations (Garcia-Cisneros et al., 2015).

Some singular facts may then be pointed out for a better interpretation of our results. Since all individuals from the studied population share a common genotype (besides somatic mutations), then all inter-individual variability found in somatic indices, and other studied life traits, is attributable to environmental or stochastic factors. Thus, this population of *C. tenuispina* can be considered as a unique natural model for studying inter-individual variability unlinked to genetic factors.

The temporal trends in the NW Mediterranean population indices of *C. tenuispina* show a mixed pattern of yearly recurring events with other of higher inter-annual variability. Gonad development seems to be a fixed character following a regular pattern across years, whereas fissiparous reproduction, pyloric caeca index (a measure of food availability), population density and size distribution are more irregular, probably due to the influence of environmental changes. Thus, our results agree with the view that, whereas sexual reproduction cycles are broadly regulated by underlying genetic patterns, adapted to natural periodicities; asexual reproduction may otherwise be an adaptive response to changes in environmental conditions (Mladenov 1996).

Echinoderms from subtropical and temperate latitudes usually show markedly seasonal gonadal cycles (Xu & Barker, 1990; Rubilar et al., 2005; Georgiades et al., 2006; Pastor-de-Ward et al., 2006; Mercier & Hamel, 2013), probably determined by genetic traits selected for adapting to the prevalent conditions in their original distribution areas. Recent colonizers of the Mediterranean (during the last interglacial periods) may retain these genetically programmed reproductive traits (Wangensteen et al., 2013b), despite their failure to attain regular successful reproduction events, if other compensation mechanisms are acting which are able to sustain populations. Asexual reproduction by fissiparity seems to be a crucial such compensation mechanism in the case of NW Mediterranean populations of *C. tenuispina*. The observed correlation of the gonadal cycle of *C. tenuispina* with photoperiod indicates the regulation of gametogenesis timing by photoperiod, with gonads developing during winter months, coinciding with the timing of populations from Brazil (Alves et al., 2002) or Bermuda (Crozier, 1921), which supports this hypothesis. Therefore, photoperiod could regulate the timing for gonad development, while other factors may modulate the chance of successfully completing the sexual reproductive cycle (e.g. increasing the fecundity by producing larger gonads or influencing larval survival or settlement success; Kettle & Lucas, 1987; Yund 2000). Other species of *Coscinasterias* also show regulation of gonad development by photoperiod (Georgiades et al., 2006). Moreover, photoperiod is known to regulate gametogenesis in shallow water starfish species (Pearse & Walker, 1986), whereas other traits such as pyloric caecum index or body size were not influenced by photoperiod (Xu & Barker, 1990). However, previous studies on *C. muricata* detected mature gonads only in well-fed individuals (Sköld et al., 2002). This is in agreement with our finding of individuals with gonads having higher pyloric caeca index values

than individuals that were unable to develop gonads (Fig. 10), and reinforces the view that producing gonads is an expensive energy investment.

Genetic uniformity and absence of new recruits of small size classes in our locality (Fig. S1) demonstrate the absence of new larval settlers in this area. No specific studies have been carried out to explain the lack of sexual reproduction in NW Mediterranean populations of *C. tenuispina*. However, a plausible cause is the cold temperature prevailing during winter months in this region, which would prevent larvae to achieve complete development. Other echinoderms of tropical affinities with overlapping distribution area, such as the sea urchin *Arbacia lixula*, has been proposed to be also limited by cold temperature, which would increase larval mortality and thus prevent successful recruitment events in NW Mediterranean (Wangensteen et al., 2013a). Similar considerations are probably valid for the larvae of *C. tenuispina*. However, while the sexually reproducing *A. lixula* could be positively selected for better reproductive individuals, improving and genetically adapting its sexual cycle generation after generation, *C. tenuispina* would remain asexual in the same area, without any genetic change, because no recombination or selection would be possible in this monoclonal population.

Besides the environmental conditions, two main additional drawbacks have been related to asexually reproducing populations: the accumulation of deleterious somatic mutations and the lack of genetic diversity for adaptive potential (Becks & Agrawal, 2012; Honnay & Bossuyt, 2005). Most natural asexual populations are supposed to overcome these handicaps by performing sporadic episodes of sexual reproduction. However, this case is unlikely for *C. tenuispina* in NW Mediterranean, since the absence of females evidently prevents any sexual success. Despite this limitation, in our studied population, some clonal individuals continue to grow mature gonads and therefore, spend energy in potential for sexual reproduction, with null success. Besides the possibility to retain the genetically programmed reproductive traits, this could allow for future potential sexual reproduction events in the population, provided that the sex ratio changes due to colonization by females from other populations; however, some energy surplus is being presently wasted in reproductive potential by this population, even though slim chances of a hypothetical future regaining of successful sexual reproduction can be expected. The population of Llançà thrives in an area where environmental conditions prevent the completion of the sexual cycle. Genetic diversification (including the appearing of females) in these conditions could only be possible if a larval supply existed, originated in distant areas where sexual reproduction is possible, from which new settlers could be provided. However, that does not seems to be the case for our studied population, which is probably too geographically distant from other populations where environmental conditions favour sexual reproduction. Conversely, the presence of both males and females within the same population in Canary Islands allows for successful sexual reproduction and therefore the higher genetic diversity previously found in these Islands (Garcia-Cisneros et al., 2015).

It is evident that asexual reproduction has an impact on sex ratio of fissiparous starfish. Studies on Brazilian *C. tenuispina* showed that this ratio was skewed toward majority of males or only males (Alves et al., 2002). Similar results were noted in Barker (2013) who pointed out that other species of the genus *Coscinasterias* had usually biased sex ratios (Crump & Barker, 1985). In Japan, all-male populations of *C. acutispina* have been reported, whereas other populations in the same study were biased towards females (Seto et al., 2000). Dramatic changes in sex ratio are possible in some populations; e.g., a population of *C. calamaria* from New Zealand, having originally more females, was reported to have changed towards only males in 2002 (Sköld et al., 2002). These changes suggest that all these populations, despite being mainly asexual, are subject to the arrival of new genets by either recruitment of new larvae or adult dispersal. Though biases toward males are more commonly found in most fissiparous echinoderms, including holothuroids (Conand et al., 2002), ophiuroids (Chao & Tsai, 1995, McGovern, 2002) and asteroids (Crump & Barker, 1985; Alves et al., 2002; Sköld et al., 2002; Rubilar et al.,

2005), females can also reproduce asexually and all-female populations has been occasionally found (Mladenov & Emson, 1988). In the ophiuroid *Ophiactis savigni*, fission was proven to be more frequent in males than females (McGovern, 2002). Although no conclusive studies have explained this trend in sex ratio toward males, it was proposed that sperm dilution could be a limiting factor for fertilization in broadcast spawning species, so that increasing the number of males could be a strategy to increase fertilization success during the sporadic sexual events (Yund, 2000).

Although fissiparity events happened in our study population throughout the sampled period, a prevalence of recently split individuals during late summer and early autumn could be observed during two consecutive years (Fig. 5), and a significant correlation of the ratio of recently divided individuals with the average temperature during the previous month was found (Fig. 6). The percentage of recently split individuals was negatively correlated with fed-condition when the average values of PCI and fission were considered. However, with our data we cannot tell if low nutritional condition promoted fission or were simply affected after fission. Nutritional status tends to decrease after asexual reproduction, probably due to the energy transfer needed to regenerate new tissues and organs. Furthermore, loss of arms could limit the covered area and the movement of the individual, impairing its ability to find new preys. Both factors, temperature and nutritional conditions, have been already proposed to regulate fission in *C. acutispina* and *C. muricata* (Sköld et al., 2002; Haramoto et al., 2007; Seto et al., 2013), whereas in *C. calamaria* food availability was negatively correlated with fission, as in our case (Crump & Barker, 1985). On the other hand, the temporal patterns of the number of madreporites contributed little to understanding the asexual reproductive cycle of our population.

Despite the correlation found between high temperature and fissiparity rates, the populations of *C. tenuispina* from the Canary Islands, which are subject to higher average temperatures along the year, reported less clonality (Garcia-Cisneros et al., 2015). However, individuals from constant warmer waters were significantly larger than those found in NW Mediterranean (Fig. S2). Previous studies with *C. tenuispina* also reported increased fission frequency during summer in Bermuda (Crozier, 1921), where temperature ranges by nearly 10 ºC (18 - 28 ºC). However, in Brazil, a probably spurious inverse correlation was found with temperature (Alves et al., 2002), but the authors pointed out very low temperature variations during their study and, thus, other factors could had more influence on asexuality. Therefore, in populations with temperature fluctuations, peaks of high temperature could cause stress and induce fission, while in populations with constant temperate or warm water, individuals could be growing more constantly without fission events. Consequently, biotic or abiotic factors other than temperature could be the main triggers of fission events in those populations with constant temperatures.

Little is known about how fission influences survival rates in starfish. Fission decreases starfish mobility and size, and therefore, ability to find food. A decrease in nutritional condition after fission would be the consequence of that period of scarcity. Even though there is not information about survival rates after fission in *Coscinasterias*, a significant survival decrease after fission was reported in *Allosticaster capensis*, when food availability also decreased (Rubilar et al., 2011).

Asexual reproduction is usually linked to episodes of food abundance in other opportunistic metazoan groups typically reproducing by asexual mechanisms, such as aphids (Simon et al., 2002; Halkett et al., 2004) or cladocerans (Hebert et al., 1993; Hebert & Finston, 2001). However, this does not seem to be the case for carnivorous echinoderms such as starfish, perhaps because food scarcity is not usually a problem for these non-specific predators in natural ecosystems. Our observations are in agreement with this view, since many individuals of *C. tenuispina* in our sampling location were found in active predation behavior.

The higher density values found in the Inshore area were probably related to a higher number of rocks and boulders near the coastal line, providing more available refuges for the starfish and their prey (therefore, more available food in this area). However, no evidences of these causes were tested. On the other hand, due to the shallow depth of the Inshore area, individuals were subjected to a more unstable environment, with more influence of waves, tides and temperature changes. This instability may also be the cause for increased fission rates and density reduction during Levanter winds events. We are not able to know if density drops after windstorms were due to mortality or because the waves buried the individuals, making them harder to find. Measured density values of $1 - 4$ individuals m^{-2} was similar to those reported for *C. muricata* in New Zealand ($2 - 8$ individuals m^{-2}) which produced high mortality rates on their preys, but not enough to modulate the ecosystem (Witman & Grange, 1998). On the other side, densities from Gran Canaria, in the Canary Islands, were reported to be much lower, with 0.06 individuals m^{-2} (Donet, 2007). However, as mentioned above, individuals from Canary Islands were significantly larger than those from NW Mediterranean, and therefore probably their trophic influence on the ecosystem could be higher. In any case, no significant correlations between fission rates and population densities were found in our study.

Overall, our data show that factors regulating asexual reproduction and alternative reproductive strategies in starfish are complex and are still far from being fully understood. Further experiments should be designed in order to study the influence of other factors not explicitly tested here, such as food availability or hydrodynamism intensity. Asexual populations of *C. tenuispina*, which are widespread throughout the Western Mediterranean basin, can be considered an excellent natural model for testing hypotheses related with reproductive ecology of asexual organisms in order to increase our knowledge on reproductive strategies of invertebrates.

Acknowledgements

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Capítulo 5

Long telomeres are associated with clonality in wild populations of the fissiparous starfish *Coscinasterias tenuispina*

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Abstract

Telomeres usually shorten during an organism's lifespan and have thus been used as an aging and health marker. When telomeres become sufficiently short, senescence is induced. The most common method of restoring telomere length is via telomerase reverse transcriptase activity, highly expressed during embryogenesis. However, although asexual reproduction from adult tissues plays an important role in the life cycles of certain species, its effect on the aging and fitness of wild populations, as well as its implications for the long-term survival of populations with limited genetic variation, is largely unknown. Here we compare relative telomere length of fifty-eight individuals from four populations of the asexually-reproducing starfish *Coscinasterias tenuispina*. Additionally, 12 individuals were used to compare telomere lengths in regenerating and non-regenerating arms, in two different tissues (tube feet and pyloric caecum). The level of clonality was assessed by genotyping the populations based on twelve specific microsatellite loci and relative telomere length was measured via qPCR. The results revealed significantly longer telomeres in Mediterranean populations than Atlantic ones as demonstrated by the Kruskal-Wallis test($K = 24.17$, significant value: p-value < 0.001), with the former also characterized by higher levels of clonality derived from asexual reproduction. Telomeres were furthermore significantly longer in regenerating arms than in non-regenerating arms within individuals (pyloric caecum tissue: Mann-Whitney test, $V = 299$, p-value $< 10^{-6}$; and tube feet tissue Student's-t $= 2.28$, p-value $= 0.029$). Our study suggests that one of the mechanisms responsible for the long-term somatic maintenance and persistence of clonal populations is telomere elongation.

Keywords: Aging, Asexual Reproduction, Clonal, Microsatellites, Population Genetics, Regeneration.

Resumen

Los telómeros, en general, se van acortando a lo largo de la vida de los organismos y, por tanto, su longitud se ha utilizado como un marcador de envejecimiento y salud. Cuando los telómeros son suficientemente cortos, se induce la senescencia. El mecanismo más común para restaurar la longitud de los telómeros es la actividad de la telomerasa transcriptasa inversa, altamente expresada durante la embriogénesis. Aunque la reproducción asexual a partir de tejidos maduros juega un papel importante en los ciclos de vida de ciertas especies, es en gran parte desconocido su efecto sobre el envejecimiento y sobre la eficacia biológica de las poblaciones naturales, así como sus implicaciones en la supervivencia a largo plazo de aquellas que presentan una variación genética limitada. En este estudio se compara la longitud relativa de los telómeros de cincuenta y ocho individuos de cuatro poblaciones de la estrella de mar asexual *Coscinasterias tenuispina*. Además, doce individuos fueron también utilizados para comparar las longitudes de los telómeros entre brazos en regeneración y sin regeneración, en dos tejidos diferentes (pies ambulacrales y ciego pilórico). El nivel de clonalidad se evaluó mediante el genotipado de las poblaciones basado en doce loci microsatélite, y la longitud relativa de los telómeros se midió mediante qPCR. Los resultados revelaron telómeros significativamente más largos en las poblaciones mediterráneas que en las poblaciones atlánticas, tal como confirmó el test Kruskal-Wallis ($K = 24,17$, $p < 0,001$), siendo los niveles de clonalidad más elevados en las poblaciones mediterráneas. Los telómeros eran, además, significativamente más largos en los brazos en regeneración que en los brazos sin regeneración (ciego pilórico: Mann-Whitney, $V = 299$, $p < 10^{-6}$; y pies ambulacrales: t de Student $= 2,28$, $p = 0,029$). Nuestro estudio sugiere que el alargamiento de los telómeros es uno de los mecanismos responsables del mantenimiento somático a largo plazo y de la persistencia de poblaciones clonales.

Palabras clave: Envejecer, reproducción asexual, clonalidad, microsatelites, regeneración.

Introduction

Although aging is observed in most organisms, there is a large degree of variation in the rate at which it occurs, at both the species and individual level. Telomere length has frequently been used as an aging marker because telomere caps normally become shorter during an organism's lifetime, primarily during DNA replication but also in association with other factors such as stress (von Zglinicki, 2002; Epel *et al.*, 2004; Kotrschal *et al.*, 2007). Critically short telomeres trigger a signal prompting the cell to permanently stop dividing, which leads to the induction of cellular senescence (Herbig *et al.*, 2006). Furthermore, long telomeres have been found to correlate with good health and higher life expectancies in several species, thereby also serving as an indicator of somatic fitness, which represents the boundary of aging diseases (Bize *et al.*, 2009; Horn *et al.*, 2010; Barrett *et al.*, 2013).

During fission or fragmentation in asexual organisms, two or more separate individuals are formed, resulting in clonal offspring with genotypes identical to the parent and to each other. In wild asexual populations and after recurrent fissions, it is difficult to determine the age of an individual by morphological means, not only for the potential clone itself but also the original parental half. In these cases, genetic analyses can reveal the level of clonality within a population as well as the extent of a clone, thus providing information regarding its age and longevity (Ally *et al.*, 2010). Indeed, extremely large and long-lived clonally propagating populations exist, such as some sea grass species, with clones that are estimated to be 1,000 yr (Reusch *et al.*, 1999) or more (Arnaud-Haond *et al.*, 2012). In the case of a terrestrial tree, the age of some clones have been estimated as old as 10,000 yr (Ally *et al.*, 2010) and in cold waters, clonal individuals of the coral *Lophelia pertusa* are estimated to be 4,500-6,000 yr (Dahl *et al.*, 2012). These estimations obtained for several plants and animal groups may in some way indicate the existence of mechanism to largely delay, or even resist, aging in particular clones although the evolutionary significance of these long-term resistance has not been clarify yet.

In sexually-reproducing species, telomere length is restored during embryogenesis by the reverse transcriptase telomerase (Schaetzlein *et al.*, 2004). However, little is known about aging in asexual organisms that propagate via fission or budding; many questions remain unanswered as to whether they are able to fully maintain and/or restore their telomeres to persist over time or whether they undergo somatic aging (Sköld and Obst, 2011). In a study using laboratory cultures of two invertebrate species, upregulation of telomerase has been shown to at least partly restore telomeres in the clones of a flatworm (Tan *et al.*, 2012). Telomerase is also upregulated during budding in the colonial ascidian *Botryllus schlosseri* (Laird and Weissman, 2004). On the other hand, experiments involving another colonial ascidian, *Diplosoma listerianum,* found that telomerase activity declined, telomeres shortened and the growth rate slowed after prolonged asexual duplication, indicative of long-term senescence in the studied clones (Sköld and Obst *et al.,* 2011). There is however very limited information available regarding the effect of prolonged periods of asexual duplication on telomere length and aging in wild populations. Whether molecular aging occurs and how it could potentially be delayed in wild clones is still unknown.

Most asteroids have the ability to regenerate their body parts after autotomy or injuries, and about 26 species can reproduce asexually via fission (Emson and Wilkie, 1980). All four known species of the cosmopolitan genus *Coscinasterias,* commonly found in shallow waters, can reproduce both sexually and asexually via fission (Alves *et al.*, 2001; Lawrence 2013, among other references). When individuals of *Coscinasterias* reproduce sexually, they release planktotrophic larvae that remain in the water column for several weeks, with a high potential to colonize new habitats via dispersal (Karako *et al.*, 2002). The species *Coscinasterias tenuispina* (Lamark 1816), widely distributed throughout the Atlantic Ocean and Mediterranean Sea, presents in some cases populations either consisting of individuals of only one gender (usually males) or with an unbalanced proportion of males and females (Alves *et al.*, 2002, authors'

Figure 1 (A) Map of sampling locations, including those in Mediterranean Sea and Northeastern Atlantic Ocean. Circles highlight the three sampling areas. Two different populations were sampled in Tenerife. (B) Schematic anatomy of *Coscinasterias tenuispina* showing normal and regenerating arms.

unpublished data). The absence of one gender in some populations of this species, as well dominance of a few genotypes - according to allozyme analyses (Ventura *et al.*, 2004) - suggests that maintenance of these populations takes place solely via asexual reproduction.

The aim of the present study was to assess the effect of asexual reproduction on relative telomere length in wild populations of the starfish *C. tenuispina*, and its implication for the long-term survival of populations with limited genetic variation. For this purpose, telomere length and its relationship with different levels of genetic diversity related to asexuality were assessed using populations from two Mediterranean and two Atlantic sites. Additionally, we explored the potential existence of mechanisms for telomere length control in somatic tissues by comparing regenerating and non-regenerating arms within a set of individuals.

Material and Methods

Sampling

Starfish of the species *C. tenuispina* (Supplementary material FS. 1) were collected from four different European sites (Fig. 1A), two in the Atlantic basin and two in the Mediterranean basin, with between 13 and 17 individuals collected per locality. The two Mediterranean sites, Llançà (Costa Brava, Northwestern Mediterranean) and Taormina (east of Sicily, Central Mediterranean), hereafter referred to as LLA and TAO, respectively, were sampled in autumn 2011. Both Atlantic sites, Bocacangrejo and Abades (BOCA and ABA, separated by 33 km), were located near Tenerife (Canary Islands) and were sampled in the spring (June) of 2012 (Table 1). These four sampling locations were selected based on the abundance of the studied species and on the varying prevalence of individuals undergoing fission. The starfish were sampled at between 0 and 20 m depth by snorkeling or SCUBA-diving. Immediately after their removal from the sea, the animals were photographed on a millimeter-scaled table in order to determine body size. Tube feet, used for the starfish locomotion and substrate attachment, from the middle part of the longest arms of each individual were also collected (Fig. 1B) and preserved in either RNA*later* (Invitrogen) for telomere length analysis or absolute ethanol for microsatellite genotyping. The animals were then released back into the sea. All tissue samples were stored at -20° C once in the laboratory prior to analysis. Body size was assessed as the longest diameter across the starfish using the ImageJ software program.

Additionally, both tube feet and pyloric caeca tissue (stomach extensions for processing and storage organ) were collected from the middle part of the longest (non-regenerating) and shortest (regenerating arm; when the length of this arm was less than a 50 % of the longest arm) arms of 12 asymmetric specimens sampled at site LLA (Fig. 1B, Supplementary material FS. 1). Pyloric caeca tissue was considered in the present analysis because it is a distinct tissue that has further been shown to be involved in arm regeneration (Hernroth *et al.*, 2010). Although gonads may be also considered for the telomere length analysis, they cannot be used in this study because more than 50 % of the individuals of the species lack gonads, even during the reproductive season (Crozier 1920).

Genetic analyses using microsatellites

Twelve microsatellite markers (m.ten1, m.ten6, m.ten13, m.ten14, m.ten19, m.ten24, m.ten25, m.ten27, m.ten30, m.ten31, m.ten32, m.ten40) specifically designed for *C. tenuispina* (Garcia-Cisneros *et al.*, 2013) were employed for population genetic analyses (Table 1). Extractions and amplifications were performed using a REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). For PCR reactions we added 4 µl of PCR Ready Mix, 4 pmol of each primer, between 10 ng and 50 ng of DNA, and ultrapure water for molecular use to a final reaction volume of 10 µl. Forward primers for each locus were labeled with a fluorescent dye as described in Garcia-Cisneros et al. (2013). Amplifications were performed on an S1000™ Thermal Cycler Dual 48/48 Reaction Module (BioRad Laboratories), with an initial step of 1 min denaturation at 96 °C followed by 35 cycles of 96 °C for 1 min, 49 °C for 30 seconds and 72 °C for 20 seconds, and a final step at 72 °C for 3 min. Amplification products were analyzed on an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems Life Technologies) by the Scientific-Technical Services of the University of Barcelona. Allele size was estimated relative to an internal size standard 70-400 ROX (Bioventures Inc.) using the software program Peak-Scanner-96 (Applied Biosystems). The prevalence of identical genotypes, considered as potential clones, within and between populations was tested using GenoDive (Meirmans and Van Tienderen, 2004) and MLGsim (Stenberg *et al.*, 2003). Both approaches were applied to count the number of individuals with identical Multi Locus Genotypes (MLGs) and to calculate the likelihood of observing identical MLGs in a population due to sexual events (P_{sex}). MLGsim calculations of the P_{sex}, and their significance values, were based on 1,000 random simulations. MLGsim set a critical value for MLGs due to sexual events at a minimum of 0.040. Values obtained for MLGs of *C. tenuispina* were significantly lower than the critical value of 0.040 (see Table 1) pointing to clonal reproduction as the origin of identical MLGs. Genetic diversity was measured by calculating allelic richness, including all individuals from the populations even if they were considered the same clone, genotype diversity (synonym of clonal diversity) after rarefaction for each population, heterozygosity expected and observed and the inbreeding coefficient (F_{is}) using the Hierfstat and Vegan packages (Dixon, 2003; Goudet, 2005) in the software program R v. 3.0.0.

In order to assess genetic differentiation between populations, we calculated values of the *D* estimator (Jost, 2008), a statistic to measure differences in genetic structure between populations based on the allele frequencies, using the R package DEMEtics (Gerlach *et al.*, 2010). The significance of these *D* values was evaluated by performing 10,000 permutations including all the individuals from the populations (Gerlach *et al.*, 2010).

Telomere measurements

The relative telomere lengths in either tube feet or pyloric caeca tissue were measured for each specimen via a qPCR method (Farzaneh-far *et al.*, 2008), and modified and optimized in our

Population	Code	Coordinates	n	MLG	Clonal MLG	Single MLG	Allelic richness r[13]	R (Clonal richness)	Genotype diversity r[13]	Ho/He	F
Llança, Costa Brava	LLA	42°23' N, 3°09' E	17	1	1	0	1.33	0	1.00 (±0.0)	0.33/0.17	-
Taormina, Sicily	TAO	37°51' N, 15°18' E	15	4	3	1	1.75	0.21	3.80 (±0.4)	0.24/0.27	0
Bocacangrejo, Tenerife	BOCA	28°24' N, 16°19' W	13	8	2	6	2.06	0.58	7.54 (±0.5)	0.53/0.27	-
Abades, Tenerife	ABA	28°08' N, 16°26 W	13	7	1	6	2.14	0.5	6.54 (±0.5)	0.43/0.25	-
TOTAL			58	20	7	13			0.33		

Table 1. Geographical coordinates and genetic information of the sampled *Coscinasterias tenuispina* populations. Data presented include: Sampl[e]... Number of different Multi Locus Genotypes (MLGs), Clonal MLGs (found in more than one individual) and Single MLGs (found in only one indiv[idual])... well as Allelic richness after rarefaction to 13, Clonal richness (R), Genotype diversity measured after rarefaction, observed and expected hete[rozygosity] (Ho/He) and inbreeding coefficient (F_{is}).

laboratory (Gothenburg, Sweden). Telomere length assessment via qPCR has already been validated (Grabowski *et al.*, 2005; O'Callaghan *et al.*, 2008). However, although qPCR telomere measurements are normalized based on single copy genes in humans and other model species, the lack of genomic data for non-model species such as *C. tenuispina* hinders the identification of nuclear genes without paralogs for normalization. For this reason, telomeric DNA measurements in the present study were performed relative to the total quantity of DNA in the samples.

Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions, and DNA concentrations assessed with a NanoDrop (Thermo Scientific) in triplicate to obtain an accurate value of DNA quantity. Absorbance ratios measuring DNA purity at 260/280 averaged approximately 1.9 ± 0.1, with the 260/230 absorbance ratio also indicating acceptable sample purity (1.8 ± 0.2). Small differences in absorbance ratios were not correlated with final telomere length (260/280: $R^2 = 0.006$, p = 0.96; 260/230: $R^2 = 0.001$, p = 0.99).

The amount of DNA to be added in the qPCR reaction was estimated from a standard curve obtained via a dilution series of a mixed sample DNA pool (10 − 0.0001 ng). The reaction efficiency calculated from the standard curve was E = 104.1 %, $R^2 = 0.997$. For the sample qPCR reaction, all DNA samples were adjusted to the same concentration, and 0.5 ng of DNA was added for each PCR mix; this produced a concentration value falling well within the linear range of the mixed sample standard curve.

Telomere analyses examining a broad range of species have indicated that the TTAGGG sequence is conserved among deuterostomes (Gomes *et al.*, 2010); the primers used for the qPCR reaction in *C. tenuispina* were therefore those described for humans in Farzaneh-far *et al.* (2008):

(forward) 5' CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT 3', and

(reverse) 5' GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT 3'.

Concentrations used for the forward and reverse primers were 100 nM and 200 nM, respectively. A 10 µl KAPA SYBR FAST qPCR Kit (Kapa Biosystems) was employed as a Mastermix, with 3 µl of water added for a final PCR volume of 20 µl following the protocol described by Carney Almroth *et al.* (2012). Telomeres were amplified in triplicate for each sample to ensure accurate measurements using the following qPCR protocol cycle: 3 min at 95 °C followed by 25 cycles of 15 sec at 95 °C and 1 min at 56 °C. The final step comprised 81 cycles of temperature increase from 55 °C to 95 °C in order to generate a melt curve, indicating the presence of a single product. Relative telomere length is represented as a Cycle Threshold Value (Ct Value), which is inversely proportional to telomere amount; longer telomeres thus produce an earlier detectable signal than shorter telomeres. All measurements were analyzed in seven PCR plates, with the mixed sample DNA dilution series included on each plate as an internal standard; Ct values for these differed by less than 1.5 % between plates and were adjusted for.

Telomere length verification was performed in an independent laboratory (Umeå, Sweden) using a similar protocol for telomere length qPCR (Cawthon 2002). DNA from 11 samples were send to Umeå and DNA concentration were re-measured by the Nanodrop instrument (Thermo scientific). Each qPCR reaction contained: 17.5 ng DNA (diluted in TE/E.coli buffer), 0.1 µM forward primer, 0.9 µM reverse primer, 1X PCR Buffer 2, 1.7 mM MgCl2, 2.5 mM DTT, 0.2 mM dNTP, 150 nM ROX, 0.2X SYBR and 0.625 U AmpliTaq Gold (Applied Biosystems).

Telomere primer sequence (5'–3') Forward: CGGTTTGTTTGGGTTTGGGTTT GGGTTTGGGTTTGGGTT, Reverse: GGCTTGCCTTACCCTTACCCTTACCCTT ACCCTTACCCT. Each sample was analyzed two separate times in triplicates on the ABI7900HT instrument (Applied Biosystems).

Statistical analyses

To compare body size between specimens from different populations, a non-parametric Mann-Whitney test was performed since the data did not match our prior expectation of homoscedascity (Bartlett test = 35.97, and signification value, p-value < 10^{-7}). As body size data also did not match normality (Shapiro-Wilk test; W = 0.93, p-value = 0.0048), a Spearman correlation was used to test if telomere lengths were correlated to body size. A mixed model analysis of variance, with telomere length (expressed as the Ct value) as a dependent variable, was performed using "basin" (Atlantic and Mediterranean basins) as a fixed factor, and "population" and "MLGs" as random factors. A non-significant effect was found for the MLGs (p-value > 0.98) and was therefore not considered for further analyses (see Supplementary material FS. 3). Since our data did not match normality (Shapiro-Wilk test; W = 0.96, p-value < 0.01), a Kruskal-Wallis test of the telomere length was performed between the different sites, and a Mann-Whitney test between basins. Spearman correlation was used to test the relationship between genetic diversity and mean telomere length.

Potential differences in telomere length between regenerating and non-regenerating arms within individuals were tested using a mixed model involving the logarithmic transformation of Ct values, "tissue type" as a factor, and interaction with "regeneration". Moreover, we separately tested the effects of regeneration for both tissue types via a paired-data student's t-test for tube feet, and a paired Mann-Whitney test for pyloric caeca telomere length since the data did not match normality (non-normal distribution; Shapiro-Wilk test; W = 0.8482, p-value = 0.002).

All statistical analyses of telomere length and box plots were performed in R v. 3.0.0.

Results

Genetic diversity and genetic distance between localities

The low and significant values (p-values) of the P_{sex} (Table 2) indicated that the identical genotypes observed among individuals are a consequence of clonal propagation in this species. The studied populations of *C. tenuispina* presented different levels of allelic richness and clonality as defined by identical genotypes (Table 1). Populations from the Atlantic Ocean were genetically more diverse than those from the Mediterranean Sea, presenting twelve out of the thirteen single multilocus genotypes (MLGs) found in the whole study area. Furthermore, excess of heterozygotes were found in all populations except Taormina. The *D* estimator, used to assess differences in genetic structure between populations, revealed significant genetic differences among all four populations here analyzed (Table 3). Large and significant differences were recorded between the two Mediterranean populations, with the latter characterized by a higher prevalence of genetic clones compared to the Atlantic populations. Indeed, only one multilocus genotype was detected at Llançà (Table 1).

Clonal MLG	Locality	n	P_{sex}	p-value
MLG 1	LLA	17	$< 10^{-14}$	0.000*
MLG 2	TAO	5	0.000	0.000*
MLG 4	TAO	3	$< 10^{-8}$	0.007*
MLG 8	TAO	6	$< 10^{-15}$	0.000*
MLG 9	BOCA	2	$< 10^{-10}$	0.000*
MLG 11	BOCA	5	0.000	0.000*
MLG 12	ABA	7	$< 10^{-14}$	0.000*

Table 2. Different Multi Locus Genotypes (MLGs) found in more than one individual in the four localities. Llançà (LLA), Taormina (TAO), Boca Cangrejo (BOCA), and Abades (ABA). Data presented include the number of individuals sharing the same MLG (n), the probability of obtaining the same MLG from different sexual events (P_{sex}), and the associated p-value of P_{sex}. * Significant when p-values < 0.01.

	TAO	LLA	BOCA
LLA	0.167*		
BOCA	0.159*	0.233*	
ABA	0.143*	0.148*	0.138*

Table 3. Values of the D genetic differentiation estimator between populations of *Coscinasterias tenuispina*. Llançà (LLA), Taormina (TAO), Boca Cangrejo (BOCA), and Abades (ABA). * Indicates significant p-values < 0.01.

Differences in telomere length and body size between and within populations

Telomeres were significantly longer in the Mediterranean than in the Atlantic starfish populations as shown by the Kruskal-Wallis test ($K = 24.17$, significant value: p-value < 0.001) (Figs. 2A, 2B). When the four populations were analyzed separately, significantly longer telomeres were again observed in the two Mediterranean populations (Kruskal-Wallis test; $K = 37.03$, p-value < 0.001), with individuals from Llançà presenting the longest telomeres (Fig. 2B). Telomere length measurements were double-checked and verified by an independent laboratory (Umeå, Sweden), and the result showed a strong correlation between the measurements between both independent sets of analyses (value of the correlation for the regression: $R^2=0.88$), (Supplementary material FS 2).

Genotype diversity (also expressed as clonal diversity) in this species depends on the relative ratio of fission (asexual reproduction) versus sexual reproduction. Here, populations with lower genotype diversity, and therefore higher fission rates had longer telomeres at the population level, as demonstrated by a significant correlation (Pearson correlation: $R = 0.99$, p-value < 0.007). In Figure 3, it is presented the high correlation between mean of genotype diversity per population and the Ct vale (which is inversely proportional to telomere length).

Populations analyzed showed differences in the mean body size of the starfish, demonstrated by a significant value of the Mann-Whitney test (W = 740, p-value < 10⁻⁷), with considerably larger specimens observed in the Atlantic populations (mean body size = 6.36± SD 3.8 cm) than in those from the Mediterranean Sea (mean body size = 2.81± SD 1.1 cm). However, telomere length did not depend on the starfish body size, as demonstrated by the absence of correlation between these two variables (Correlation value: rho = 0.065, p-value = 0.63, non-significant) (Supplementary material FS. 3).

Telomere length in relation to arm regeneration

Our results showed that telomere length was significantly longer in regenerating (short) arms than in non-regenerating (long) arms (Fig.1B, SF. 1), as demonstrate by the significance of the different tests applied (F = 52.26, p-value < 0.001), for both tissue types analyzed, tube feet (Student's-t = 2.28, p-value = 0.029) and pyloric caecum tissue (Mann-Whitney, V = 299, p-value < 10⁻⁶) (Fig. 4). Pyloric caecum telomeres were always longer in regenerating arms in all individuals, while eight out of twelve specimens displayed longer telomere lengths in regenerating arm tube feet.

Discussion

Life expectancy in clonal lineages remains unclear due the lack of understanding of different phenomena that would influence on its time survival. Firstly, we do not know the real consequences of the accumulation of somatic mutations and their deleterious effects on the individual and their clonal offspring. Secondly, we ignore the real effect in wild populations of the lack of genetic diversity for adaptive potential, and finally the mechanisms to avoid senescence. The first two difficulties are usually overcome when the species are able to maintain sex, even at low rates or sporadic events, by combining genomes and eliminating phenotypic expression of deleterious mutations when recessive. Here, in this study, we shed some light on the third problem, evidencing telomere elongation during asexual reproduction of the starfish *Coscinasterias tenuispina*.

Figure 2. Box plots of telomere qPCR Ct values obtained from tube feet for the *Coscinasterias tenuispina* populations from different seas and localities: A) Ct values of grouped Atlantic and Mediterranean populations, and B) Ct values of each separate locality. Llançà (LLA), Taormina (TAO), Abades (ABA), and Bocacangrejo (BOCA). Lower Ct values indicate longer telomeres. Boxes are represented by the first and third quartile, the dark line is the median, and dots are outliers.

Figure 3. Correlation between relative telomere length in tube feet (telomere qPCR Ct Value) and genotype diversity for the four *Coscinasterias tenuispina* populations analyzed. Horizontal and vertical bars represent the standard error for genotype diversity and relative telomere length, respectively. The relative telomere length was represented as the mean value per locality.

Figure 4. Box plot of *Coscinasterias tenuispina* telomere qPCR Ct values in regenerating and non-regenerating arms for two different tissues from individuals of Llança: pyloric caecum (Pyl) and tube feet (TF). Pyloric caecum from non-regenerating arms (Pyl NR); Pyloric caecum from regenerating arms (Pyl R); Tube feet from non-regenerating arms (TF NR); Tube feet from regenerating arms (TF R). Boxes are represented by the first and third quartile, the dark line is the median, and dots are outliers.

Our work represents the first study to explore the potential implications of asexual reproduction on relative telomere length in wild populations of a clonal starfish. To date, most ecological studies examining telomere length and/or telomerase activity have focused on obligate sexually-reproducing species or clonal organisms maintained in laboratory cultures (see examples in Klapper *et al.*, 1998; Ojimi and Hidaka, 2010; Horn *et al.*, 2010; Sköld and Obst, 2011; Carney Almroth *et al.*, 2012; Tan *et al.*, 2012), and no previous study based on telomeres and aging has been conducted on wild clonal populations of any species.

Longer telomeres were recorded in the Mediterranean populations of the starfish *C. tenuispina*, while these specimens also significantly smaller in size. Although shorter telomeres were observed in the Atlantic populations characterized by a larger mean body size, our results did not detect any correlation between the two variables. This finding is consistent with the lack of age-related telomere shortening demonstrated for other marine species, including sea urchins and lobsters, and may be attributed to high phenotypic plasticity in body size and/or to continuous telomerase activity throughout their life-span (Klapper *et al.*, 1998; Ebert *et al.*, 2008). Nevertheless, telomere length is regarded as an indicator of health and somatic fitness, and its variation observed in our populations may be influenced by both inherited and/or environmental components (Epel *et al.*, 2004). Thus, populations of *C. tenuispina* comprising only one clone may be healthily maintained, including those in Llançà, which were also characterized by the longest telomeres.

The longer telomeres found in regenerating compared to longer non-regenerating arms are indicative of telomere elongation and the preservation of chromosome ends in somatic tissue over the asexual cycle. These results may explain the positive correlation between telomere length and level of clonality. Asexual reproduction via fission has been proposed as more prevalent in small specimens of *Coscinasterias* (Emson and Wilkie, 1980), which is also consistent with our results and observations. It is therefore possible that the key to retain long telomeres in these starfish is to frequently undergo fission. On the other hand, fission may be influenced by the environment, either directly, e.g. by different temperature regimes between the Mediterranean and Atlantic basins here analyzed, or indirectly, e.g. by growth limitation and therefore it could be more prevalent in such situations (Haramoto *et al.* 2007). Therefore, in *C. tenuispina,* longer telomeres and greater somatic fitness may be triggered by unstable environmental conditions in wild populations.

Elongation of telomeres in populations of *C. tenuispina* may be one of the mechanisms related to the absence of senescence and genetic defects associated to prolonged periods of asexual propagation. Studies investigating terrestrial species have demonstrated that both telomere length and telomere erosion are predictors of survival and somatic fitness (Bize *et al.*, 2009; Horn *et al.*, 2010), with the combination of long telomeres and telomere elongation in regenerating tissues potentially providing these clonal starfish a high probability of survival. Although the results of a previous study examining a colonial ascidian suggest that passing through a sexual reproductive phase is required in order to avoid senescence after prolonged periods of asexual budding (Sköld *et al.*, 2011), it is not known how generally this finding can be applied to other species. Despite the fact that asexual reproduction facilitates clonal dispersion and renewal, it depends on mitotic divisions, which may increase the accumulation of somatic mutations, the Muller ratchet phenomena. Different studies with asexual species already revealed accumulation of somatic mutations on non-synonymus position in clonal lineages compared to their non-clonal sibling species (Paland and Lynch 2006; Barraclough *et al.,* 2007). Unfortunately, the negative effects of deleterious mutations in wild populations of clonal species have never been fully investigated or proved, and these effects have been only supposed, but asexual lineages persist over short evolutionary periods (Schwander and Crespi 2009). However, older clones of aspen species has been found to exhibit a significant reduction in reproductive performance associated with male sexual fitness decline, suggesting that at least some long-lived

clonal organisms may be vulnerable to senescence over long periods of time (Ally *et al.*, 2008). A hypothesis to explain the excess of heterozygosity found in the studied populations of *C. tenuispina* may be a positive selection of heterozygotes to keep genetic diversity, besides having greater individual adaptability by high phenotype plasticity (Hörandl, 2009; Goudie *et al.,* 2012). Nevertheless, our current results cannot actually test this hypothesis, and the heterozygotes excess found in all populations may be results from other stochastic processes that have not been controlled in this study. In other organisms as plants that maintain asexual lineages has been observed that polyploidy and hybridization commonly generate heterozygotes. In other species as fur seals, inbreeding is avoided by an active selection from females for non-relative males or heterozygotes (Hoffman *et al.,* 2007). However, we do not have evidences of any of these processes, and positive selection of heterozygotes is the only hypothesis that can be presented here, but further studies on this particular point may shed light on it.

Although the molecular mechanisms responsible for telomere elongation and its preservation in *C. tenuispina* remain unknown, studies examining other asexual and sexual organisms indicate a pivotal role for telomerase in the telomere length regulation of somatic cell lineages. In *Asterias rubens*, a sexually reproducing starfish, telomerase activity is high throughout the animal irrespectively of mitotic activity and there was no difference in telomerase activity nor telomere length between regenerating and non-regenerating arms (Hernroth *et al*., 2010), but clonal starfishes may have higher telomerase activities after fission as other asexual species. In flatworms, maintenance of somatic telomere length seems to be an adaptation of asexual but not sexual strains, and is based on different levels of telomerase activity (Tan *et al.*, 2012). Furthermore, in the colonial ascidian *Botryllus schlosseri*, telomerase activity is up-regulated in early bud rudiments, and declines during zooid development (Laird and Weissman, 2004). Differences in the relative abundance of somatic versus stem cells might also determine variation in telomere length (Ojimi and Hidaka, 2010), but we cannot either discard recombination between homologous telomeres as a means to elongate telomeres (Liu *et al.*, 2007). Even in plants, telomere restoration is only present in meristomatic and reproductive tissues, with exceptions in long-lived species (Flanary and Kletetschka, 2005; Watson and Riha, 2010; among other references), while in some algae it has been described telomerase activity during all life cycles, but large differences in telomerase activity have been found across algae groups (Fulnecková *et al.,* 2013; Ševčíková *et al*., 2013). Further comparison of telomerase and stem cells in relation to regeneration in the analyzed populations of *C. tenuispina* would thus be of future interest.

The results presented here reveal the need for further research exploring the ecological and evolutionary significance of asexual reproduction and telomere elongation in clonal lineages. Future empirical studies measuring the success of Atlantic and Mediterranean populations should consider additional variables such as sexual reproductive success, as well as an evaluation of whether genetic diversity is fundamental to the maintenance of clonal populations, concepts that have been only theoretical explored for few authors as Weissman *et al.* (2009) and Marriage and Orive (2012). Those theoretical models for asexual species have evaluated and proposed that clones with large population sizes exhibit high successful levels (Weissman *et al.*, 2009; Marriage and Orive, 2012). Nevertheless, further analysis regarding possible somatic deleterious mutations, as well as health and population size monitoring, is essential in order to understand the life expectancy of clonal populations.

Data archiving

Data available from the Dryad Digital Repository: http://dx.doi.org/ 10.5061/dryad.305c3.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Discusión y conclusiones
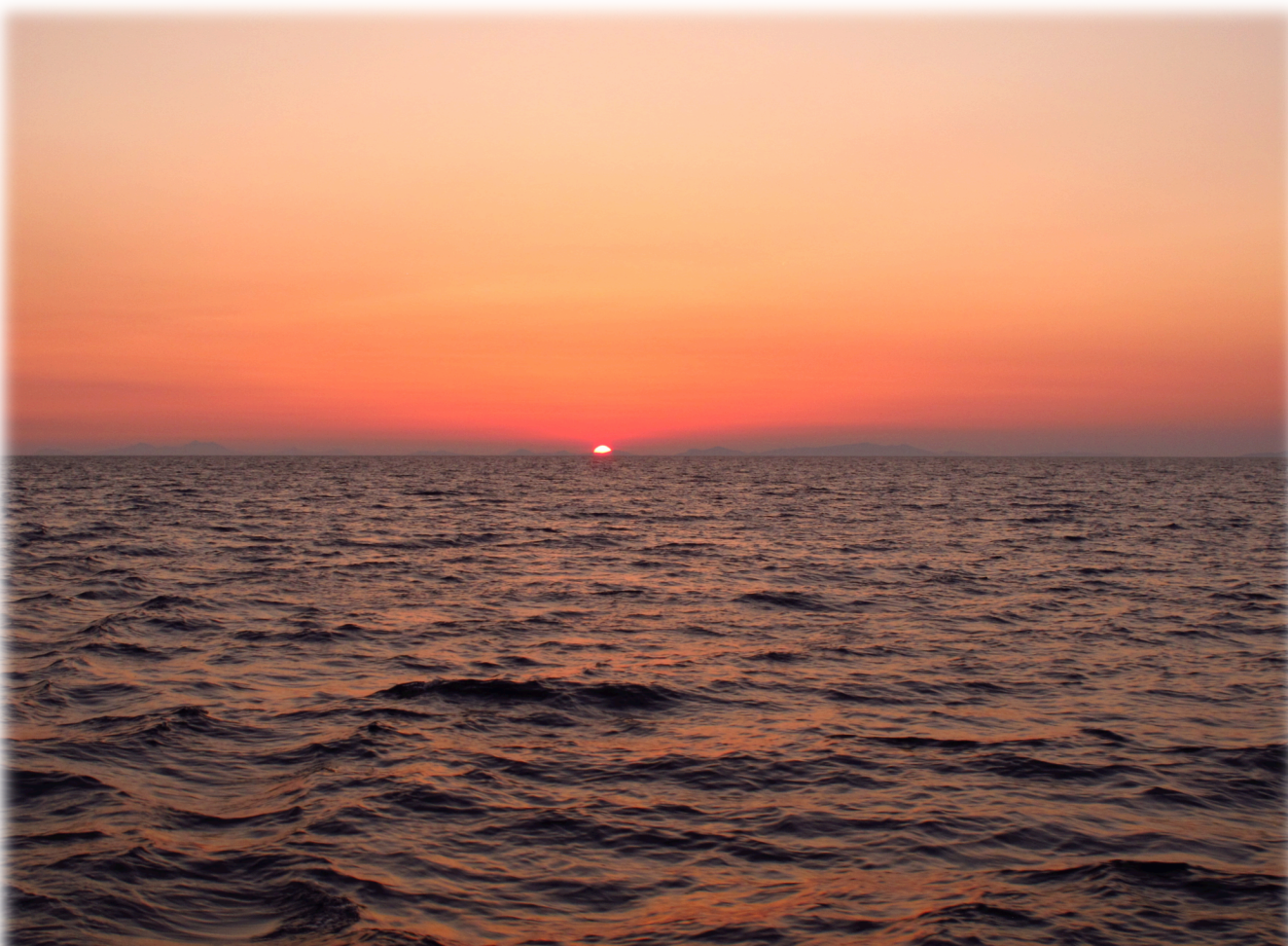
Discusión general

Echinaster sepositus y *Coscinasterias tenuispina* son dos de las estrellas de mar más comunes del litoral del Mediterráneo y del Atlántico templado. A pesar de compartir buena parte de su área de distribución geográfica, como se demuestra en esta tesis doctoral, su historia evolutiva, características biológicas y estrategias vitales son muy diferentes. La divergencia en sus estrategias reproductivas, así como sus particularidades ecológicas condicionan la estructura de sus poblaciones y patrones de conectividad.

Diversidad genética y tamaño efectivo poblacional

Tanto *Echinaster sepositus* como *Coscinasterias tenuispina*, presentan valores bajos de diversidad genética para el gen COI, si las comparamos con otros asteroideos de distribución atlanto-mediterránea (Pérez-Portela *et al.*, 2010, para *Marthasterias glacialis*), y en general con otros equinodermos de distribución similar (Duran *et al.*, 2004b, para *Paracentrotus lividus*; Boissin *et al.*, 2008, para *Ophioderma longicauda*; Borrero-Pérez *et al.*, 2011, para *Holothuria (H.) mammata*; Wangensteen *et al.*, 2012, para *Arbacia lixula*; Pérez-Portela *et al.*, 2013, para *Ophiotrix spp*; Valente *et al.*, 2014, para *Holothuria (R.) polii*; ver Tabla 1). Esta baja diversidad se observa también en la diversidad alélica de los microsatélites nucleares, comparada con la que aparece en los escasos estudios disponibles de asteroideos de distribución atlanto-mediterránea (Zulliger *et al.*, 2009, para *Astropecten aranciacus*) o con otras estrellas de mar en general (Yasuda *et al.*, 2009, para *Acanthaster plancii*). Sin embargo las causas de esta baja diversidad genética son diferentes para las dos especies estudiadas.

Equinodermos	H_d	π	Referencia
Arbacia lixula	0.912	0.00658	Wangensteen *et al.* 2012
Paracentrotus lividus	0.961	0.0071	Duran *et al.* 2004b
Holothuria mammata	0.92	0.0071	Borrero-Pérez *et al.* 2011
Holothuria poli	0.930	0.0053	Valente *et al.* 2014
Ophiotrix fragilis	1	0.012	Pérez-Portela *et al.* 2013
Ophiotrix sp. II	0.998	0.0189	Pérez-Portela *et al.* 2013
Opioderma longicauda	0.980	0.019	Boissin *et al.* 2011
Marthasterias glacialis	0.911	0.0081	Pérez-Portela *et al.* 2010
Echinaster sepositus	0.619	0.00129	
Coscinasterias tenuispina	0.645	0.00452	

Tabla 1. Diversidad haplotípica total (H_d) y diversidad nucleotídica total (π) de COI para diferentes equinodermos Atlanto-Mediterráneos.

Mientras los valores de diversidad genética para *E. sepositus* se muestran bastante homogéneos dentro de las cuencas estudiadas, *C. tenuispina* es más heterogénea y presenta un máximo de

diversidad en las Islas Canarias. Las áreas de mayor diversidad genética suelen relacionarse con las zonas de origen de las especies, aquellas desde donde expandieron sus áreas de distribución (Arrigo *et al.,* 2010). En el caso de *C. tenuispina,* otras hipótesis alternativas también podrían explicar este máximo de diversidad, como por ejemplo, una mayor tasa de reproducción sexual en Canarias. Las condiciones climáticas y la disponibilidad de alimento pueden también modular el ciclo reproductor de *C. tenuispina* (Ventura *et al.,* 2004); en respuesta a situaciones de estrés relacionadas con factores ambientales, la reproducción asexual puede ser la forma dominante (Mladenov, 1996), lo que tiene consecuencias en la diversidad genética de las poblaciones. En Canarias, *C. tenuispina* se encuentra en una zona donde las temperaturas son templadas y relativamente constantes a lo largo del año, sin llegar nunca a los mínimos que se dan en algunas áreas mediterráneas y del mar Cantábrico, ni sufrir tampoco grandes oscilaciones de temperatura entre el invierno y el verano. Estas condiciones permiten potencialmente completar el ciclo sexual de los individuos y por tanto, la recombinación genética que genera nuevos genotipos, que a su vez incrementan la diversidad genética de las poblaciones. Las grandes diferencias en la prevalencia de un tipo de reproducción u otro son las que determinan las diferencias observadas entre la diversidad genética de las poblaciones.

El caso de *Echinaster sepositus,* las causas inferidas para explicar la baja diversidad son diferentes. A pesar de la homogeneidad general, se dan unos ligeros máximos de diversidad genética en la cuenca occidental del Mediterráneo. Podría tratarse, por lo tanto, de la zona geográfica de origen de la especie, desde donde se expandió a otras áreas, pero también es cierto que dicha cuenca recibe tanto los migrantes del Atlántico como los del Mediterráneo oriental y, con ellos, un posible incremento de su variabilidad genética.

Es curioso observar que justo en el Mediterráneo occidental, zona donde parece que se dan las condiciones óptimas para las poblaciones de *E. sepositus*, las poblaciones de *C. tenuispina* parecen encontrarse en su límite de su distribución, tal y como indican sus elevadas tasas de clonalidad y la ausencia de reproducción sexual en esta cuenca (capítulos 2 y 3).

Determinados procesos demográficos, como son las expansiones (aumento drástico en el tamaño efectivo poblacional) o los cuellos de botella (reducción drástica en el tamaño efectivo poblacional), pueden afectar de forma importante a la diversidad genética de las poblaciones de ambas especies (Avise, 2000). Además de estos eventos demográficos, hay otros procesos que influyen en el mantenimiento y/o pérdida de la diversidad genética a lo largo del tiempo, como la deriva genética, que depende directamente del tamaño poblacional efectivo (Nei & Tajima, 1981). Nuestros datos muestran una posible expansión reciente de las poblaciones de *E. sepositus,* datada hace tan solo 9 – 12 mil años, precisamente después del último máximo glacial ocurrido aproximadamente hace 20 mil años, y que tuvo un gran impacto en las especies bentónicas litorales de la costa Europea (Maggs *et al.,* 2008). Teniendo en cuenta los bajos valores de diversidad de las poblaciones actuales de esta especie, la expansión debió producirse a partir de tamaños efectivos poblacionales muy pequeños (capítulo 2), y parece que no han llegado aún al equilibrio. Este resultado contrasta con los otros estudios de equinodermos en la misma área geográfica. La mayoría de equinodermos del Mediterráneo han pasado por expansiones demográficas históricamente mucho más tempranas. Estas expansiones se han datado desde hace 300 – 600 mil años en el caso de *Holothuria mammata* (Borrero-Pérez *et al.,* 2011), 200 – 400 mil años en *Holthuria polii* (Valente *et al.,* 2014), y más de 90 mil años en el caso de *Arbacia lixula* (Wangensteen *et al.,* 2012); hasta dos expansiones fueron datadas para *Marthasterias glacialis* hace aproximadamente 61 – 87 mil años, la primera, y 48 – 127 mil años la segunda (Pérez-Portela *et al.,* 2010). La baja diversidad genética de *E. sepositus* es comparable con la de las especies que han colonizado nuevas regiones después del último periodo glacial (Ben-Shlomo *et al.,* 2006). Las pocas diferencias entre las poblaciones de esta estrella, considerando tanto los microsatelites como el COI (en este caso, entre poblaciones de la misma cuenca) también apoyan la hipótesis

de que la expansión reciente haya, potencialmente, borrado la huella de una estructura genética pasada en la especie (Maggs *et al.*, 2008; Hellberg, 2009).

En el caso de *Coscinasterias tenuispina*, debido a su elevada tasa de clonalidad, datar procesos demográficos del mismo modo que en una especie de reproducción exclusivamente sexual, es mucho más complejo y puede llevar a errores. Para realizar el cálculo de la variación demográfica se usa, entre otros métodos, la distribución de la distancia genética entre pares de alelos a lo largo del tiempo. Las poblaciones van incorporando mutaciones a una tasa determinada, las cuales quedan fijadas o se pierden al azar. El tamaño efectivo poblacional está estrechamente relacionado con la deriva genética, un proceso que por azar hace que los alelos menos frecuentes tengan más probabilidad de desaparecer en la siguiente generación. Así en poblaciones de tamaño efectivo grande, la probabilidad de pérdida de diversidad genética es menor que en las poblaciones de tamaño reducido. Desde un punto de vista teórico, las poblaciones estrictamente clonales o con elevada clonalidad, tienden a tamaños poblacionales efectivos de infinito (Balloux *et al.*, 2003), ya que durante el proceso de reproducción asexual (como es la fisión en *Coscinasterias*) se transmite el 100% del genoma a los descendientes, lo que reduce la pérdida de diversidad por deriva genética a 0. Así, en estas poblaciones clonales, incluso las compuestas por un número reducido de individuos, el tamaño efectivo poblacional teórico es infinito, sin ser afectadas por procesos de deriva a lo largo del tiempo (Delmotte *et al.*, 2002; Balloux *et al.*, 2003).

Para datar una expansión demográfica es necesario aplicar un reloj molecular, y por métodos de coalescencia, se puede saber en qué momento ocurrió la expansión considerando el número de generaciones. Si se desea conocer el valor en años, es necesario saber el número de generaciones anuales de la especie concreta. En muchos invertebrados marinos, y en la mayoría de equinodermos, se utiliza por defecto el valor de una generación por año (Bermingham, 1997; Grosberg *et al.*, 1998; Lessios *et al.*, 1999, 2001; Uthicke & Benzie, 2003). En el caso de *C. tenuispina*, debido a la reproducción asexual, esta asunción parece muy arriesgada. En cualquier caso, cada individuo va acumulando mutaciones somáticas a lo largo del tiempo y, por tanto, transmitiéndolas a la siguiente generación asexual (Muller, 1964; Kondrashov, 1994). Waters & Roy (2003), en su trabajo sobre la filogenia del género *Coscinasterias*, determinaron los tiempos de divergencia tanto para las 4 especies del género, como entre las poblaciones de *C. tenuispina* de ambos lados del Atlántico, asumiendo el reloj molecular para COI de equinoideos (1.6 - 3.1 % por MY; Lessios *et al.*, 1999, 2001). Sus resultados, aplicando este reloj, explican la divergencia de las cuatro especies en función de eventos geológicos e históricos. Los capítulos 3 y 4 de esta tesis doctoral demuestran que la incidencia de la reproducción asexual en *Coscinasterias tenuispina* es muy elevada y, que puede mantener poblaciones monoclonales y pasar largos periodos en ausencia de reproducción sexual. Este hecho es probable que suceda en otras especies del género y, por tanto, estos resultados apoyarían la hipótesis de que el reloj molecular podría relacionarse más con el número de divisiones celulares de la línea germinal que con el número de generaciones asexuales sucedidas (Crow, 1997; Thomas *et al.*, 2006, 2010).

Estructura genética y dispersión larvaria

Para la mayoría de invertebrados marinos bentónicos, la fase larvaria es la etapa más importante en la dispersión de la especie, y sus características están relacionadas con la conectividad de las poblaciones, ya que los adultos suelen tener poca o nula conectividad (Cowen & Sponaugle, 2009). Factores como la capacidad de producción de gametos, el tiempo de supervivencia larvaria en la columna de agua, la capacidad de asentamiento de la larva, y la depredación sobre los juveniles, junto con las barreras oceanográficas, entre otros, determinan en gran manera la dispersión efectiva en las especies. Las características reproductivas de *Echinaster sepositus*, que presenta una larva lecitotrófica de vida corta, y las de *Coscinasterias*

tenuispina, con muchas poblaciones mantenidas únicamente con reproducción asexual, pero que genera una larva planctotrófica con gran capacidad de dispersión durante la reproducción sexual, son factores que juegan un papel importante en el flujo genético y la conectividad de sus poblaciones, lo que finalmente influye de manera determinante en la estructura genética de las dos especies.

A pesar de que ambas estrellas comparten parte de su área de distribución, las poblaciones de *E. sepositus* y *C. tenuispina* muestran una estructura genética muy diferente. Por ejemplo, en el Atlántico noroccidental, *C. tenuispina* presenta diferencias claras en la estructura genética de sus poblaciones (Canarias *versus* Gijón), mientras que las de *E. sepositus* son genéticamente más similares, sobretodo en el COI (Canarias *versus* Roscoff). Un caso extremo sería el de *C. tenuispina* en la cuenca occidental del Mediterráneo, representada por una gran población compuesta por un solo linaje clonal. En el resto de cuencas, la elevada tasa de asexualidad de esta estrella hace que se mantengan las diferencias entre poblaciones a lo largo de las generaciones cuando el linaje asexual predominante es diferente en cada población, y por tanto este hecho provoca que la estructura genética sea marcada. En poblaciones monoclonales, la deriva genética es nula, mientras que en poblaciones con tamaño efectivo pequeño, el efecto de la deriva genética puede ser muy fuerte, favoreciendo la diferenciación genética entre poblaciones rápidamente (Balloux *et al.*, 2003; Marriage & Orive, 2012); la deriva genética actúa como fuerza opuesta al flujo genético, que homogeneiza la estructura de las poblaciones.

No solo es el potencial de dispersión de la larva el que determina la diferente estructuración genética de las poblaciones (capítulos 2 y 3). *E. sepositus*, que tiene una larva lecitotrófica, con un corto periodo de vida en la columna de agua (Nachtsheim, 1914), debería tener una elevada estructura genética entre poblaciones geográficamente cercanas, y sin embargo no sucede así (capítulo 2). Por otra parte, en *C. tenuispina*, con una larva planctotrófica que vive varias semanas en la columna de agua (Barker, 1978; Shibata *et al.*, 2011), se esperaría poca diferenciación en la estructura genética de sus poblaciones cercanas, ya que a mayor tiempo de vida larvaria, mayor potencial de dispersión y, por tanto, más flujo genético que homogeniza las poblaciones (Siegel *et al.*, 2003). Este trabajo de tesis reafirma lo que otros trabajos recientes apuntaban, y es que la naturaleza y biología de la larva de las especies no siempre se correlaciona directamente con la estructura genética de sus poblaciones, y son numerosas las excepciones presentes en la literatura (Cowen, 2002; Leis, 2006; Woodson & McManus, 2007). Como ya se ha comentado en la introducción, otros procesos, como la circulación marina tanto a pequeña escala como a gran escala y sustrato disponible juegan también un papel fundamental en la dispersión y conectividad de poblaciones (Pineda *et al.*, 2010).

Sin embargo, en el caso de *C. tenuispina*, existe un factor adicional que determina su estructura genética, y es su reproducción asexual. Debido a la elevada tasa de asexualidad en todas las poblaciones analizadas, es difícil interpretar el peso que tiene el potencial de dispersión de la larva en la estructura genética de la especie. Aún así, el flujo genético detectado entre las poblaciones de Canarias y las del Mediterráneo oriental, confirman el potencial de la larva de esta especie para conectar poblaciones distantes.

Además de los fenómenos de dispersión, el tipo de larva condiciona otros aspectos de la biología de las especies. Las características de una larva lecitotrófica permiten que, tras la fase de asentamiento, los nuevos reclutas dispongan de recursos tróficos para empezar a desarrollarse (Byrne & Cerra, 2000; Villinski *et al.*, 2002). Además, su corta vida larvaria posibilita que muchos individuos recluten cerca de sus progenitores, asegurándose un lugar viable para la especie, lo que se denomina "efecto tampón" (Uthicke *et al.*, 2009). De todos modos, la presencia de este "efecto tampón" en *Echinaster sepositus* entraría en conflicto con algunos de los resultados del capítulo 2. La reproducción asexual de *C. tenuispina* podría tener un papel de "tampón" parecido al de las larvas lecitotróficas, ya que la fisión de los individuos permite que la siguiente "generación" se encuentre en la misma zona donde han crecido los adultos originales,

asegurándose un nicho ecológico confortable. Esta hipótesis también sería compatible con la distribución por contagio frecuente en ambas especies, tal y como establecen para *E. sepositus* Villamor & Becerro (2010). Pero con esta tan -a priori- escasa capacidad de dispersión, ¿cómo y de qué forma encuentran estas especies nuevos lugares aptos para colonizar?

La amplia distribución de *Echinaster sepositus,* aunque más restringida que la de *Coscinasterias*, podría depender de su potencial de crear gametos y larvas con diferente flotabilidad. Mientras Villamor (2010) y Riesgo *et al.* (2011), afirman que, en condiciones experimentales de desove inducido, las larvas de *E. sepositus* no flotan, Mitic (1992) y Turner *et al.* (2010) defienden lo contrario. Las larvas lecitotroficas presentan altos contenidos en lípidos (Byrne & Cerra, 2000; Villinski *et al.,* 2002; Prowse *et al.,* 2009) que además de actuar como reserva energética para su desarrollo, también confieren flotabilidad a las larvas (Prowse *et al.,* 2009). Por lo tanto, los diferentes resultados respecto a la flotabilidad podrían ser debidos a la diferente composición lipídica de las larvas en el momento de desove, resultante de las condiciones experimentales a las cuales han estado sometidas. La poca estructuración genética que presenta *E. sepositus* dentro de cada una de las cuencas mediterráneas podría estar relacionada con un mayor potencial de dispersión, resultante de larvas con mayor flotabilidad de la esperada y permitiendo un mayor flujo genético del esperado. No obstante, un aumento poblacional muy acentuado y reciente podría haber homogenizado las diferencias genéticas entre áreas y, además, podrían no haber pasado suficientes generaciones para conseguir llegar al equilibrio. En cualquier caso, ambas hipótesis, de un mayor flujo genético o de la expansión reciente, no son excluyentes una de la otra.

C. tenuispina dispone de un gran potencial colonizador que depende principalmente de su reproducción asexual, ya que le permite establecerse rápidamente en un área a partir de un número muy reducido de individuos, o incluso de uno solo (Agrawal, 2006; Kronauer *et al.,* 2012). Además, cuando se reproduce sexualmente, libera larvas planctotróficas que pueden dispersarse grandes distancias. No obstante, ninguno de estos mecanismos puede explicar de manera clara la amplia distribución de un mismo clon en toda la cuenca occidental del Mediterráneo. *C. tenuispina* es una especie con buena movilidad, pero no parece posible que el simple desplazamiento de los adultos pueda abarcar todo el rango de distribución del clon. Una posibilidad sería que utilizara el denominado mecanismo de *rafting*, es decir, la dispersión de adultos a través de substratos móviles, que es un sistema habitual de dispersión para muchos invertebrados bentónicos, sésiles y móviles (Grantham *et al.,* 2003). Aunque no está descrito en asteroideos, sí que hay otros equinodermos de pequeño tamaño que utilizan el *rafting* como mecanismo -habitual o esporádico- de dispersión (Hendler *et al.,* 1999; Roy & Sponer, 2002; Grantham *et al.,* 2003). El *rafting* se ha descrito también como un mecanismo importante para la dispersión en largas distancias tanto para organismos clonales como aclonales (Jackson & Coates, 1986).

Las barreras biogeográficas que se encuentran a lo largo del rango de distribución de ambas estrellas, como son el estrecho Sículo–Tunecino y el frente Almería–Orán, que marca la verdadera barrera entre el Océano Atlántico y el Mar Mediterráneo (Patarnello *et al.,* 2007; Calderón *et al.,* 2008; Galarza *et al.,* 2009), restringen el flujo genético y limitan la conectividad entre las poblaciones, tanto para *E. sepositus* como para *C. tenuispina*, del mismo modo que sucede con otras especies marinas (Patarnello *et al.,* 2007). Las barreras biogeográficas no se deben entender como una línea divisoria entre dos áreas, ya que suelen ser permeables; debido a las variaciones de las corrientes marinas, las poblaciones que se encuentran cercanas a las barreras pueden tener la influencia de ambos lados de la misma (Béranger *et al.,* 2005). Esto parece que ocurre en la población de *E. sepositus* de Taormina, en Sicilia, que tiene mayor afinidad a las poblaciones del Mediterráneo occidental, aunque pertenezca geográficamente al oriental. En el caso de *C. tenuispina*, los individuos encontrados en la misma población de Taormina forma parte claramente de la cuenca oriental.

Otras posibles barreras biogeográficas influyen también en la distribución global de *C. tenuispina*, como es el caso del propio océano Atlántico, de la pluma del Amazonas–Orinoco y de la surgencia de agua fría de Cabo Frío (Brasil). La barrera oceánica no parece ser un problema para *C. tenuispina*, puesto que se trata de una especie anfiatlántica, y su área de distribución debería ser consecuencia de un gran potencial dispersión (en principio, larvario), que es el que se precisa para poder cruzar el océano Atlántico (Luiz *et al.*, 2012). La pluma del Amazonas–Orinoco, en la costa este americana, limita la conectividad de las poblaciones y favorece la adaptación de la especie a las diferentes condiciones ecológicas de las dos ecoregiones que separa la barrera (Spalding *et al.*, 2007). El hecho de que los haplotipos de las poblaciones de *Coscinasterias tenuispina* de Florida y de Brasil sean relativamente lejanos entre sí y que, a su vez, sean más cercanos cada uno de ellos a los haplotipos encontrados en las poblaciones de Canarias, indica la posibilidad de que esta especie haya cruzado el Atlántico varias veces desde su potencial área de origen (Atlántico Este, zona templada-cálida). En cualquier caso, los datos moleculares, basados en microsatélites no muestran evidencias de que actualmente haya flujo genético a través del Atlántico y de que las poblaciones permanezcan conectadas; por tanto, estas poblaciones pueden estar evolucionando independientemente unas de otras. Si este proceso se mantiene sin que haya una reconexión, acabarán siendo especies diferentes.

Entre el Atlántico y el Mediterráneo, la dirección de migración dominante tanto para *C. tenuispina* como para *E. sepositus* es la oeste-este, de entrada hacia el Mediterráneo. La masa de agua atlántica, más ligera, entra en el Mediterráneo por la zona superficial, a través del estrecho de Gibraltar (Millot, 1999) y esto facilita que la migración dominante sea en esta dirección (Schunter *et al.*, 2011). Pero entre las dos cuencas mediterráneas, la estructura genética y la conectividad es diferente para las dos especies. Por un lado, los resultados de coalescencia mediante inferencia bayesiana (LAMARC) obtenidos para *E. sepositus* indican un flujo genético mayor desde el Mediterráneo oriental al occidental. En el caso de *C. tenuispina*, las diferencias poblacionales entre cuencas se deben principalmente a la ausencia del clon occidental en el Mediterráneo oriental. En general, las poblaciones del Mediterráneo oriental de esta estrella tienen más conectividad con las de las Islas Canarias que con las del Mediterráneo occidental. Evidentemente, ambas áreas tienen que conectarse a través de la cuenca occidental, y esta conexión debería darse a través de la costa del norte de África, donde las condiciones ambientales podrían posibilitar la reproducción sexual de la especie y la dispersión larvaria. Aunque no hay datos del flujo entre las cuencas mediterráneas, la relación de Canarias con el Mediterráneo oriental parece indicar que el flujo genético neto para esta especie a lo largo de todo el Mediterráneo es mayor de oeste a este.

Ciclo biológico

A pesar del desarrollo completo de unas pocas gónadas durante los meses invernales, localizadas en los brazos de algunos ejemplares, no hay ninguna evidencia de reproducción sexual en la población estudiada de *C. tenuispina*, un dato también apoyado por los análisis moleculares durante un periodo de más de dos años. No se han observado ni hembras ni reclutas, al menos de un genotipo diferente, durante toda la duración del estudio (más de dos años, ver capítulo 4), y parece que la población se mantiene únicamente por procesos de reproducción asexual. Estas características podrían ser extrapolables al resto de poblaciones clonales de la cuenca occidental del Mediterráneo.

Los individuos de *C. tenuispina* observados en Canarias son de mayor tamaño que los individuos del Mediterráneo (capítulos 3 y 4). También es en Canarias donde se ha constatado un desarrollo gonadal más completo, incluso con hembras presentes en algunas poblaciones; parece, por tanto, que con muchas reservas, el tamaño podría relacionarse con la reproducción sexual. Esta relación del tamaño con la sexualidad ya se ha visto en otras especies de

equinodermos, como es el caso de la ofiura clonal *Ophiactis savignyi*, en la que las hembras necesitan alcanzar tamaños grandes para llegar a formar gónadas maduras (McGovern, 2002).

Coscinasterias tenuispina y *Echinaster sepositus* tienen unas características biológicas muy diferentes en el Mediterráneo noroccidental, y esto es especialmente evidente en lo que respecta a su ciclo vital. Estas diferencias están, posiblemente, asociadas a su origen evolutivo. En el caso de *E. sepositus*, los datos moleculares indicaron un potencial origen Mediterráneo (posiblemente Mediterráneo occidental), y en el caso de *C. tenuispina* en el Atlántico oriental (alrededor de las Islas Canarias, y en general en el área de la Macaronesia). Los estudios llevados a cabo por Villamor (2010) y Riesgo *et al.* (2011), mostraron que *E. sepositus* presenta su máximo gonadal en los meses de verano, al contrario de lo que sucede con *C. tenuispina*. El momento del potencial desove de cada especie podría estar relacionado con las características larvarias: la larva de *C. tenuispina*, que tiene que sobrevivir y alimentarse en la columna de agua, y por tanto, aprovecha el incremento de biomasa de plancton que sucede en los meses de invierno y primavera en el Mediterráneo noroccidental, de esta manera incrementa sus posiblidades de éxito, tal y como hacen otros equinodermos (López *et al.*, 1998; Siokou-Frangou *et al.*, 2010). En el caso de *E. sepositus*, la aparición de larvas coincide con la máxima estratificación en la columna de agua; esta estratificación podría ser favorable para una larva lecitotrófica que dispone de recursos suficientes para sobrevivir y desarrollarse durante un corto periodo larvario y de post-asentamiento (Byrne & Cerra, 2000; Villinski *et al.*, 2002; Prowse *et al.*, 2009), y, además, facilitaría el efecto "tampón" anteriormente comentado (Uthicke *et al.*, 2009).

Cuadro 3: Limitaciones metodológicas

Los diferentes marcadores moleculares y las técnicas de estudio utilizadas en esta tesis, no están exentos de limitaciones. Estas limitaciones van desde la toma de muestras biológicas, ya que los ejemplares y poblaciones de las especies objeto no siempre se encuentran en las zonas que *a priori* permitirían completar un diseño muestral óptimo, a la interpretación de los resultados obtenidos a través del análisis de los marcadores moleculares. Además, los marcadores utilizados, que son adecuados para nuestros objetivos, pueden no serlo para responder a algunas de las nuevas preguntas que se plantean una vez resueltos los objetivos iniciales. A continuación se detallan algunos de los puntos críticos que han podido limitar nuestras conclusiones, y las medidas adoptadas para desarrollar de manera más adecuada la investigación propuesta:

Muestreo y toma de datos biológicos

Tanto *Echinaster sepositus* como *Coscinasterias tenuispina* presentan una distribución parcheada (Barker, 2013; Turner, 2013); los organismos se encuentran agregados de tal manera que pueden ser muy abundantes en una zona costera y desaparecer absolutamente en otra zona situada solo a decenas de metros de la primera. Del mismo modo, allí donde están presentes, la densidad de individuos puede ser muy variable. Todo esto incrementa muchísimo el esfuerzo necesario para una correcta toma de muestras y hace difícil en muchos casos completar el diseño inicial de muestreo y que se había planteado como óptimo para abordar los objetivos del trabajo. También es importante destacar que, como sucede en la mayoría de los estudios ecológicos de campo, las condiciones meteorológicas han sido limitantes, dificultando el trabajo tanto en los muestreos extensivos para genética de poblaciones de ambas especies, como en el seguimiento del ciclo biológico de *C. tenuispina*.

Marcadores genéticos

Cada señal de microsatélite en el cromatograma (que representa la distribución del tamaño del alelo correspondiente) tiene una morfología diferente, apareciendo dobles y triples picos, o formas diversas (Figura 6), lo cual incrementa la subjetividad a la hora de medir los picos,

sobretodo cuando no se hace con todos los individuos en el mismo momento. Así mismo, debido al gran número de ejemplares estudiados, éstos han tenido que ser analizados en diversas placas, lo que obligó a calibrar el análisis tanto de los genotipos de individuos y poblaciones, como del criterio de lectura de cromatogramas. La dificultad de genotipar correctamente, sumada a los errores de amplificación, ya ha sido objeto de diferentes trabajos (Dewoody *et al.*, 2006; Amos *et al.*, 2007). Que una misma persona realice el genotipado para un mismo trabajo estandariza el sistema de toma de datos y evita en gran medida la posible desviación de resultados. Para resolver estos problemas, en el caso de *Echinaster sepositus* se repitieron muestras en placas diferentes, pudiendo calibrar correctamente los datos obtenidos para diversos tiempos. En *Coscinasterias tenuispina*, se utilizaron dos individuos procedentes de Canarias a modo de control en todo el análisis para calibrar los resultados entre áreas, ya que las estrellas recolectadas en Brasil fueron amplificadas y genotipadas en un laboratorio diferente al del resto de ejemplares.
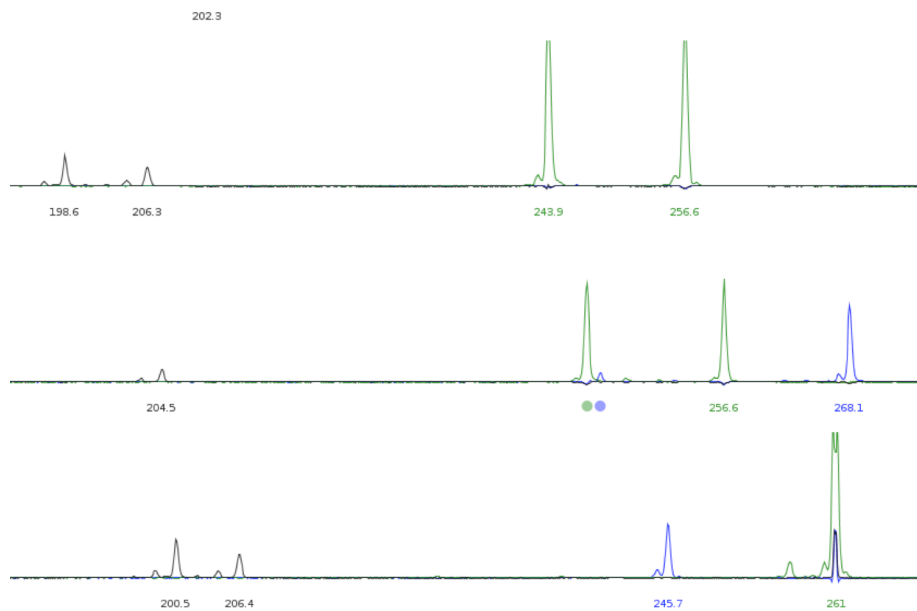
Figura 6. Ejemplo de la señal de un cromatograma indicando el valor de un microsatélite mediante picos de diferente color, dependiendo del fluorocromo, y diferente morfología, dependiendo del alelo.

El análisis bioinformático de datos presenta también dificultades metodológicas. El primer problema es que para los microsatélites nucleares se desconocen los modelos evolutivos de mutación. Aunque se aplican principalmente dos modelos, el *"Step Mutation Model"* (SMM) y el *"Infinite Allele Model"* (IAM), no se ha conseguido demostrar todavía cuál de ellos se ajusta mejor cada uno de los microsatélites (Putman & Carbone, 2014). Probablemente, el mejor ajuste sea una mezcla de ambos modelos, y dependerá de cada marcador. Existe toda una serie de análisis poblacionales que requieren conocer el modelo evolutivo correspondiente para los marcadores, y que por tanto no se pudieron aplicar en microsatélites. Por este motivo, el uso combinado del gen Citocromo C Oxidasa I y de los microsatélites, permitió realizar un análisis mucho más completo sobre la filogeografía y la genética de poblaciones de las especies, que usando solo microsatélites.

Medida de telómeros

En el caso de los telómeros, la mayor dificultad está en la determinación de su longitud absoluta, y se han descrito diversos métodos de medida. Algunos de ellos se basan en *Southern Blots*, *Single Telomere Length Analyses* (STELA) y PCR cuantitativa (qPCR). Las diferencias entre los resultados obtenidos aplicando cada uno de estos métodos e incluso dentro del mismo método, trabajando en diferentes laboratorios, han sido claras y pueden consultarse en el trabajo de Martin-Ruiz *et al.* (2014). Es por este motivo por el que, en el último capítulo de este trabajo, la medición de los telómeros de la estrella *C. tenuispina* y los análisis posteriores se han hecho en dos laboratorios diferentes, con protocolos distintos, para así asegurar la veracidad de los resultados.

Longevidad y tamaño

Como se ha explicado en los apartados anteriores, muchas poblaciones de *C. tenuispina* del Mediterráneo occidental y del Atlántico norte son clonales. Una de las preguntas que surge como resultado de la observación de estas poblaciones mantenidas exclusivamente por reproducción asexual a lo largo de los años (capítulo 4) es ¿cuánto tiempo puede vivir un linaje clonal de *C. tenuispina*? La pregunta sigue sin respuesta, del mismo modo que se desconoce si existe un límite en el número de divisiones por las que puede pasar un individuo en condiciones naturales. En especies con reproducción asexual, la posible "inmortalidad" ha sido motivo de gran debate, y existen múltiples evidencias en la literatura de especies con linajes asexuales muy longevos sin (o con muy pocos) signos de envejecimiento (por ejemplo, Judson & Normark, 1996; Schön *et al.,* 1998; Reusch *et al.,* 1999; Bosch, 2009; Ally *et al.,* 2010).

Los resultados del capítulo 5 indican que existe una elongación de los telómeros de *C. tenuispina* relacionado con procesos de fisión, un hecho que hasta el momento parecía ocurrir casi de modo exclusivo durante el desarrollo embrionario. Este proceso de elongación de los telómeros durante la fisión demuestra que esta especie dispone de al menos un mecanismo para evitar daños en el material genético a lo largo de las diferentes generaciones asexuales, lo que permitiría a los linajes clonales mantenerse a lo largo del tiempo. En la zona límite de su distribución, en la que la estrella no completa su ciclo sexual y sus poblaciones se mantienen por reproducción asexual, la mayor longitud de los telómeros estaría relacionada con la mayor clonalidad de los individuos. Paradójicamente, hay estudios que relacionan longitud de los telómeros y el estrés (tanto cronificado como puntual) apuntando a que éste provoca una baja actividad de la telomerasa y una escasa longitud de los telómeros (Epel *et al.,* 2004). No tenemos datos suficientes para saber qué influencia tiene el estrés en la estructura de los telómeros de la población de la zona estudiada, aunque seguramente existe una estrecha relación entre el estrés propio de condiciones límite, la asexualidad de la especie y la longitud de los telómeros.

Conclusiones

- *Echinaster sepositus* y *Coscinasterias tenuispina* presentan una distribución geográfica que se solapa parcialmente, aunque el patrón filogeográfico de cada una de ellas es totalmente diferente, debido a sus características biológicas y a su historia evolutiva.

- Ambas especies presentan menor diversidad genética que la que se conoce para el resto de equinodermos de distribución atlanto-mediterránea; las causas de esta baja diversidad parecen ser diferentes para cada una de ellas.

- En *Echinaster sepositus,* una expansión muy reciente a partir de un tamaño poblacional muy reducido (y un bajo número de alelos) podría explicar la poca diversidad genética en esta especie, que aún no ha alcanzado el equilibrio poblacional.

- La elevada tasa de clonalidad en todas las poblaciones de *Coscinasterias tenuispina* y la presencia de poblaciones formadas por un solo clon, serían la causa de su baja diversidad genética.

- La baja diversidad genética es la responsable de que ambas especies puedan, *a priori,* tener un bajo potencial de adaptación, y puedan verse afectadas por cambios ambientales, tal y como sucede en las dos poblaciones de *Echinaster sepositus* cercanas a zonas contaminadas.

- Las áreas de mayor diversidad para cada una de las especies no coinciden y, por tanto, su origen geográfico y posteriores expansiones a otras áreas son diferentes: *Echinater sepositus* parece tener origen en el Mediterráneo occidental y *Coscinasterias* tenuispina en el Atlántico Oeste.

- Las barreras marinas originadas por el frente Almería - Orán y por el estrecho Sículo - Tunecino, dan lugar a una restricción del flujo genético en ambas especies, aunque parece que sigue existiendo cierto grado de permeabilidad e intercambio genético.

- El flujo genético en *Echinaster sepositus* sigue la dirección de la corriente superficial de entrada de agua atlántica hacia el Mediterráneo. Dentro del Mediterráneo, el flujo genético va de la cuenca oriental a la occidental.

- El elevado potencial de dispersión de la larva de *Coscinasterias tenuispina* le ha permitido cruzar grandes barreras geográficas, como es el caso del océano Atlántico (en ambas direcciones), así como mantener cierto grado de flujo genético entre poblaciones muy distantes, como son las islas Canarias y el Mediterráneo oriental.

- Aun teniendo potenciales de dispersión larvaria muy diferentes, ninguna de las dos especies estudiadas presenta patrones de aislamiento por distancia. Este resultado apoya la idea de que en el medio marino existen barreras más importantes al flujo genético que la distancia geográfica.

- *Coscinasterias tenuispina* se reproduce asexualmente a lo largo de toda su área de distribución, y ésta parece ser la estrategia reproductiva principal en las poblaciones situadas en los límites de distribución de la especie. Estas zonas frontera están principalmente relacionadas con temperaturas mínimas más bajas.

- *Coscinaterias tenuispina* es capaz de mantener poblaciones monoclonales densas a lo largo del tiempo. La inestabilidad ambiental, tanto térmica como física, parece inducir la fisión en los individuos de esta especie.

- El potencial para la reproducción sexual de los machos de *Coscinaterias tenuispina* se mantiene en ausencia de hembras; la capacidad para desarrollar gónadas maduras se relaciona con la condición alimentaria de cada individuo.

- *Coscinasterias tenuispina* tiene mecanismos para evitar o posponer la senescencia en los linajes clonales mediante la elongación de los telómeros, lo que le permite evitar algunos de los efectos deletéreos tras generaciones consecutivas de fisión.

- Alargar la existencia de un linaje clonal y mantener el potencial reproductor del mismo permite que, cuando las condiciones ambientales sean las adecuadas, la especie pueda volver a reproducirse sexualmente, dispersarse grandes distancias y colonizar nuevas áreas.

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Apéndice I
Material suplementario

Capítulo 1

Class	Order	Species	Number of reads	Plate %	Average read length	% GC	Total SSR Loci with more than 8 repeats	% c con micro
Asteroidea	Forcipulatida	*Coscinasterias tenuispina*	220654	12.5	339.84	34.15	1974	
Asteroidea	Spinulosida	*Echinaster sepositus*	101340	16.67	238.5	41.3	261	
Echinoidea	Arbacioida	*Arbacia lixula*	315499	16.67	273.2	39.4	14041	

Online Resource 1. Reads sequenced with 454 from the three species, the different used plate percentages, average of read length (bp), percentage of GC, t of SSR with 8 repeats or more found in all sequences and percentage of reads containing at least one microsatellite.

Ratios of microsatellites types

Legend:
- dinucleotides
- trinucleotides
- tetranucleotides
- pentanucleoties
- hexanucleotides
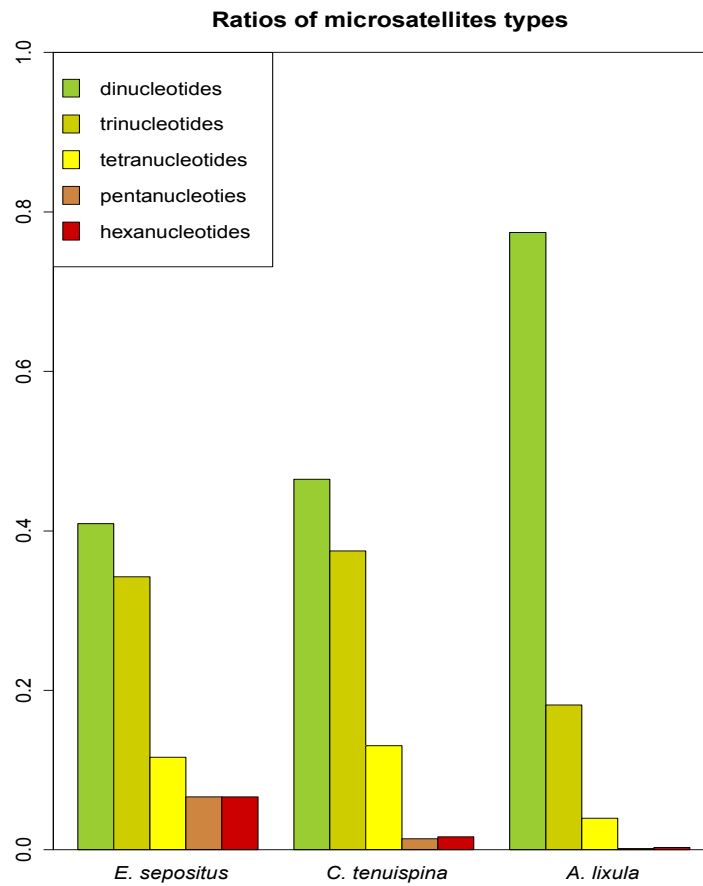
Online Resource 2. Ratio of the different microsatellite types, with at least 8 repeats and 30 bases flanking the microsatellite. The total number of found in the three species were: 181 for *Echinaster sepositus*, 1.364 for *Coscinasterias tenuispina* and 13.433 for *Arbacia lixula*.
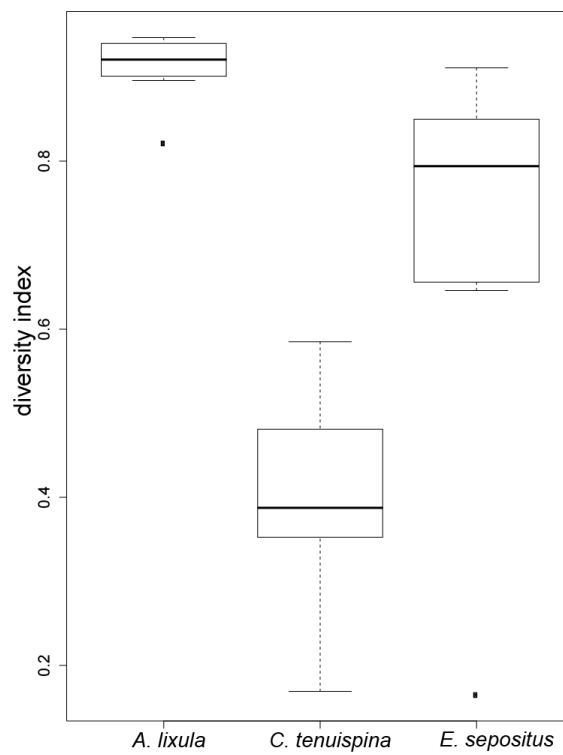
Online Resource 3. Alleles diversity of the polymorphic SSR in the three species. Boxes represents the 50 % of the SSR while the whiskers the 25 % e
highlight straight line represents the median diversity value.

Capítulo 2

Supplementary material

Tables

	CAB	CAR	PAL	BLA	PSA	ROS	MAR	LIV	TAO	TAB	MON	RHO	CRO	GIG
CABRERA	-	0.013	0.11	0.091	0.297	0.011	0.34	0.02	0.199	0.286	0.526	0.448	0.525	0.198
CARBONERAS	0.000	-	-0.037	-0.066	0.059	-0.014	0.103	-0.001	0.031	0.021	0.299	0.24	0.298	0.324
CARTAGENA	0.048	-0.025	-	-0.053	0	0.003	0.016	0.013	-0.028	-0.03	0.162	0.116	0.16	0.515
BLANES	0.030	-0.033	-0.031	-	-0.009	0.032	0.048	0.048	0.012	-0.054	0.247	0.199	0.245	0.418
ST. FELIU	0.144	0.028	-0.005	-0.008	-	0.143	-0.019	0.15	0.001	-0.072	0.112	0.088	0.111	0.641
ROSES	0.000	-0.014	-0.005	0.011	0.089	-	0.15	-0.041	0.042	0.141	0.279	0.215	0.278	0.414
MARSEILLE	0.192	0.061	0.007	0.026	-0.021	0.112	-	0.153	-0.009	-0.041	0.051	0.032	0.05	0.741
LIVORNO	0.008	-0.006	0.003	0.023	0.100	-0.043	0.124	-	0.044	0.153	0.274	0.209	0.273	0.418
TAORMINA	0.118	0.015	-0.027	0.002	-0.005	0.029	-0.015	0.034	-	-0.011	0.082	0.046	0.081	0.634
TABARKA	0.105	-0.009	-0.034	-0.044	-0.060	0.070	-0.043	0.088	-0.020	-	0.123	0.098	0.121	0.611
MONASTIR	0.411	0.264	0.177	0.221	0.127	0.309	0.073	0.324	0.121	0.183	-	0.2	0	1
RHODAS	0.365	0.219	0.131	0.184	0.100	0.249	0.045	0.260	0.068	0.146	-0.012	-	0.2	0.928
ROGOZNICA	0.402	0.256	0.170	0.213	0.121	0.301	0.068	0.316	0.115	0.172	-0.024	-0.014	-	1
LOS GIGANTES	0.088	0.124	0.207	0.140	0.265	0.207	0.344	0.227	0.309	0.208	0.583	0.555	0.574	-
ROSCOFF	0.109	0.206	0.307	0.251	0.390	0.250	0.466	0.264	0.408	0.380	0.686	0.653	0.680	0.033

Supplementary Table S1. Pairwise comparisons between populations of *E. sepositus* based on COI sequences. Φ_{ST} values (below the diagonal) and Jost's D values (above the diagonal) are presented. Significant p-values (p-value < 0.01) are in bold.

	CAB	CAR	PAL	BLA	PSA	ROS	MAR	LIV	TAO	TAB	MON	RHO	CRO	GIG
CABRERA	-	0.002	0.06	-0.006	0.004	0.003	0.007	0.125	-0.001	0.018	0.036	0.021	0.02	0.02
CARBONERAS	0.013	-	0.054	-0.003	0	0.002	0.007	0.133	-0.004	0.006	0.027	0.013	0.019	0.03
CARTAGENA	0.154	0.139	-	0.056	0.056	0.062	0.025	0.087	0.042	0.084	0.069	0.082	0.055	0.05
BLANES	0.01	0.02	0.185	-	0	-0.008	0.002	0.117	-0.013	0.023	0.041	0.031	0.019	0.05
ST. FELIU	0.024	0.014	0.136	0.054	-	-0.004	0.004	0.108	0.002	0.007	0.019	0.009	0.032	0.02
ROSES	0.033	0.035	0.171	0.026	0.014	-	0.01	0.102	-0.007	0.017	0.028	0.015	0.029	0.04
MARSEILLE	0.035	0.042	0.081	0.053	0.019	0.058	-	0.072	0.004	0.023	0.037	0.025	0.016	0.03
LIVORNO	0.347	0.369	0.219	0.332	0.297	0.277	0.21	-	0.119	0.165	0.146	0.131	0.102	0.15
TAORMINA	0.013	0.004	0.122	-0.01	0.024	0.001	0.036	0.42	-	0.022	0.022	0.02	0.023	0.03
TABARKA	0.067	0.034	0.229	0.097	0.058	0.084	0.102	0.33	0.098	-	0.029	0.031	0.016	0.06
MONASTIR	0.118	0.1	0.191	0.118	0.076	0.1	0.126	0.352	0.073	0.11	-	0.017	0.025	0.06
RHODAS	0.082	0.056	0.21	0.133	0.042	0.066	0.096	0.333	0.068	0.109	0.049	-	0.023	0.06
ROGOZNICA	0.078	0.07	0.166	0.094	0.115	0.098	0.076	0.282	0.084	0.05	0.064	0.074	-	0.07
LOS GIGANTES	0.1	0.145	0.156	0.201	0.114	0.162	0.151	0.411	0.134	0.246	0.204	0.224	0.261	-
ROSCOFF	0.064	0.046	0.134	0.089	0.06	0.045	0.075	0.33	0.034	0.136	0.11	0.087	0.124	0.11

Supplementary Table S2. Pairwise comparisons between populations of *E. sepositus* based on microsatellite loci. F_{st} values (below the diagonal) a[nd] values (above the diagonal) are presented. Significant p-values (p-value < 0.01) are in bold.

LAMARC priors	Theta	Growth	Migration		
			Western Mediterranean	Eastern Mediterranean	Atlantic
Western Mediterranean	$1e10^{-6} - 10$	$200 - 15{,}000$	-	$3{,}000 - 10{,}000$	$2{,}000 - 9{,}000$
Eastern Mediterranean	$1e10^{-4} - 10$	$1{,}000 - 15{,}000$	$1 - 4{,}000$	-	Invalid
Atlantic	$1e10^{-5} - 10$	$50 - 15{,}000$	$1e10^{-10} - 2{,}500$	Invalid	-

Supplementary Table S3. Priors selected for final analysis in LAMARC. Values of theta were logarithmically transformed.

	Gelman and Rubin's diagnosis		
	Point est.	Upper C.I.	ESS
Ln. Data. Likelihood	1.00	1.01	3,394
Theta for western Mediterranean (WM)	1.01	1.03	3,560
Theta for eastern Mediterranean (EM)	1.05	1.06	1,700
Theta for Atlantic Ocean (AT)	1.01	1.03	2,135
Migration rate into WM from EM	1.01	1.02	4,301
Migration rate into WM from AT	1.00	1.00	4,239
Migration rate into EM from WM	1.00	1.00	2,594
Migration rate into AT from WM	1.08	1.12	2,554
Growth for WM	1.00	1.00	36,81
Growth for EM	1.00	1.00	3,579
Growth for AT	1.00	1.01	2,763
Multivariate psrf = 1.02			

Supplementary Table S4. Gelman and Rubin's test and effective sample size (ESS) analysis between replicas of the Bayesian analysis performed using LAMARC.

Figures
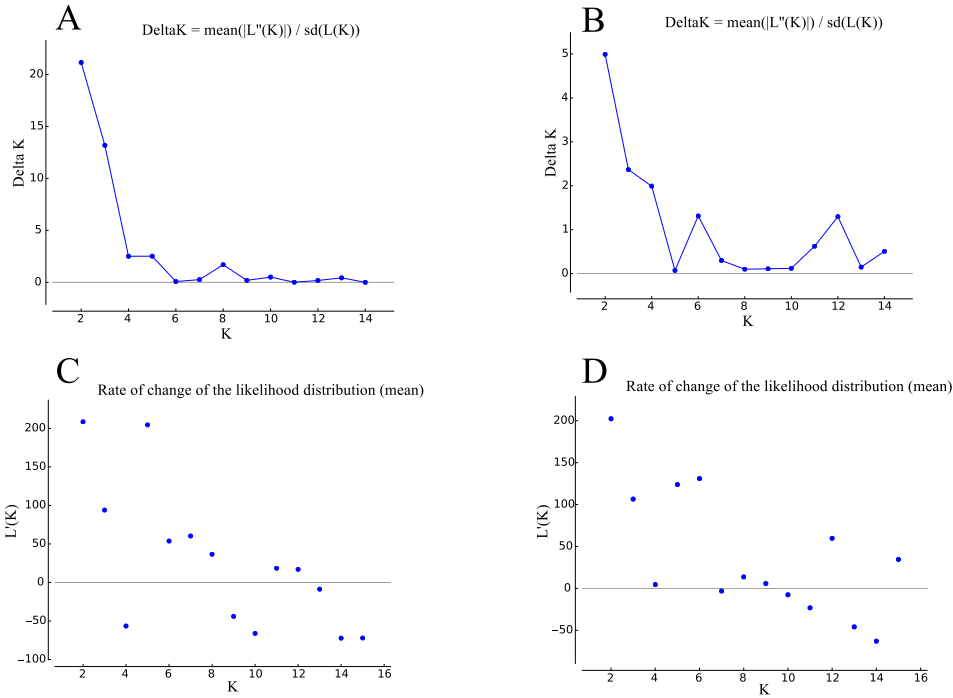
Supplementary Figure S1. Values of ΔK (Delta K) and L'(K) along the different K values obtained using STRUCTURE from two different datasets: A and C, from microsatellites; and B and D, from microsatellites and COI sequences combined.
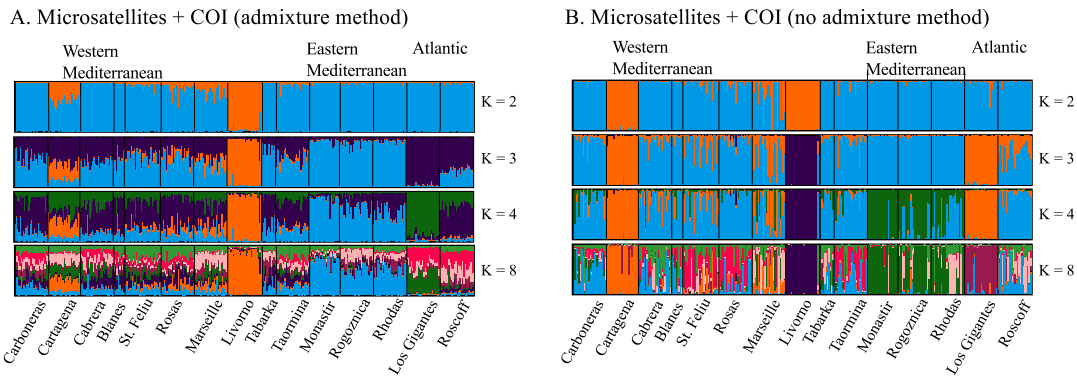
Supplementary Figure S2. Comparison of STRUCTURE results from the combined dataset of COI and microsatellites applying different methods: A) an admixture method, and B) a non-admixture method. The analysis was always with location as a prior.
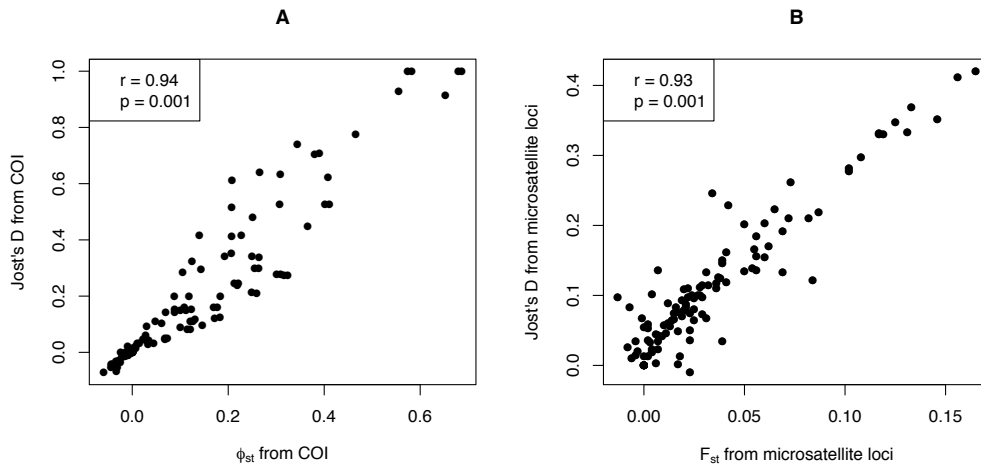
Supplementary Figure S3. Correlations between: (A) Φ_{ST} and Jost's D for COI data and (B) F_{ST} and Jost's D from microsatellite loci. Note the different scales of the graphs.
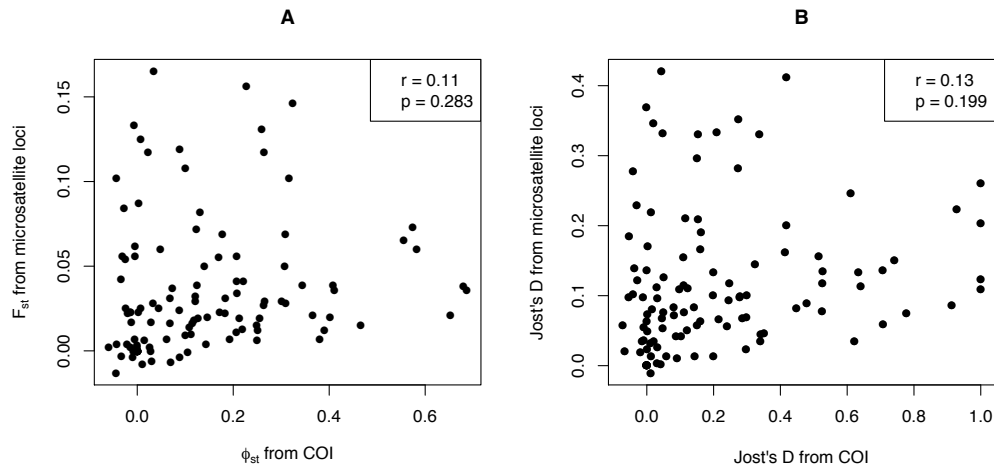
Supplementary Figure S4. Correlation between pairwise comparisons of populations using COI and microsatellite loci. A) Φ_{ST} of COI and F_{ST} of microsatellites, and B) Jost's D from COI and microsatellites.

A)
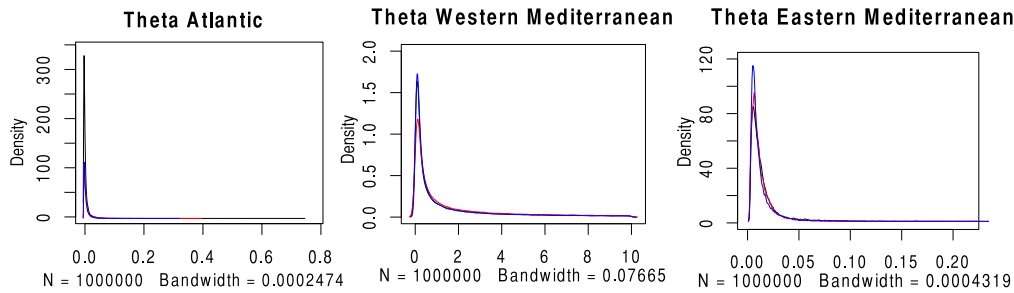
B)
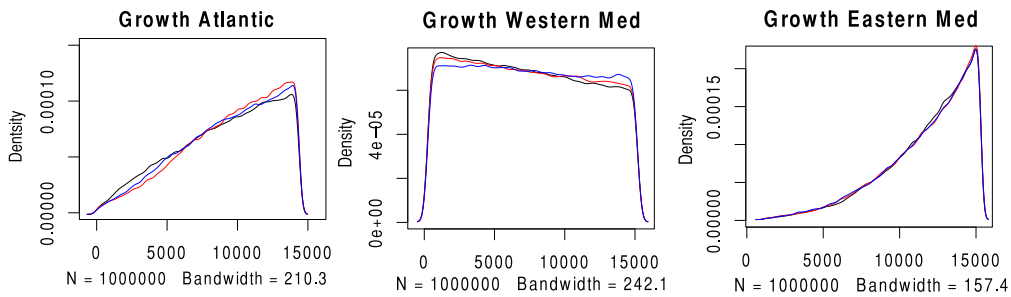
C)
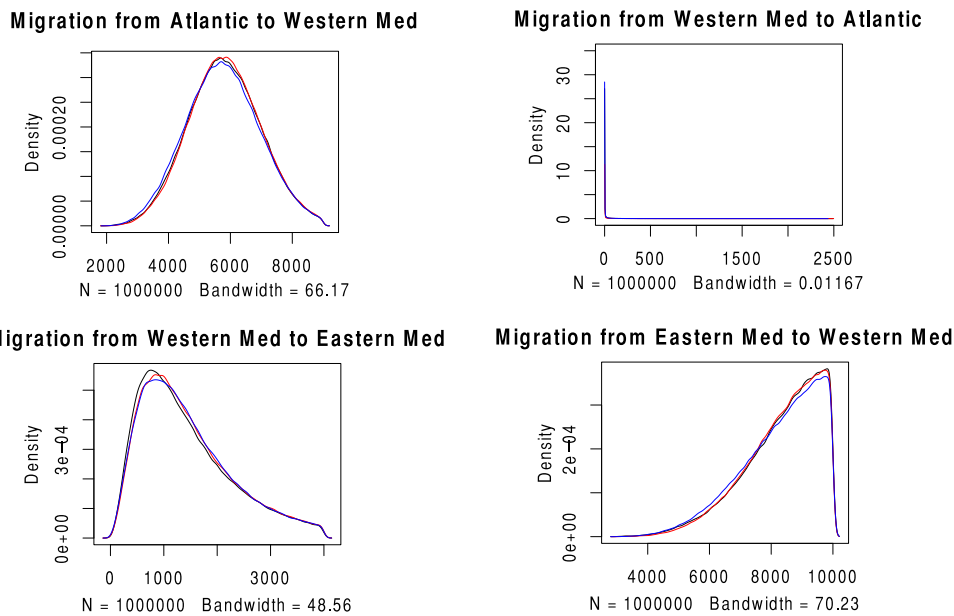
Supplementary Figure S5. Plots of MCMC values from LAMARC replicates for the Atlantic, western Mediterranean and eastern Mediterranean basins: A) *theta* values, B) Growth, and C) migration between basins and sub-basins.

Ln.Data.Likelihood.
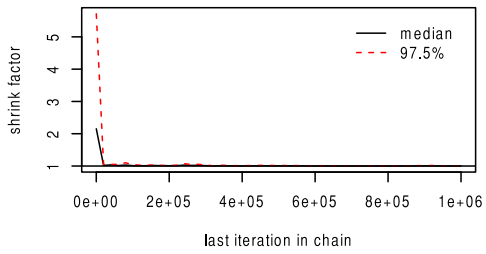
Theta.for.East Med
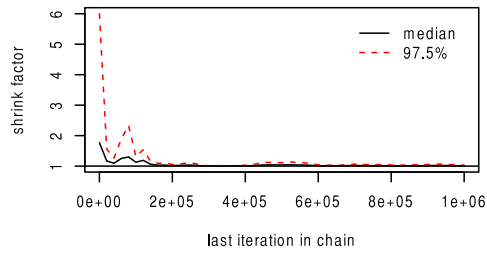
Theta.for.West Med
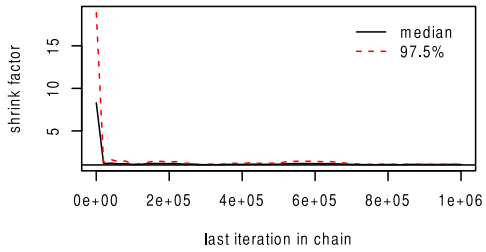
Theta.for.Atl
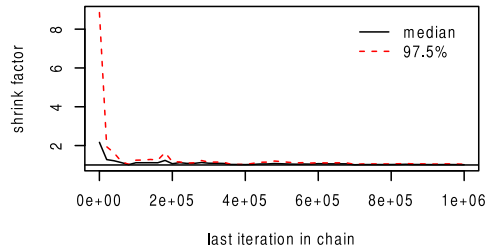
Migration.rate.into.West Med.from.East Med

Migration.rate.into.West Med.from.Atl

Migration.rate.into.East Med.from.West Med

Migration.rate.into.Atl.from.West Med

Growth.for.West Med

Growth.for.East Med
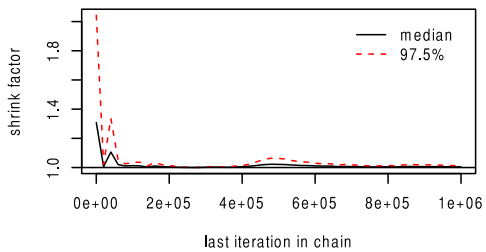
Growth.for.Atl
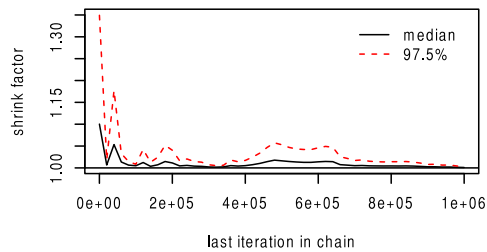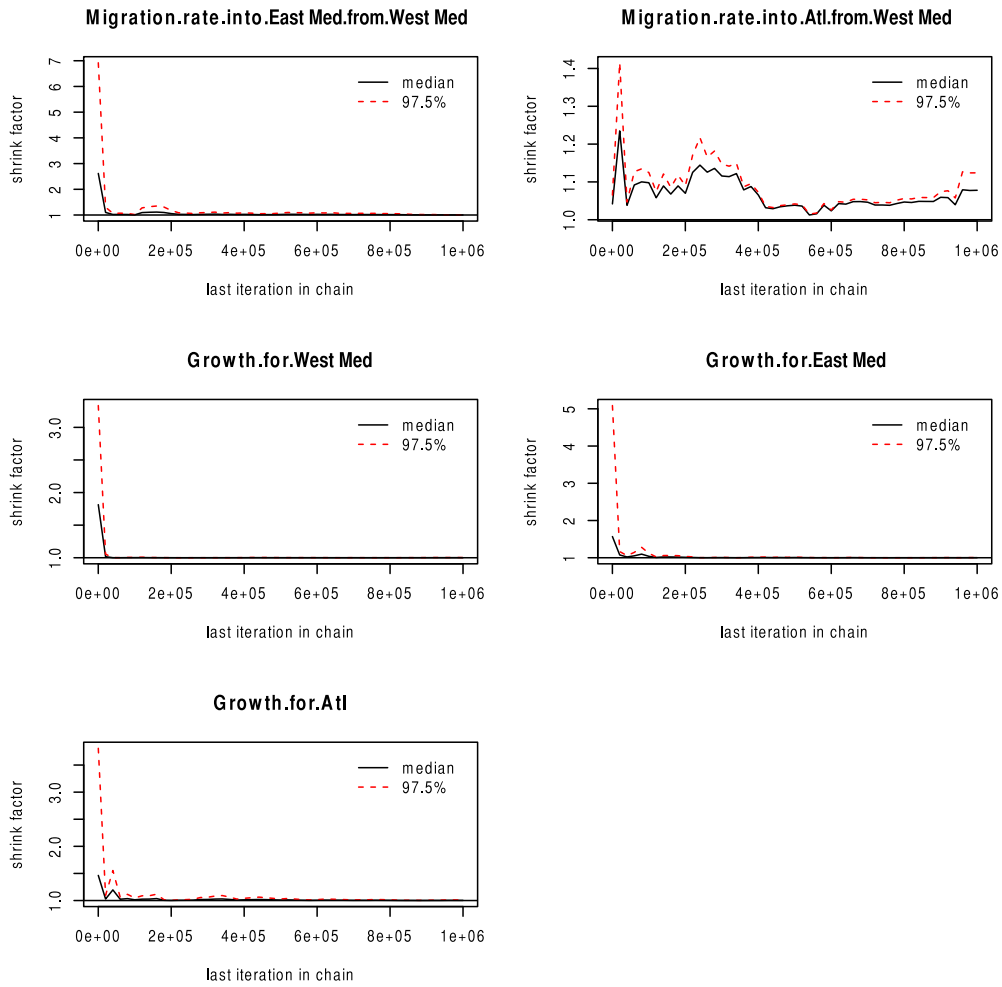
Supplementary Figure S6. Values of Gelman and Rubin's test that show whether the shrink factor converges between replicas or fluctuates.

Capítulo 3

Supplementary material

	Correlation values (R^2)				
	Range Tº	Max Tº	Min Tº	Mean Tº	Salinity
R	-0.196	0.141	0.309	0.365	-0.229
div	-0.21	0.302	0.435	**0.514**	-0.206
eve	**0.598**	-0.102	**-0.722***	**-0.612**	0.31

Supplementary Table S1. Correlation of clonal richness (R), diversity (div) and evenness values (eve) with the range of temperature between the coldest and warmest season (Range Tº), maximum and minimum temperatures (Max. Tº and Min Tº, respectively), mean temperature over the year (Mean Tº), and mean salinity over the year (Salinity). Temperatures expressed in degrees Celsius (ºC). * indicates p-value < 0.05 after Benjamini-Yecutieli correction and values in bold indicate correlations higher than 0.5.

Locality	Range Tº	Max Tº	Min Tº	Mean Tº	Salinity
ALICANTE	12.769	26.733	13.963	19.608	41.203
LLANCÀ	10.844	22.507	11.663	16.781	41.788
NAPOLS	12.366	26.386	14.021	19.771	41.988
SICILY	13.595	27.783	14.188	19.863	41.746
KNIDOS	9.757	26.11	16.352	20.536	42.115
PLAKIAS	9.594	25.471	15.877	20.448	42.239
ABADES	4.78	23.808	19.028	21.175	37.858
BOCACANGREJO	4.958	23.94	18.982	21.291	37.802
LANZAROTE	5.116	23.287	18.171	20.534	37.737
TAZACOSTE	4.909	24.613	19.704	22.098	37.727
GIJÓN	8.279	20.516	12.237	15.617	34.932
FLORIDA	14.943	31.132	16.189	23.931	36.249
FERRADURA	4.471	26.211	21.74	23.835	35.786
ITAIPÚ	4.068	26.307	22.239	24.113	35.793
PRAINHA	4.303	25.891	21.588	23.712	35.793
TARTARUGA	4.755	26.554	21.799	24.044	35.786

Supplementary Table S2. Temperature and salinity values for each location. Range of temperature between the coldest and the warmest season (Range Tº), maximum and minimum temperatures (Max. Tº and Min Tº, respectively), mean temperature over the year (Mean Tº), and mean salinity over the year (Salinity). Temperatures expressed as degrees Celsius (ºC).

Locus	Num	Eff_num	Ho	He	H't	F_{IS}	% missing
m.ten 1	4	1.485	0.44	0.333	0.62	-0.321	0.000
m.ten 6	3	1.391	0.411	0.285	0.499	-0.442	0.000
m.ten 14	5	1.45	0.384	0.317	0.403	-0.211	0.000
m.ten 31	2	1.398	0.494	0.287	0.479	-0.724	0.003
m.ten 30	3	1.381	0.416	0.28	0.579	-0.489	0.000
m.ten 32	2	1.459	0.503	0.318	0.49	-0.582	0.000
m.ten 19	5	1.182	0.199	0.157	0.219	-0.269	0.000
m.ten 40	4	1.19	0.261	0.162	0.439	-0.614	0.000
m.ten 24	4	1.187	0.213	0.161	0.59	-0.328	0.006
m.ten 25	2	1.505	0.553	0.339	0.511	-0.632	0.000
m.ten 27	5	1.297	0.353	0.234	0.623	-0.507	0.067
m.ten 13	2	1.065	0.041	0.063	0.142	0.348	0.006
Overall	3.417	1.332	0.356	0.245	0.466	-0.455	

Supplementary Table S3. Genetic descriptors of the twelve microsatellites used in this study for the whole dataset. Total number of alleles (Num), effective number of alleles (Eff_num), observed (Ho) and expected heterozygosity (He), corrected total heterozygosity (H't), inbreeding values (F_{IS}), percentage of missing data (% missing).

	Alicante	Llançà	Napoli	Plakias	Sicily	Knidos	Abades	Bocacangrejo	Lanzarote	Tazacoste	Gijon	Florida	Ferradura	Itaipú	Prainha
Alicante	-	0.354	0	0.018	0	0	0	0.003	0	0.003	**1**	**1**	**1**	**1**	**1**
Llançà	0.4103	-	0.354	0.333	0.354	0.354	0.335	0.332	0.339	0.33	**1**	**1**	**1**	**1**	**1**
Napoli	0	**0.36**	-	0.018	0	0	0	0.003	0	0.003	**1**	**1**	**1**	**1**	**1**
Plakias	0.108	**0.306**	0.061	-	0.018	0.018	0.006	0.005	0.009	0.005	**1**	**1**	**1**	**1**	**1**
Sicily	0	**0.410**	0	0.108	-	0	0	0.003	0	0.003	**1**	**1**	**1**	**1**	**1**
Knidos	0	**0.410**	0	0.108	0	-	0	0.003	0	0.003	**1**	**1**	**1**	**1**	**1**
Abades	0.061	**0.306**	0.006	0.012	0.061	0.061	-	-0.006	-0.006	-0.006	**1**	**1**	**1**	**1**	**1**
Bocacangrejo	0.046	**0.311**	0.004	0.016	0.045	0.046	-0.033	-	-0.008	-0.009	**1**	**1**	**1**	**1**	**1**
Lanzarote	0.034	**0.325**	-0.009	0.024	0.034	0.034	-0.035	-0.046	-	-0.008	**1**	**1**	**1**	**1**	**1**
Tazacoste	0.052	**0.305**	0.008	0.014	0.051	0.052	-0.032	-0.040	-0.046	-	**1**	**1**	**1**	**1**	**1**
Gijón	**1**	**0.687**	**1**	**0.875**	**1**	**1**	**0.952**	**0.920**	**0.956**	**0.918**	-	**1**	**1**	**1**	**1**
Florida	**1**	**0.587**	**1**	**0.787**	**1**	**1**	**0.881**	**0.840**	**0.899**	**0.833**	1	-	**1**	**1**	**1**
Ferradura	**0.955**	**0.617**	**0.935**	**0.800**	**0.955**	**0.955**	**0.867**	**0.841**	**0.879**	**0.837**	**0.957**	**0.904**	-	**1**	0.157
Itaipú	**0.954**	**0.612**	**0.934**	**0.795**	**0.954**	**0.954**	**0.862**	**0.837**	**0.875**	**0.832**	**0.956**	**0.899**	**0.879**	-	**1**
Prainha	**0.768**	**0.492**	**0.703**	**0.630**	**0.768**	**0.768**	**0.637**	**0.644**	**0.662**	**0.637**	**0.775**	**0.610**	0.237	**0.662**	-
Tartaruga	**1**	**0.636**	**1**	**0.831**	**1**	**1**	**0.923**	**0.883**	**0.9301**	**0.879**	**1**	**1**	-0.017	**0.931**	0.314

Supplementary Table S4. Pairwise distances between sampled locations based on the COI sequences. F_{ST} values are represented below the diagonal, a values above the diagonal. Significant values, when p-value < 0.01, are in bold.

	Alicante	Llançà	Napols	Plakias	Sicily	Knidos	Abades	Bocacangrejo	Lanzarote	Tazacoste	Gijon	Florida	Ferradura	Itaipú	Prai
Alicante	-	-0.007	-0.006	**0.130**	**0.161**	**0.187**	**0.151**	**0.261**	**0.186**	**0.099**	**0.331**	**0.303**	**0.414**	**0.413**	
Llançà	0.001	-	-0.006	**0.131**	**0.161**	**0.187**	**0.152**	**0.267**	**0.187**	**0.101**	**0.331**	**0.303**	**0.414**	**0.413**	
Napols	0.006	0.005	-	**0.134**	**0.164**	**0.190**	**0.152**	**0.270**	**0.187**	**0.102**	**0.335**	**0.303**	**0.416**	**0.416**	
Plakias	**0.542**	**0.552**	**0.553**	-	**0.072**	**0.156**	**0.197**	**0.226**	**0.226**	**0.159**	**0.324**	**0.371**	**0.459**	**0.437**	
Sicily	**0.459**	**0.468**	**0.450**	**0.469**	-	**0.061**	**0.137**	**0.179**	**0.150**	**0.094**	**0.188**	**0.338**	**0.391**	**0.389**	
Knidos	**0.366**	**0.372**	**0.356**	**0.483**	**0.268**	-	**0.148**	**0.179**	**0.164**	**0.100**	**0.196**	**0.396**	**0.442**	**0.424**	
Abades	**0.335**	**0.343**	**0.323**	**0.515**	**0.306**	**0.241**	-	**0.185**	**0.095**	**0.037**	**0.276**	**0.331**	**0.435**	**0.458**	
Bocacangrejo	**0.403**	**0.420**	**0.399**	**0.429**	**0.317**	**0.284**	**0.267**	-	**0.160**	**0.124**	**0.283**	**0.326**	**0.443**	**0.452**	
Lanzarote	**0.373**	**0.382**	**0.361**	**0.554**	**0.345**	**0.263**	**0.160**	**0.243**	-	**0.036**	**0.171**	**0.308**	**0.388**	**0.432**	
Tazacoste	**0.227**	**0.236**	**0.219**	**0.457**	**0.272**	**0.160**	0.065	**0.195**	0.062	-	**0.169**	**0.291**	**0.395**	**0.427**	
Gijon	**0.701**	**0.706**	**0.711**	**0.818**	**0.523**	**0.454**	**0.562**	**0.492**	**0.427**	**0.386**	-	**0.425**	**0.406**	**0.495**	
Florida	**0.548**	**0.559**	**0.534**	**0.640**	**0.506**	**0.483**	**0.408**	**0.387**	**0.388**	**0.373**	**0.705**	-	**0.349**	**0.292**	
Ferradura	**0.608**	**0.616**	**0.600**	**0.688**	**0.527**	**0.543**	**0.516**	**0.490**	**0.499**	**0.493**	**0.688**	**0.473**	-	**0.101**	
Itaipú	**0.691**	**0.697**	**0.692**	**0.772**	**0.639**	**0.623**	**0.631**	**0.578**	**0.620**	**0.605**	**0.800**	**0.555**	**0.291**	-	
Prainha	**0.548**	**0.555**	**0.537**	**0.613**	**0.467**	**0.493**	**0.472**	**0.448**	**0.465**	**0.456**	**0.650**	**0.411**	0.029	**0.185**	
Tartaruga	**0.626**	**0.633**	**0.619**	**0.701**	**0.544**	**0.558**	**0.540**	**0.506**	**0.518**	**0.513**	**0.691**	**0.503**	-0.003	**0.311**	

Supplementary Table S5. Pairwise genetic distances between sampled locations based on the microsatellite data. F_{ST} values are represented below the dia... Jost's D values above the diagonal. Significant values, when p-value < 0.01, are in bold.

	Harmonic sample size	Estimated Ne^	95% CIs for Ne^
ALICANTE	24	Infinite	[Infinite]
LLANCÀ	25	Infinite	[Infinite]
NAPOLS	25	Infinite	[Infinite]
SICILY	22.8	0.5	[0.3-0.8]
KNIDOS	15	Infinite	[Infinite]
PLAKIAS	19	0.8	[0.5-1.3]
ABADES	14	1.4	[0.8-2.4]
BOCACANGREJO	24	1.4	[0.9-2.1]
LANZAROTE	16	1.2	[0.8-1.9]
TAZACOSTE	19	0.5	[0.3-0.8]
GIJÓN	30	Infinite	[Infinite]
FLORIDA	7.4	1.8	[0.7-29.3]
FERRADURA	20	1.8	[0.8-5.9]
ITAIPÚ	23.5	Infinite	[Infinite]
PRAINHA	23.8	1.9	[0.9-5.8]
TARTARUGA	23	Infinite	[Infinite]

Supplementary Table S6. Estimated effective population size (Ne^) for populations of *C. tenuispina* calculated using the linkage disequilibrium and heterozygote excess methods.
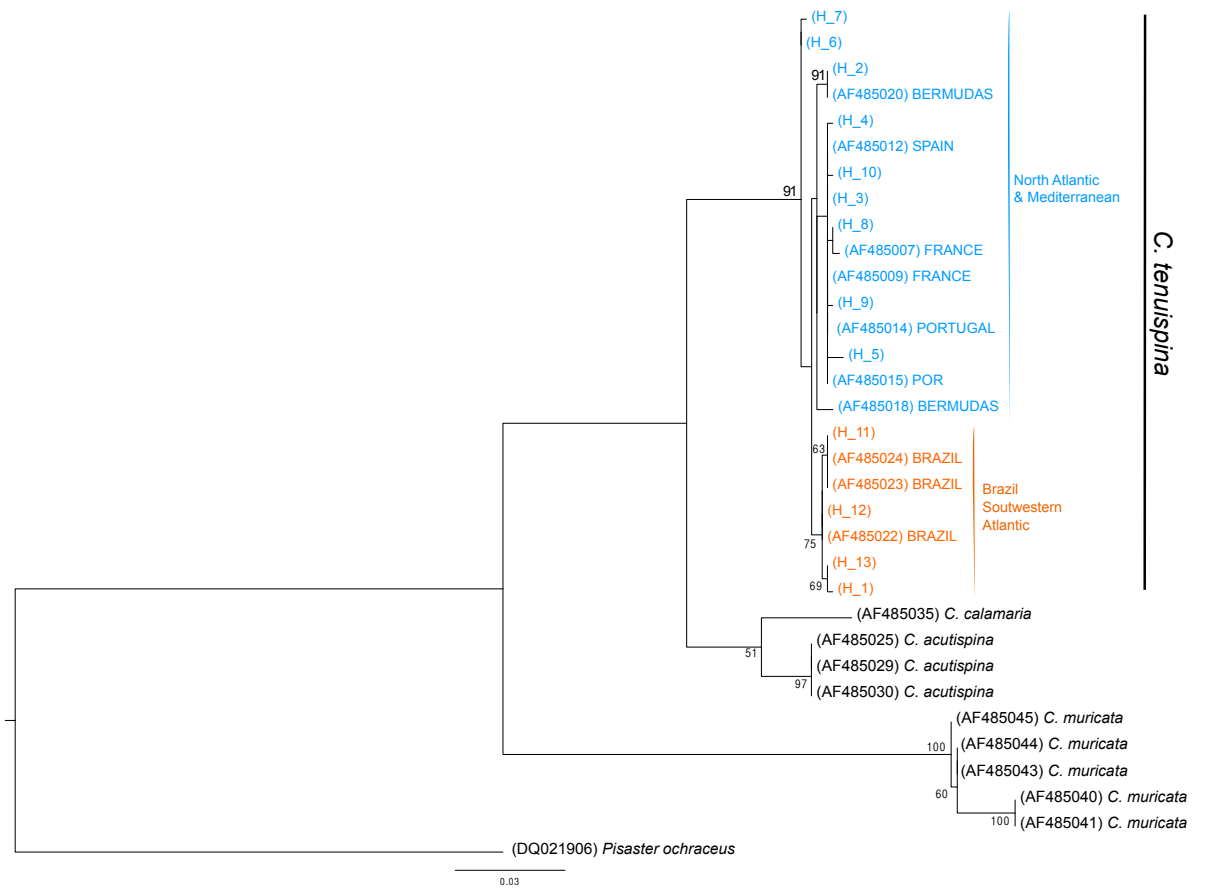
Supplementary Figure S1. Maximum-likelihood tree for all COI haplotypes of C. tenuispina. Sequences of COI available from Genbank of other Co[...] species were included as outgroups. In blue, sequences of C. tenuispina from individuals in the North Atlantic and Mediterranean Sea; in orange, sample[...] south-western Atlantic. Accession numbers of samples obtained from Genbank appear in brackets. Bootstrap values are on the nodes of the clades when ≥ 60.
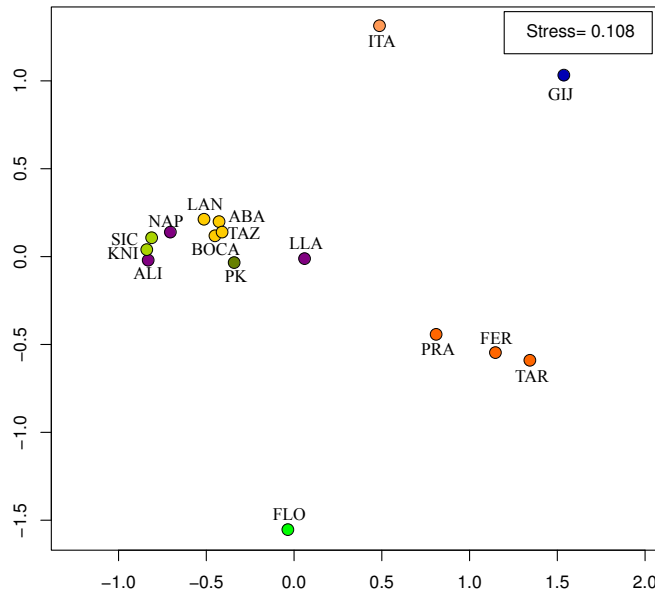
Supplementary Figure S2. Multidimensional scaling (MDS) based on the F_{ST} pairwise differences from COI sequence data with the first two dimensions plotted. Populations are represented by their codes.

Fst COI vs. microsatellites
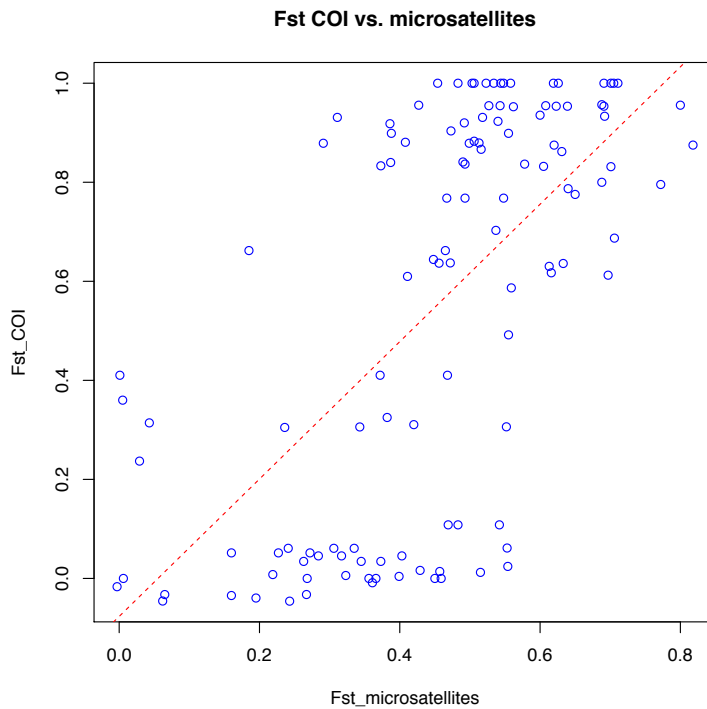
Supplementary Figure S3. Correlation between F_{ST} from COI and F_{ST} from microsatellites ($R^2 = 0.638$; p-value < 0.001).

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Capítulo 4

Supplementary material
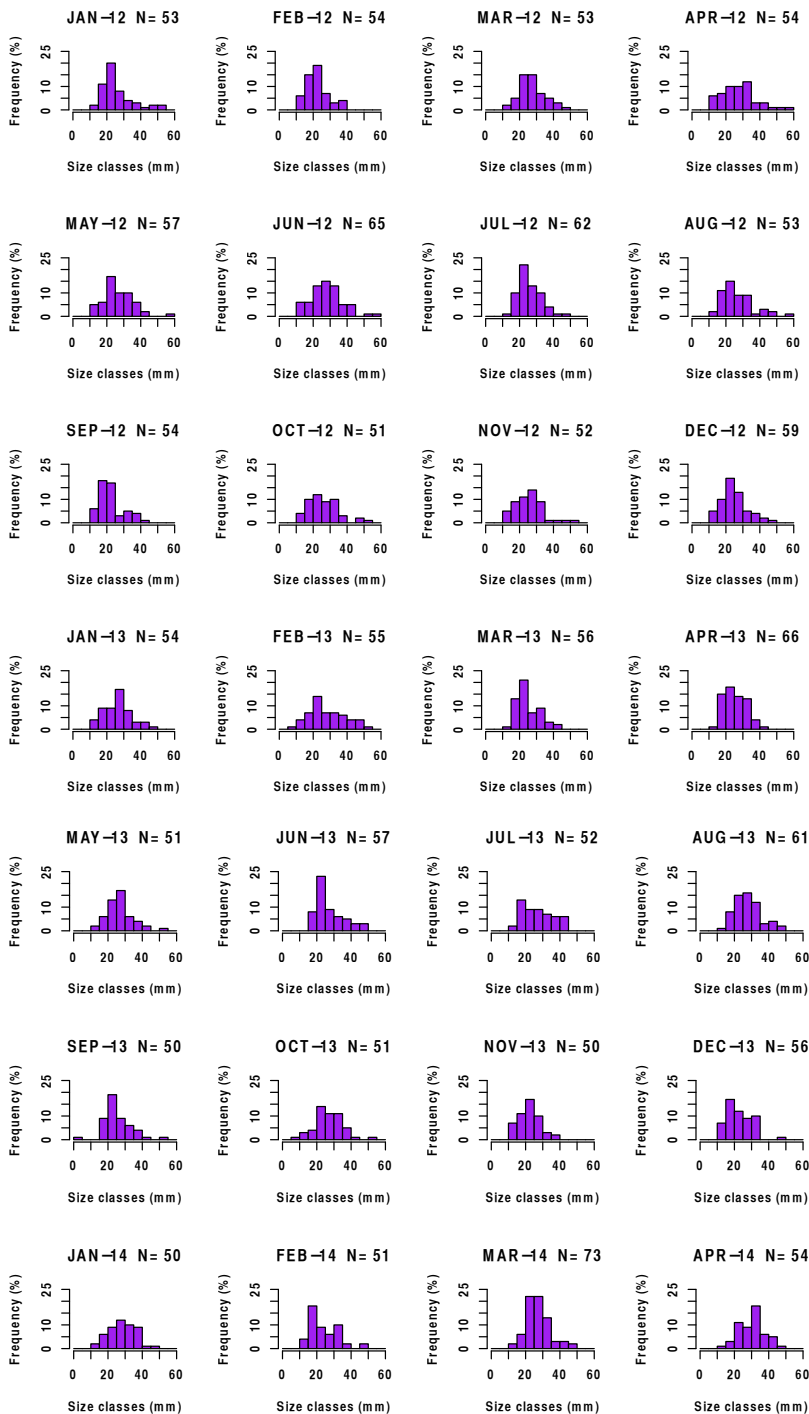
Supplementary figure S1. Histograms of size structure of the population per month.
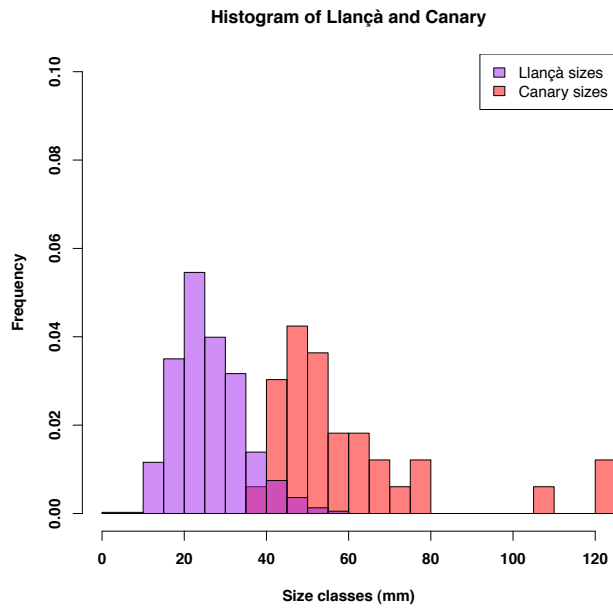
Supplementary figure S2. Histogram of sizes at Llançà and Canary Islands.
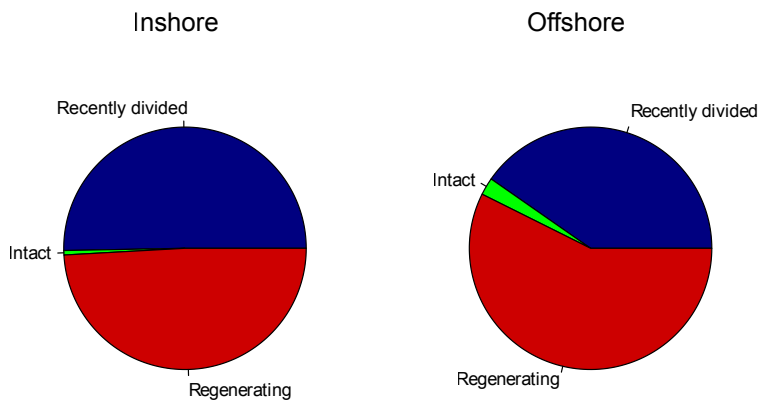
Supplementary figure S3. Pie charts showing the regeneration state for individuals of both areas.
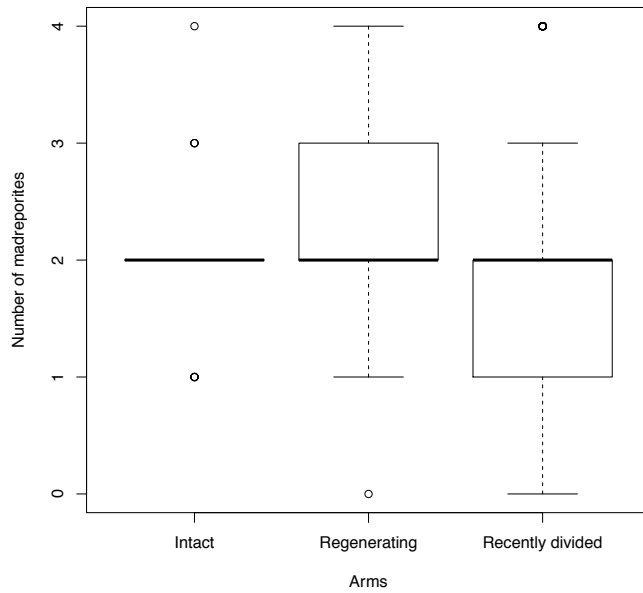
Supplementary figure S4. Number of madreporites in individuals of different regeneration state.

Supplementary figure S5. Histology sections from *C. tenuispina* male gonads with the three mature stages found. A) Growing, B) pre-mature and C) mature. *S*, spermatozoa and *NT*, nutritive tissue.

Capítulo 5

Supplementary material
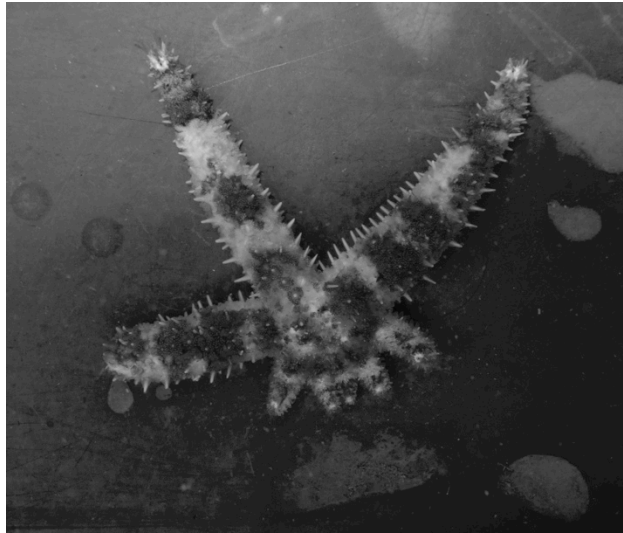
SF 1. Picture of the aboral side of a *Coscinasterias tenuispina* individual in black and white image. The individual had three non-regenerating arms, that were longer, and five shorter regenerating arms.
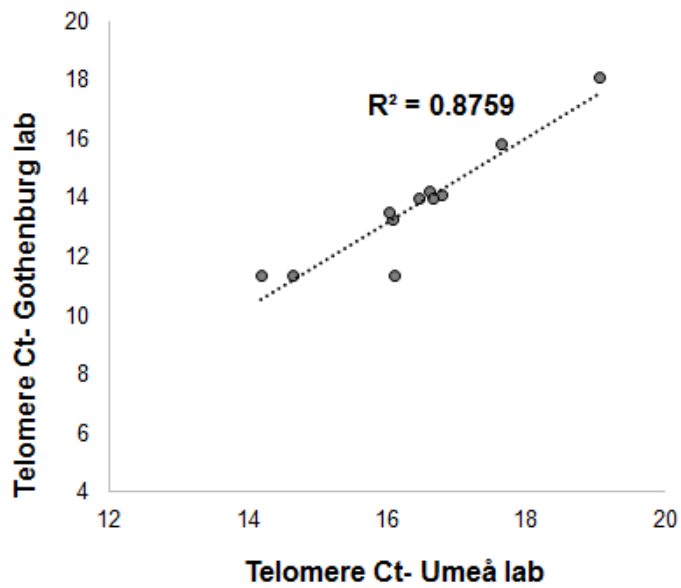
SF 2. Telomere length PCR verification. Eleven samples were send to an independent laboratory in Umeå, Sweden, and reanalyzed for telomere length. The samples telomere Ct-values were compared between Gothenburg and Umeå laboratories.
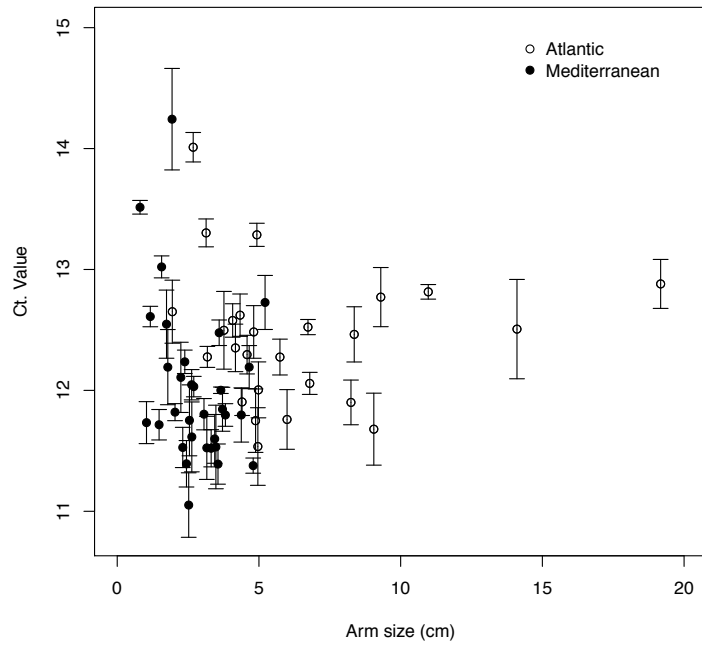
SF 3. Dot plot representing the mean Ct. value, at the y-axis, and the longest arm length. at the x-axis. Full filled dots represent Mediterranean individuals while white dots individuals from the Atlantic. Vertical bars represent the standard deviation from the three replicas analysis per each individual.

Apéndices II
Publicaciones

TECHNICAL NOTE

Characterization of thirty two microsatellite loci for three Atlanto-Mediterranean echinoderm species

**Alex Garcia-Cisneros · Claudio Valero-Jiménez ·
Creu Palacín · Rocío Pérez-Portela**

Abstract Thirty two microsatellites were optimized from 454 pyrosequencing libraries for three Atlanto-Mediterranean echinoderms: *Coscinasterias tenuispina*, *Echinaster sepositus* and *Arbacia lixula*. We observed different frequency of microsatellite types (di-, tri-, tetra- and penta-nucleotide) throughout the genome of the species, but no significant differences were observed in allele richness among different microsatellite repeats. No loci showed linkage disequilibrium. Heterozygosity deficit and departure from Hardy–Weinberg equilibrium were observed for some loci, in two species, probably due to high levels of inbreeding. Heterozygosity excess observed in *C. tenuispina* could be explained by selection against homozygotes and/or outcrossing.

Keywords Pyrosequencing · Inbreeding · Clonality · Conservation · Starfish · Sea urchin

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During last century, Mediterranean Sea has suffered an extensive loss of biodiversity due to high anthropogenic pressures and environmental perturbations (Coll et al. 2010). Introduction of non-native species, increase in water temperature and extensive gaps in the distribution of natural populations due to urbanization, are among the most important environmental pressures (Thibaut et al. 2005; Lejeusne et al. 2010).

In this study we described new microsatellite loci for three of the most common Atlanto-Mediterranean echinoderms with important implications for conservation; the starfishes *Echinaster sepositus* and *Coscinasterias tenuispina*, and the sea urchin *Arbacia lixula*. *E. sepositus* is an emblematic species along the Atlanto-Mediterranean area but some populations at the North-Western Mediterranean have suffered a severe decline (Villamor and Becerro 2010; authors' pers. obs.). This species is now scarce in areas with high anthropogenic pressure and affluence of divers, and larger populations are only observed within marine protected areas. Due to the short-distance dispersal of its lecithotrophic larva, studies about populations' connectivity, inbreeding and genetic structure are crucial to design future management strategies for restoring their populations (Jones et al. 2007).

On the other hand, mitochondrial data suggested a recent colonization of the Mediterranean from the Atlantic Ocean by the thermophilous species *A. lixula* and *C. tenuispina* (Wangensteen et al. 2012; authors' unpublished data), and whose densities may increase dramatically in the foreseeable future. Global warming might facilitate population blooms and thus turn these species into an ecological problem. Both species can modify sublittoral habitats because of their voracity generating barren grounds when populations reach high densities (Guidetti et al. 2003; Bonaviri et al. 2011). Populations' monitoring, including

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Table 1 Characteristics of 32 microsatellite markers for three echinoderm species

Species	Locus (dye), GenBank accession number	F and R primer sequence	Repeat motif	T_a (°C)	Size range (bp)	Population 1				Population 2			
						N	N_A	H_O/H_E	H–W	N	N_A	H_O/H_E	H–W
C. tenuispina	m.ten1 (6'FAM), KC699497	F: TCAAGGCTGTGTAGTACTCT / R: TCAATCAAACTGTGTACCTT	(ATT)*12	51	171–174	22	2	0.045/0.045	1.0	16	2	0.812/0.498	0.014
	m.ten6 (NED), KC699498	F: CATGAGAGCTTACAGAAAAG / R: CTTAGGTGTAATGAAGTGCT	(TAA)*7	51	160–163	21	2	0.952/0.511	0.001*	16	2	0.812/0.498	0.014
	m.ten13 (6'FAM), KC699499	F: GACAGAGTGCTTCTTAATG / R: AGTTCTGGAATAAACTACCC	(ATAC)*12	51	360–364	19	1	0/0	–	15	2	0/0.331	0.001*
	m.ten14 (HEX), KC699500	F: CACTCTGAGCCTATAAGAGA / R: GTTAATTTCTCCCTACCT	(TAA)*7	51	137–138	22	2	1.0/0.512	0.001*	11	1	0/0	–
	m.ten19 (HEX), KC699501	F: CTGCTGGCTCCAGCTGCTAT / R: TCAACCAGGTCGTTGATCTTTGT	(GATT)*8	51	133–150	22	1	0/0	–	12	2	0.583/0.4311	0.487
	m.ten25 (HEX), KC699502	F: TAACTGTTGAATCCATCCT / R: CCTGTCATGATTATGTTTGT	(GTA)*10	51	295–298	22	1	0/0	–	16	2	1.0/0.217	0.001*
	m.ten24 (HEX), KC699503	F: CTCATAAGGGTGCTGTTT / R: ATGAATCATACGTGTGTGG	(GT)*11	51	365–367	22	1	0/0	–	16	2	0.437/0.353	0.543
	m.ten27 (6'FAM), KC699504	F: CTTCATAAGAGGTTAGTTGG / R: TCCAAGTCATGGAATAACTA	(AT)*9	53	293–295	13	1	0/0	–	10	2	0.6/0.442	0.480
	m.ten30 (NED), KC699505	F: GGTACCAGTCGTCATAAATA / R: AGGTCCACACTACAGAT	(AGTC)*17	51	397–409	22	3	1.0/0.638	0.001*	16	2	0.812/0.497	0.014
	m.ten31 (6' FAM), KC699506	F: GTGAGTGAAGCCAGAAACTT / R: ACATTTGGAATGTTTCCATC	(TGTT)*9	51	298–302	18	1	0/0	–	16	2	1.0/0.516	0.001*
	m.ten32 (6'FAM), KC699507	F: ATGAGAGTGGATGACTGACA / R: CCATAAGCTTAGCACTACAGG	(TAGA)*8	51	245–249	19	2	0.947/0.512	0.002*	14	2	0.571/0.423	0.505
	m.ten33 (HEX), KC699508	F: CTGTTGAATCCATCCTGTT / R: GCCCTGTCATGATTATGTTT	(GTA)*10	51	290–296	19	2	0.789/0.490	0.012	16	4	1.0/0.647	0.001*
	m.ten40 (6'FAM), KC699509	F: CCAGCTTGTTTCCATCCAAGGC / R: TCTGCACCTCGGGGCGCATAGA	(AG)*11	51	151–154	19	1	0/0	–	16	4	0.312/0.635	0.001*
E. sepositus	mES 2 (JOE), KC699510	F: CGTATTTATGTGCAGTTG / R: ATCATCCCATTAGAGGTTTA	(TTA)*9	51	232–254	25	7	0.520/0.619	0.012	11	8	0.636/0.740	0.272
	mES 4 (6'FAM), KC699511	F: GCCAAAGATGCCATAAAT / R: CTGTAGGCTAGCTGAGTTT	(CAA)*6	51	115–148	26	9	0.692/0.788	0.087	16	8	0.688/0.823	0.295
	mES 11 (FAM), KC699512	F: GTTGTAGTGATTTCCTGATG / R: CCGTGTTGAGAATATGTAA	(TTA)*8	51	128–256	21	3	0.143/0.138	1.000	8	3	0.250/0.242	1.000
	mES 23 (6'FAM), KC699513	F: ATCATTGTTCTTCAGTTTCC / R: TTGTTAAATAGTCCCCAACT	(TG)*10	51	85–91	19	5	0.611/0.607	0.771	1	2	1.00/1.00	1.000
	mES 24 (HEX), KC699514	F: AGAGATCATTAACCCATTCA / R: ACTAGTATGTATCCGTTGGC	(TTCA)*12	51	87–195	26	10	0.115/0.838	0.000*	15	7	0.333/0.860	0.000*
	mES 25 (HEX), KC699515	F: TAATTGATCCCATTCCTGTA / R: TCACTGTATCCAGATTTCCT	(TAAA)*10	51	154–199	25	11	0.680/0.873	0.118	14	16	1.00/0.955	1.000

Table 1 Characteristics of 32 microsatellite markers for three echinoderm species

Species	Locus (dye), GenBank accession number	F and R primer sequence	Repeat motif	T_a (°C)	Size range (bp)	Population 1				Population 2			
						N	N_A	H_O/H_E	H–W	N	N_A	H_O/H_E	H–W
	mES 29 (6FAM), KC699516	F: ACTAGAATGTGGAGTGACAG	(AC)*12	51	203–288	26	13	0.833/0.891	0.465	16	12	0.938/0.885	0.876
		R: GTCGCTTAGGAAACATCT											
	mES 30 (HEX), KC699517	F: AAAGGTCTCTTTGAAGGTGTT	(CTG)*8	51	262–290	26	8	0.269/0.767	0.000*	14	6	0.286/0.745	0.001*
		R: TTCAGGTAGTTGAAGAATTGC											
	mES 38 (HEX), KC699518	F: CCAGTTGACCCATCATAAAT	(GCA)*9	51	256–317	25	9	0.320/0.796	0.000*	16	7	0.688/0.784	0.656
		R: GTGATTATGTCCAAAGTGC											
A. lixula	ALM 2 (6-FAM), KC699519	F: TGCTAAACGGCAACAATGAA	(AATC)*12	56	283–355	23	12	0.739/0.756	0.5071	18	17	0.889/0.881	0.667
		R: TGGTCGCTAATGGAGGTTTC											
	ALM 4 (6-FAM), KC699520	F: TGAGACAACGGGAAAGTCAA	(AATC)*14	56	239–308	23	17	0.435/0.912	0.000*	18	18	0.778/0.910	0.000*
		R: CGATGGTCCTAGAGGTGACA											
	ALM 5 (6-FAM), KC699521	F: GTGGAATGGTGATGGAAAGG	(AGAT)*14	57	120–228	23	16	0.696/0.903	0.000*	18	14	0.722/0.866	0.008
		R: TCACGCCTGTTGAAATATCC											
	ALM 7 (HEX), KC699522	F: CATGGTTCATTTCTGCCTCA	(AATC)*11	56	228–352	23	6	0.826/0.708	0.0835	18	13	0.500/0.866	0.000*
		R: GAATGGTTGACTTATTGGACGTT											
	ALM 8 (6-FAM), KC699523	F: CCATCCATTCATTCACTACTTCA	(AGGT)*11	57	78–173	23	16	0.478/0.881	0.0906	18	14	0.444/0.886	0.000*
		R: ACAGATGGGTGGGTGGAG											
	ALM 9 (HEX), KC699524	F: TGTACGTACGTTGGCTGACGA	(AACT)*10	58	221–275	23	11	0.261/0.857	0.000*	18	8	0.278/0.816	0.000*
		R: GCTCACATACAGCTCCCATGTT											
	ALM 11 (HEX), KC699525	F: CAGCTGAATCCGATGGTGTA	(AAAATC)*9	57	350–469	23	9	0.261/0.871	0.000*	18	8	0.222/0.841	0.000*
		R: TCACGTGCGAGATGTTCTTC											
	ALM 14 (NED), KC699526	F: GCCTTATCATTAGGTGCAGGT	(AGT)*16	57	181–259	23	18	0.609/0.911	0.000*	17	18	0.471/0.903	0.016
		R: CCGTCTAAGTGGAGAGCTATGG											
	ALM 15 (HEX), KC699527	F: GAGGGCTTCATCCAACAATG	(ACT)*15	58	75–125	23	14	0.478/0.797	0.000*	16	12	0.667/0.833	0.005
		R: TAATTGGCGGCGTATATTG											
	ALM 17 (NED), KC699528	F: GGATCCTACCATGAATTGTTACAT	(AC)*16	51	177–356	23	13	0.799/0.911	0.259	18	11	0.625/0.865	0.007
		R: AATCAACCTGCTCCGTGAAT											

T_a annealing temperature, N number of individuals, N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity and H–W

p value of the Hardy–Weinberg equilibrium test (*) significant after Bonferroni corrections

recruitment and connectivity studies between Atlantic sources and Mediterranean stocks based on microsatellites, is highly recommendable to evaluate the potential threat of these species for Mediterranean ecosystems.

We used 454 pyrosequencing to isolate novel microsatellite loci in *C. tenuispina*, *E. sepositus* and *A. lixula*. Genomic DNA was extracted using QIAamp® DNA Mini Kit (QIAGEN) to a final DNA concentration of 5 ng/μl and distributed in three physically separated lanes of a plate. Pyrosequencing was performed on a Roche Life Science 454 GS-FLX System at the Scientific-Technical Services of University of Barcelona. Sequences were trimmed to remove regions with a greater than 0.5 % chance of error per base using GENEIOUS version 5.5 (Drummond et al. 2011). Total number of sequences which passed quality filtering, number of microsatellites detected, and reads mode length were variable between species, and all details are summarized in Online Resource 1. Sequences were searched for perfect microsatellites (di-, tri-, tetra- and pentanucleotides) with at least eight repeats and enough priming regions with QDD1 v. 1.3 (Meglécz et al. 2010). Primers were designed with the software PRIMER 3 (Rozen and Skaletsky 2000).

Amplification success and polymorphism were tested in two populations per species: Costa Brava (42°29′N, 3°10′E) and Tenerife (28°25′N, 16°19′W) in *C. tenuispina*, Costa Brava (41°46′N, 3°05′E) and Marseille (43°16′N, 49°34′E) for *E. sepositus*, and Costa del Sol (36°34′N, 4°34′W) and Costa Brava (42°24′N, 3°07′E) in *A. lixula*. Total DNA was extracted from feet tube and amplified using the REDExtract-N-Amp Tissue PCR Kit (Sigma Aldrich). Forward primers were labelled with a fluorescent dye as shown in Table 1. PCR amplifications were performed as described in Valero-Jiménez et al. (2012). Allele length was estimated relative to the internal size standard 70-500 ROX (Bioventures) using the software Peak-Scanner (Applied Biosystems).

Dinucleotides were the most frequent microsatellites followed by tri, tetra and pentanucleotides throughout the genome of the species (see Online Resource 2). A total of thirteen, nine and ten polymorphic microsatellite were optimized for *C. tenuispina, E. sepositus* and *A. lixula,* respectively, including a selection of different microsatellite types (see Table 1). Linkage disequilibrium, observed and expected heterozygosity, and deviation from Hardy–Weinberg equilibrium were calculated with ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010). Bonferroni corrections of the *p* values for multiple tests were run.

No evidence of linkage disequilibrium was detected across all pairwise comparisons. Failed amplifications due to presence of null alleles were not detected for any loci. Nineteen markers showed Hardy–Weinberg disequilibrium after Bonferroni corrections. Heterozygosity deficit

observed in two species may be explained by high levels of inbreeding, as demonstrated in other marine invertebrates (Pérez-Portela and Turon 2008; Calderón et al. 2009). The heterozygosity excess observed in *C. tenuispina* may be explained by clonal reproduction, selection against homozygotes and/or outcrossing (Blanquer and Uriz 2010). After confirming normality and homoscedasticity of the dependent variable, we used a two-way ANOVA to test for differences in genetic diversity (measured as allelic richness) of different microsatellite types and species. Genetic diversity values were adjusted to population size with a rarefaction index calculated in CONTRIB V1.2 (Petit et al. 1998). Our results did not show differences in genetic diversity among di, tri, tetra and pentanucleotide repeats (F = 0.233; $p = 0.872$) but diversity was significantly different among species (F = 35.69; $p < 0.0001$) (see Online Resource 3). This result suggests that different microsatellite types are equally valid in terms of genetic diversity to assess population genetics in echinoderm species.

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ORIGINAL ARTICLE

Long telomeres are associated with clonality in wild populations of the fissiparous starfish *Coscinasterias tenuispina*

This article has been corrected since Advance Online Publication and a corrigendum is also printed in this issue

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Telomeres usually shorten during an organism's lifespan and have thus been used as an aging and health marker. When telomeres become sufficiently short, senescence is induced. The most common method of restoring telomere length is via telomerase reverse transcriptase activity, highly expressed during embryogenesis. However, although asexual reproduction from adult tissues has an important role in the life cycles of certain species, its effect on the aging and fitness of wild populations, as well as its implications for the long-term survival of populations with limited genetic variation, is largely unknown. Here we compare relative telomere length of 58 individuals from four populations of the asexually reproducing starfish *Coscinasterias tenuispina*. Additionally, 12 individuals were used to compare telomere lengths in regenerating and non-regenerating arms, in two different tissues (tube feet and pyloric cecum). The level of clonality was assessed by genotyping the populations based on 12 specific microsatellite loci and relative telomere length was measured via quantitative PCR. The results revealed significantly longer telomeres in Mediterranean populations than Atlantic ones as demonstrated by the Kruskal–Wallis test ($K = 24.17$, significant value: P-value < 0.001), with the former also characterized by higher levels of clonality derived from asexual reproduction. Telomeres were furthermore significantly longer in regenerating arms than in non-regenerating arms within individuals (pyloric cecum tissue: Mann–Whitney test, $V = 299$, P-value $< 10^{-6}$; and tube feet tissue Student's $t = 2.28$, P-value $= 0.029$). Our study suggests that one of the mechanisms responsible for the long-term somatic maintenance and persistence of clonal populations is telomere elongation.

INTRODUCTION

Although aging is observed in most organisms, there is a large degree of variation in the rate at which it occurs, at both the species and individual level. Telomere length has frequently been used as an aging marker because telomere caps normally become shorter during an organism's lifetime, not only primarily during DNA replication but also in association with other factors such as stress (Von Zglinicki, 2002; Epel *et al.*, 2004; Kotrschal *et al.*, 2007). Critically short telomeres trigger a signal prompting the cell to permanently stop dividing, which leads to the induction of cellular senescence (Herbig *et al.*, 2006). Furthermore, long telomeres have been found to correlate with good health and higher life expectancies in several species, thereby also serving as an indicator of somatic fitness, which represents the boundary of aging diseases (Bize *et al.*, 2009; Horn *et al.*, 2010; Barrett *et al.*, 2013).

During fission or fragmentation in asexual organisms, two or more separate individuals are formed, resulting in clonal offspring with genotypes identical to the parent and to each other. In wild asexual populations and after recurrent fissions, it is difficult to determine the age of an individual by morphological means, not only for the potential clone itself but also the original parental half. In these cases, genetic analyses can reveal the level of clonality within a population as well as the extent of a clone, thus providing information regarding its age and longevity (Ally *et al.*, 2010). Indeed, extremely large and long-lived clonally propagating populations exist, such as some sea grass species, with clones that are estimated to be 1000 year (Reusch *et al.*, 1999) or more (Arnaud-Haond *et al.*, 2012). In the case of a terrestrial tree, the age of some clones have been estimated as old as 10 000 year (Ally *et al.*, 2010) and in cold waters, clonal individuals of the coral *Lophelia pertusa* are estimated to be 4500–6000 year (Dahl *et al.*, 2012). These estimations obtained for several plants and animal groups may in some way indicate the existence of mechanism to largely delay, or even resist, aging in particular clones although the evolutionary significance of these long-term resistance has not been clarify yet.

In sexually reproducing species, telomere length is restored during embryogenesis by the reverse transcriptase telomerase (Schaetzlein *et al.*, 2004). However, little is known about aging in asexual organisms that propagate via fission or budding; many questions

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Received 30 September 2014; revised 23 March 2015; accepted 13 April 2015; published online 20 May 2015
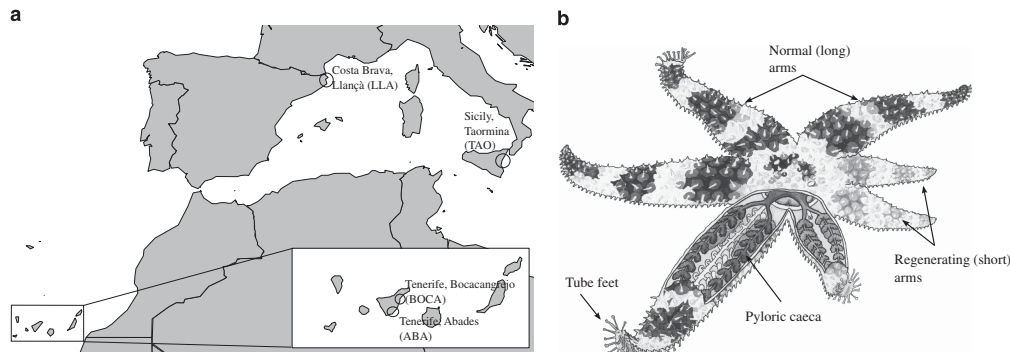
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Figure 1 (**a**) Map of sampling locations, including those in Mediterranean Sea and Northeastern Atlantic Ocean. Circles highlight the three sampling areas. Two different populations were sampled in Tenerife. (**b**) Schematic anatomy of *Coscinasterias tenuispina* showing normal and regenerating arms.

remain unanswered as to whether they are able to fully maintain and/or restore their telomeres to persist over time or whether they undergo somatic aging (Sköld and Obst, 2011). In a study using laboratory cultures of two invertebrate species, upregulation of telomerase has been shown to at least partly restore telomeres in the clones of a flatworm (Tan *et al.*, 2012). Telomerase is also upregulated during budding in the colonial ascidian *Botryllus schlosseri* (Laird and Weissman, 2004). On the other hand, experiments involving another colonial ascidian, *Diplosoma listerianum,* found that telomerase activity declined, telomeres shortened and the growth rate slowed after prolonged asexual duplication, indicative of long-term senescence in the studied clones (Sköld and Obst, 2011). There is, however, very limited information available regarding the effect of prolonged periods of asexual duplication on telomere length and aging in wild populations. Whether molecular aging occurs and how it could potentially be delayed in wild clones is still unknown.

Most asteroids have the ability to regenerate their body parts after autotomy or injuries, and about 26 species can reproduce asexually via fission (Emson and Wilkie, 1980). All four known species of the cosmopolitan genus *Coscinasterias,* commonly found in shallow waters, can reproduce both sexually and asexually via fission (Alves *et al.*, 2002; Lawrence, 2013 among other references). When individuals of *Coscinasterias* reproduce sexually, they release planktotrophic larvae that remain in the water column for several weeks, with a high potential to colonize new habitats via dispersal (Karako *et al.*, 2002). The species *Coscinasterias tenuispina* (Lamark 1816), widely distributed throughout the Atlantic Ocean and Mediterranean Sea, presents in some cases populations either consisting of individuals of only one gender (usually males) or with an unbalanced proportion of males and females (Alves *et al.*, 2002; authors' unpublished data). The absence of one gender in some populations of this species, as well dominance of a few genotypes—according to allozyme analyses (Ventura *et al.*, 2004) —suggests that maintenance of these populations takes place solely via asexual reproduction.

The aim of the present study was to assess the effect of asexual reproduction on relative telomere length in wild populations of the starfish *C. tenuispina*, and its implication for the long-term survival of populations with limited genetic variation. For this purpose, telomere length and its relationship with different levels of genetic diversity related to asexuality were assessed by using populations from two Mediterranean and two Atlantic sites. Additionally, we explored the potential existence of mechanisms for telomere length control in somatic tissues by comparing regenerating and non-regenerating arms within a set of individuals.

MATERIALS AND METHODS
Sampling
Starfish of the species *C. tenuispina* (Supplementary Figure 1) were collected from four different European sites (Figure 1a), two in the Atlantic basin and two in the Mediterranean basin, with between 13 and 17 individuals collected per locality. The two Mediterranean sites, Llançà (Costa Brava, Northwestern Mediterranean) and Taormina (east of Sicily, Central Mediterranean), hereafter referred to as LLA and TAO, respectively, were sampled in autumn 2011. Both Atlantic sites, Bocacangrejo and Abades (BOCA and ABA, separated by 33 km), were located near Tenerife (Canary Islands) and were sampled in the spring (June) of 2012 (Table 1). These four sampling locations were selected based on the abundance of the studied species and on the varying prevalence of individuals undergoing fission. The starfish were sampled at between 0 and 20 m depth by snorkeling or scuba-diving. Immediately after their removal from the sea, the animals were photographed on a millimeter-scaled table in order to determine body size. Tube feet, used for the starfish locomotion and substrate attachment, from the middle part of the longest arms of each individual were also collected (Figure 1b) and preserved in either RNA*later* (Invitrogen, Life Technologies, Carlsbad, CA, USA; www.invitrogen.com) for telomere length analysis or absolute ethanol for microsatellite genotyping. The animals were then released back into the sea. All tissue samples were stored at −20° C once in the laboratory prior to analysis. Body size was assessed as the longest diameter across the starfish using the ImageJ software program (Abramoff *et al.*, 2004).

Additionally, both tube feet and pyloric caeca tissue (stomach extensions for processing and storage organ) were collected from the middle part of the longest (non-regenerating) and shortest (regenerating arm; when the length of this arm was <50% of the longest arm) arms of 12 asymmetric specimens sampled at site LLA (Figure 1b, Supplementary Figure 1). Pyloric caeca tissue was considered in the present analysis because it is a distinct tissue that has further been shown to be involved in arm regeneration (Hernroth *et al.*, 2010). Although gonads may be also considered for the telomere length analysis, they cannot be used in this study because more than 50% of the individuals of the species lack gonads, even during the reproductive season (Crozier, 1921).

Genetic analyses using microsatellites
Twelve microsatellite markers (m.ten1, m.ten6, m.ten13, m.ten14, m.ten19, m.ten24, m.ten25, m.ten27, m.ten30, m.ten31, m.ten32 and m.ten40) specifically designed for *C. tenuispina* (Garcia-Cisneros *et al.*, 2013) were employed

Table 1 Geographical coordinates and genetic information of the sampled *Coscinasterias tenuispina* populations

Population	Code	Coordinates	n	MLG	Clonal MLG	Single MLG	Allelic richness r[13]	R (Clonal richness)	Genotype diversity r[13]	Ho/He	F_{is}
Llança, Costa Brava	LLA	42°23' N, 3°09' E	17	1	1	0	1.33	0.00	1.00 (±0.0)	0.33/0.17	−1
Taormina, Sicily	TAO	37°51' N, 15°18' E	15	4	3	1	1.75	0.21	3.80 (±0.4)	0.24/0.27	0.11
Bocacangrejo, Tenerife	BOCA	28°24' N, 16°19' W	13	8	2	6	2.06	0.58	7.54 (±0.5)	0.53/0.27	−0.29
Abades, Tenerife	ABA	28°08' N, 16°26W	13	7	1	6	2.14	0.50	6.54 (±0.5)	0.43/0.25	−0.22
Total			58	20	7	13		0.33			

Abbreviation: MLG, multilocus genotypes.
Data presented include: sample size (*n*), number of different MLGs, clonal MLGs (found in more than one individual) and single MLGs (found in only one individual), as well as allelic richness after rarefaction to 13, clonal richness (*R*), genotype diversity measured after rarefaction, observed and expected heterozigosity (*Ho/He*) and inbreeding coefficient (F_{is}).

for population genetic analyses (Table 1). Extractions and amplifications were performed using a REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St Louis, MO, USA; www.sigmaaldrich.com). For PCR reactions we added 4 μl of PCR Ready Mix, 4 pmol of each primer, between 10 and 50 ng of DNA, and ultrapure water for molecular use to a final reaction volume of 10 μl. Forward primers for each locus were labeled with a fluorescent dye as described in Garcia-Cisneros *et al.* (2013). Amplifications were performed on an S1000 Thermal Cycler Dual 48/48 Reaction Module (BioRad Laboratories, Hercules, CA, USA; www.bio-rad.com), with an initial step of 1 min denaturation at 96 °C followed by 35 cycles of 96 °C for 1 min, 49 °C for 30 s and 72 °C for 20 s and a final step at 72 °C for 3 min. Amplification products were analyzed on an ABI Prism 3730xl Genetic Analyzer (Applied Biosystems, Life Technologies, San Francisco, CA, USA; www.lifetechnologies.com) by the Scientific-Technical Services of the University of Barcelona. Allele size was estimated relative to an internal size standard 70–400 ROX (Bioventures Inc, Murfreesboro, TN, USA; www.bioventures.com) using the software program Peak-Scanner-96 (Applied Biosystems). The prevalence of identical genotypes, considered as potential clones, within and between populations was tested using GenoDive (Meirmans and Van Tienderen, 2004) and MLGsim (Stenberg *et al.*, 2003). Both approaches were applied to count the number of individuals with identical multilocus genotypes (MLGs) and to calculate the likelihood of observing identical MLGs in a population due to sexual events (P_{sex}). MLGsim calculations of the P_{sex}, and their significance values, were based on 1000 random simulations. MLGsim set a critical value for MLGs because of sexual events at a minimum of 0.040. Values obtained for MLGs of *C. tenuispina* were significantly lower than the critical value of 0.040 (see Table 1) pointing to clonal reproduction as the origin of identical MLGs. Genetic diversity was measured by calculating allelic richness, including all individuals from the populations even if they were considered the same clone, genotype diversity (synonym of clonal diversity) after rarefaction for each population, heterozygosity expected and observed and the inbreeding coefficient (F_{is}) using the Hierfstat and Vegan packages (Dixon, 2003 Goudet, 2005) in the software program R v. 3.0.0.

In order to assess genetic differentiation between populations, we calculated values of the *D* estimator (Jost, 2008), a statistic to measure differences in genetic structure between populations based on the allele frequencies, using the R package DEMEtics (Gerlach *et al.*, 2010). The significance of these *D* values was evaluated by performing 10 000 permutations including all the individuals from the populations (Gerlach *et al.*, 2010).

Telomere measurements

The relative telomere lengths in either tube feet or pyloric caeca tissue were measured for each specimen via a quantitative PCR (qPCR) method (Farzaneh-far *et al.*, 2008), and modified and optimized in our laboratory (Gothenburg, Sweden). Telomere length assessment via qPCR has already been validated (Grabowski *et al.*, 2005; O'Callaghan *et al.*, 2008). However, although qPCR telomere measurements are normalized based on single copy genes in humans and other model species, the lack of genomic data for non-model species such as *C. tenuispina* hinders the identification of nuclear genes without paralogs for normalization. For this reason, telomeric DNA measurements in the present study were performed relative to the total quantity of DNA in the samples.

Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands; www.qiagen.com) according to the manufacturer's instructions, and DNA concentrations assessed with a NanoDrop (Thermo Scientific, Waltham, MA, USA; www.thermoscientific.com) in triplicate to obtain an accurate value of DNA quantity. Absorbance ratios measuring DNA purity at 260/280 averaged approximately 1.9 ± 0.1, with the 260/230 absorbance ratio also indicating acceptable sample purity (1.8 ± 0.2). Small differences in absorbance ratios were not correlated with final telomere length (260/280: $R^2 = 0.006$, $P = 0.96$; 260/230: $R^2 = 0.001$, $P = 0.99$).

The amount of DNA to be added in the qPCR reaction was estimated from a standard curve obtained via a dilution series of a mixed sample DNA pool (10– 0.0001 ng). The reaction efficiency calculated from the standard curve was $E = 104.1\%$, $R^2 = 0.997$. For the sample qPCR reaction, all DNA samples were adjusted to the same concentration, and 0.5 ng of DNA was added for each PCR mix; this produced a concentration value falling well within the linear range of the mixed sample standard curve.

Telomere analyses examining a broad range of species have indicated that the TTAGGG sequence is conserved among deuterostomes (Gomes *et al.*, 2010); the primers used for the qPCR reaction in *C. tenuispina* were therefore those described for humans in Farzaneh-far *et al.* (2008).

(forward) 5′ CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT 3′, and (reverse) 5′ GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT 3′.

Concentrations used for the forward and reverse primers were 100 and 200 nM, respectively. A 10-μl KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA; www.kapabiosystems.com) was employed as a Mastermix, with 3 μl of water added for a final PCR volume of 20 μl following the protocol described by Carney Almroth *et al.* (2012). Telomeres were amplified in triplicate for each sample to ensure accurate measurements using the following qPCR protocol cycle: 3 min at 95 °C followed by 25 cycles of 15 s at 95 °C and 1 min at 56 °C. The final step comprised 81 cycles of temperature increase from 55 to 95 °C in order to generate a melt curve, indicating the presence of a single product. Relative telomere length is represented as a cycle threshold value (Ct value), which is inversely proportional to telomere amount; longer telomeres thus produce an earlier detectable signal than shorter telomeres. All measurements were analyzed in seven PCR plates, with the mixed sample DNA dilution series included on each plate as an internal standard; Ct values for these differed by less than 1.5% between plates and were adjusted for.

Telomere length verification was performed in an independent laboratory (Umeå, Sweden) using a similar protocol for telomere length qPCR (Cawthon, 2002). DNA from 11 samples were send to Umeå and DNA concentration were re-measured by the Nanodrop instrument (Thermo scientific). Each qPCR reaction contained: 17.5 ng DNA (diluted in TE/*E. coli* buffer), 0.1- μM forward primer, 0.9-μM reverse primer, 1 × PCR Buffer 2, 1.7 mM-MgCl2, 2.5 mM-DTT, 0.2 mM-dNTP, 150-nM ROX, 0.2X SYBR and 0.625 U AmpliTaq Gold (Applied Biosystems).

Telomere primer sequence (5′–3′) Forward: CGGTTTGTTTGGGTTTG GGTTTGGGTTTGGGTTTGGGTT, Reverse: GGCTTGCCTTACCCTTACCC TTACCCTTACCCTTACCCT. Each sample was analyzed two separate times in triplicates on the ABI7900HT instrument (Applied Biosystems).

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Statistical analyses

To compare body size between specimens from different populations, a non-parametric Mann–Whitney test was performed since the data did not match our prior expectation of homoscedascity (Bartlett test = 35.97, and signification value, P-value $< 10^{-7}$). As body size data also did not match normality (Shapiro–Wilk test; $W = 0.93$, P-value = 0.0048), a Spearman correlation was used to test if telomere lengths were correlated to body size. A mixed model analysis of variance, with telomere length (expressed as the Ct value) as a dependent variable, was performed using 'basin' (Atlantic and Mediterranean basins) as a fixed factor, and 'population' and 'MLGs' as random factors. A non-significant effect was found for the MLGs (P-value > 0.98) and was therefore not considered for further analyses (see Supplementary Figure 3). Since our data did not match normality (Shapiro–Wilk test; $W = 0.96$, P-value < 0.01), a Kruskal–Wallis test of the telomere length was performed between the different sites, and a Mann–Whitney test between basins. Spearman correlation was used to test the relationship between genetic diversity and mean telomere length.

Potential differences in telomere length between regenerating and non-regenerating arms within individuals were tested using a mixed model involving the logarithmic transformation of Ct values, 'tissue type' as a factor, and interaction with 'regeneration'. Moreover, we separately tested the effects of regeneration for both tissue types via a paired-data student's t-test for tube feet, and a paired Mann–Whitney test for pyloric caeca telomere length since the data did not match normality (non-normal distribution; Shapiro–Wilk test; $W = 0.8482$, P-value = 0.002).

All statistical analyses of telomere length and box plots were performed in R v. 3.0.0.

RESULTS

Genetic diversity and genetic distance between localities

The low and significant values (P-values) of the P_{sex} (Table 2) indicated that the identical genotypes observed among individuals are a consequence of clonal propagation in this species. The studied populations of *C. tenuispina* presented different levels of allelic richness and clonality as defined by identical genotypes (Table 1). Populations from the Atlantic Ocean were genetically more diverse than those from the Mediterranean Sea, presenting 12 out of the 13 single multilocus genotypes (MLGs) found in the whole study area. Furthermore, excess of heterozygotes were found in all populations except Taormina. The D estimator, used to assess differences in genetic structure between populations, revealed significant genetic differences among all four populations here analyzed (Table 3). Large and significant differences were recorded between the two Mediterranean populations, with the latter characterized by a higher prevalence of genetic clones compared with the Atlantic populations. Indeed, only one multilocus genotype was detected at Llançà (Table 1).

Differences in telomere length and body size between and within populations

Telomeres were significantly longer in the Mediterranean than in the Atlantic starfish populations as shown by the Kruskal–Wallis test ($K = 24.17$, significant value: P-value < 0.001) (Figures 2a and b). When the four populations were analyzed separately, significantly longer telomeres were again observed in the two Mediterranean populations (Kruskal–Wallis test; $K = 37.03$, P-value < 0.001), with individuals from Llançà presenting the longest telomeres (Figure 2b). Telomere length measurements were double-checked and verified by an independent laboratory (Umeå, Sweden), and the result showed a strong correlation between the measurements between both independent sets of analyses (value of the correlation for the regression: $R^2 = 0.88$), (Supplementary Figure 2).

Genotype diversity (also expressed as clonal diversity) in this species depends on the relative ratio of fission (asexual reproduction) versus

Table 2 Different MLGs found in more than one individual in the four localities

Clonal MLG	Locality	n	P_{sex}	P-value
MLG 1	LLA	17	$< 10^{-14}$	0.000*
MLG 2	TAO	5	0.000	0.000*
MLG 4	TAO	3	$< 10^{-8}$	0.007*
MLG 8	TAO	6	$< 10^{-15}$	0.000*
MLG 9	BOCA	2	$< 10^{-10}$	0.000*
MLG 11	BOCA	5	0.000	0.000*
MLG 12	ABA	7	$< 10^{-14}$	0.000*

Abbreviations: ABA, Abades; BOCA, Boca Cangrejo; LLA, Llançà; multilocus genotypes, MLG; TAO, Taormina.
Data presented include the number of individuals sharing the same MLG (*n*), the probability of obtaining the same MLG from different sexual events (P_{sex}) and the associated P-value of P_{sex}.
*Significant when P-values < 0.01.

Table 3 Values of the D genetic differentiation estimator between populations of *Coscinasterias tenuispina*

	TAO	LLA	BOCA
LLA	0.167*		
BOCA	0.159*	0.233*	
ABA	0.143*	0.148*	0.138*

Abbreviations: ABA, Abades; BOCA, Boca Cangrejo; LLA, Llançà; TAO, Taormina.
*Indicates significant P-values < 0.01.

sexual reproduction. Here, populations with lower genotype diversity, and therefore higher fission rates had longer telomeres at the population level, as demonstrated by a significant correlation (Pearson correlation: $R = 0.99$, P-value < 0.007). In Figure 3, it is presented the high correlation between mean of genotype diversity per population and the Ct value (which is inversely proportional to telomere length).

Populations analyzed showed differences in the mean body size of the starfish, demonstrated by a significant value of the Mann–Whitney test ($W = 740$, P-value < 10^{-7}), with considerably larger specimens observed in the Atlantic populations (mean body size = 6.36 ± s.d. 3.8 cm) than in those from the Mediterranean Sea (mean body size = 2.81 ± s.d. 1.1 cm). However, telomere length did not depend on the starfish body size, as demonstrated by the absence of correlation between these two variables (correlation value: $\rho = 0.065$, P-value = 0.63, non-significant) (Supplementary Figure 3).

Telomere length in relation to arm regeneration

Our results showed that telomere length was significantly longer in regenerating (short) arms than in non-regenerating (long) arms (Figure 1b, Supplementary Figure 1), as demonstrate by the significance of the different tests applied ($F = 52.26$, P-value < 0.001), for both tissue types analyzed, tube feet (Student's-$t = 2.28$, P-value = 0.029) and pyloric cecum tissue (Mann–Whitney, $V = 299$, P-value < 10^{-6}) (Figure 4). Pyloric cecum telomeres were always longer in regenerating arms in all individuals, while 8 out of 12 specimens displayed longer telomere lengths in regenerating arm tube feet.

DISCUSSION

Life expectancy in clonal lineages remains unclear due the lack of understanding of different phenomena that would influence on its time survival. Firstly, we do not know the real consequences of the accumulation of somatic mutations and their deleterious effects on the
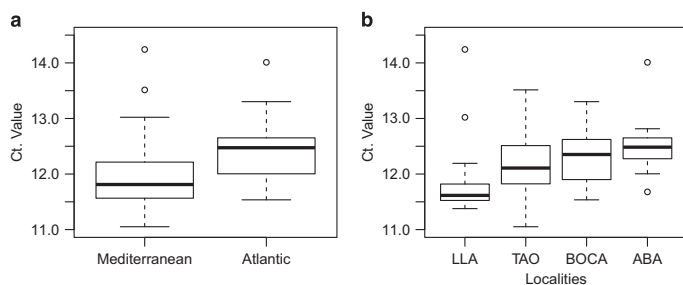
Figure 2 Box plots of telomere qPCR Ct values obtained from tube feet for the *Coscinasterias tenuispina* populations from different seas and localities: (**a**) Ct values of grouped Atlantic and Mediterranean populations, and (**b**) Ct values of each separate locality. Lower Ct values indicate longer telomeres. Boxes are represented by the first and third quartile, the dark line is the median, and dots are outliers. Llançà (LLA), Taormina (TAO), Abades (ABA) and Bocacangrejo (BOCA).
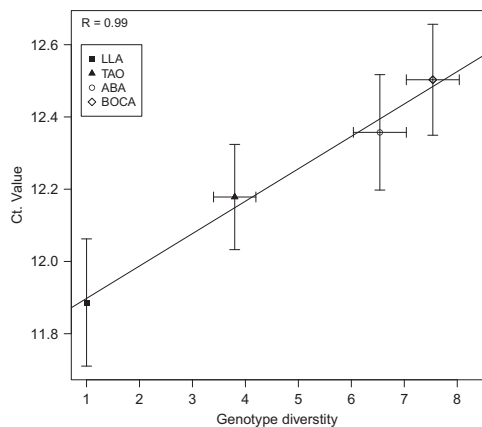

Figure 3 Correlation between relative telomere length in tube feet (telomere qPCR Ct Value) and genotype diversity for the four *Coscinasterias tenuispina* populations analyzed. Horizontal and vertical bars represent the standard error for genotype diversity and relative telomere length, respectively. The relative telomere length was represented as the mean value per locality.

Figure 4 Box plot of *Coscinasterias tenuispina* telomere qPCR Ct values in regenerating and non-regenerating arms for two different tissues from individuals of Llança: pyloric cecum (Pyl) and tube feet (TF). Boxes are represented by the first and third quartile, the dark line is the median, and dots are outliers. Pyloric cecum from non-regenerating arms (Pyl NR); pyloric cecum from regenerating arms (Pyl R); tube feet from non-regenerating arms (TF NR); tube feet from regenerating arms (TF R).

individual and their clonal offspring. Secondly, we ignore the real effect in wild populations of the lack of genetic diversity for adaptive potential, and finally the mechanisms to avoid senescence. The first two difficulties are usually overcome when the species are able to maintain sex, even at low rates or sporadic events, by combining genomes and eliminating phenotypic expression of deleterious mutations when recessive. Here, in this study, we shed some light on the third problem, evidencing telomere elongation during asexual reproduction of the starfish *C. tenuispina*.

Our work represents the first study to explore the potential implications of asexual reproduction on relative telomere length in wild populations of a clonal starfish. To date, most ecological studies examining telomere length and/or telomerase activity have focused on obligate sexually reproducing species or clonal organisms maintained in laboratory cultures (see examples in Klapper *et al.*, 1998; Horn *et al.*, 2010; Ojimi and Hidaka, 2010; Sköld and Obst, 2011; Carney

Almroth *et al.*, 2012; Tan *et al.*, 2012), and no previous study based on telomeres and aging has been conducted on wild clonal populations of any species.

Longer telomeres were recorded in the Mediterranean populations of the starfish *C. tenuispina*, while these specimens also significantly smaller in size. Although shorter telomeres were observed in the Atlantic populations characterized by a larger mean body size, our results did not detect any correlation between the two variables. This finding is consistent with the lack of age-related telomere shortening demonstrated for other marine species, including sea urchins and lobsters, and may be attributed to high phenotypic plasticity in body size and/or to continuous telomerase activity throughout their lifespan (Klapper *et al.*, 1998; Ebert *et al.*, 2008). Nevertheless, telomere length is regarded as an indicator of health and somatic fitness, and its variation observed in our populations may be influenced by both

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inherited and/or environmental components (Epel *et al.*, 2004). Thus, populations of *C. tenuispina* comprising only one clone may be healthily maintained, including those in Llançà, which were also characterized by the longest telomeres.

The longer telomeres found in regenerating compared with longer non-regenerating arms are indicative of telomere elongation and the preservation of chromosome ends in somatic tissue over the asexual cycle. These results may explain the positive correlation between telomere length and level of clonality. Asexual reproduction via fission has been proposed as more prevalent in small specimens of *Coscinasterias* (Emson and Wilkie, 1980), which is also consistent with our results and observations. It is therefore possible that the key to retain long telomeres in these starfish is to frequently undergo fission. On the other hand, fission may be influenced by the environment, either directly, for example, by different temperature regimes between the Mediterranean and Atlantic basins here analyzed, or indirectly, for example, by growth limitation and therefore it could be more prevalent in such situations (Haramoto *et al.*, 2007). Therefore, in *C. tenuispina,* longer telomeres and greater somatic fitness may be triggered by unstable environmental conditions in wild populations.

Elongation of telomeres in populations of *C. tenuispina* may be one of the mechanisms related to the absence of senescence and genetic defects associated to prolonged periods of asexual propagation. Studies investigating terrestrial species have demonstrated that both telomere length and telomere erosion are predictors of survival and somatic fitness (Bize *et al.*, 2009; Horn *et al.*, 2010), with the combination of long telomeres and telomere elongation in regenerating tissues potentially providing these clonal starfish a high probability of survival. Although the results of a previous study examining a colonial ascidian suggest that passing through a sexual reproductive phase is required in order to avoid senescence after prolonged periods of asexual budding (Sköld *et al.*, 2011), it is not known how generally this finding can be applied to other species. Despite the fact that asexual reproduction facilitates clonal dispersal and renewal, it depends on mitotic divisions, which may increase the accumulation of somatic mutations, the Muller ratchet phenomena. Different studies with asexual species already revealed accumulation of somatic mutations on non-synonymus position in clonal lineages compared with their non-clonal sibling species (Paland and Lynch, 2006; Barraclough *et al.,* 2007). Unfortunately, the negative effects of deleterious mutations in wild populations of clonal species have never been fully investigated or proved, and these effects have been only supposed, but asexual lineages persist over short evolutionary periods (Schwander and Crespi, 2009). However, older clones of aspen species has been found to exhibit a significant reduction in reproductive performance associated with male sexual fitness decline, suggesting that at least some long-lived clonal organisms may be vulnerable to senescence over long periods of time (Ally *et al.*, 2008). A hypothesis to explain the excess of heterozygosity found in the studied populations of *C. tenuispina* may be a positive selection of heterozygotes to keep genetic diversity, besides having greater individual adaptability by high phenotype plasticity (Hörandl, 2009; Goudie *et al.,* 2012). Nevertheless, our current results cannot actually test this hypothesis, and the hetero-zygotes excess found in all populations may be results from other stochastic processes that have not been controlled in this study. In other organisms as plants that maintain asexual lineages has been observed that polyploidy and hybridization commonly generate heterozygotes. In other species as fur seals, inbreeding is avoided by an active selection from females for non-relative males or hetero-zygotes (Hoffman *et al.*, 2007). However, we do not have evidences of

any of these processes, and positive selection of heterozygotes is the only hypothesis that can be presented here, but further studies on this particular point may shed light on it.

Although the molecular mechanisms responsible for telomere elongation and its preservation in *C. tenuispina* remain unknown, studies examining other asexual and sexual organisms indicate a pivotal role for telomerase in the telomere length regulation of somatic cell lineages. In *Asterias rubens*, a sexually reproducing starfish, telomerase activity is high throughout the animal irrespectively of mitotic activity and there was no difference in telomerase activity nor telomere length between regenerating and non-regenerating arms (Hernroth *et al.*, 2010), but clonal starfishes may have higher telomerase activities after fission as other asexual species. In flatworms, maintenance of somatic telomere length seems to be an adaptation of asexual but not sexual strains, and is based on different levels of telomerase activity (Tan *et al.*, 2012). Furthermore, in the colonial ascidian *Botryllus schlosseri*, telomerase activity is upregulated in early bud rudiments, and declines during zooid development (Laird and Weissman, 2004). Differences in the relative abundance of somatic versus stem cells might also determine variation in telomere length (Ojimi and Hidaka, 2010), but we cannot either discard recombina-tion between homologous telomeres as a means to elongate telomeres (Liu *et al.*, 2007). Even in plants, telomere restoration is only present in meristomatic and reproductive tissues, with exceptions in long-lived species (Flanary and Kletetschka, 2005; Watson and Riha, 2011 among other references), whereas in some algae it has been described telomerase activity during all life cycles, but large differences in telomerase activity have been found across algae groups (Fulnecková *et al.*, 2013; Ševčíková *et al.*, 2013). Further comparison of telomerase and stem cells in relation to regeneration in the analyzed populations of *C. tenuispina* would thus be of future interest.

The results presented here reveal the need for further research exploring the ecological and evolutionary significance of asexual reproduction and telomere elongation in clonal lineages. Future empirical studies measuring the success of Atlantic and Mediterranean populations should consider additional variables such as sexual reproductive success, as well as an evaluation of whether genetic diversity is fundamental to the maintenance of clonal populations, concepts that have been only theoretical explored for few authors as Weissman *et al.* (2009) and Marriage and Orive (2012). Those theoretical models for asexual species have evaluated and proposed that clones with large population sizes exhibit high successful levels (Weissman *et al.*, 2009; Marriage and Orive, 2012). Nevertheless, further analysis regarding possible somatic deleterious mutations, as well as health and population size monitoring, is essential in order to understand the life expectancy of clonal populations.

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Supplementary Information accompanies this paper on Heredity website (http://www.nature.com/hdy)