Cytogenetic and molecular analyses in *Palaemon* species

Zeltia Torrecilla Pérez

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Supervisors: Dra. Ana M. González Tizón & Dr. Andrés Martínez Lage

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Zeltia Torrecilla Pérez

Supervisors / Directores

Dr Ana M. González Tizón & Dr Andrés Martínez Lage

Reviewed by / Revisado por

Elisabetta Coluccia - Università di Cagliari

Alexandra Leitão - Qatar University

Departamento de Biología. Facultad de Ciencias

Los Dres. ANA MARÍA GONZÁLEZ TIZÓN, profesora contratada doctora, y ANDRÉS MARTÍNEZ LAGE, profesor titular de universidad, del área de genética del Departamento de Biología de la Universidade da Coruña

INFORMAN:

Que la licenciada **Zeltia Torrecilla Pérez** del programa de doctorado de Biología Celular y Molecular, ha realizado en el Departamento de Biología de la Facultad de Ciencias el trabajo con el título *Cytogenetic and molecular analyses in Palaemon species* bajo nuestra supervisión. Considerándolo finalizado permitimos su presentación bajo la modalidad internacional y compendio de artículos.

Y para que conste firmamos la presente en A Coruña a 8 de junio de 2018.

VºBª de los Directores

Fdo. Ana Mª González Tizón   Andrés Martínez Lage

La doctoranda

Fdo. Zeltia Torrecilla Pérez

To Adrián and our F_1

To my parents and my sister

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The strength of the Wolf is the Pack…

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SHORT ABSTRACTS

Abstract

In this PhD thesis, different genetic studies were carried out in two marine species of the genus *Palaemon* Weber, 1795 (Crustacea, Decapoda): the common littoral prawn *Palaemon serratus* (Pennant, 1777) and the rockpool prawn *Palaemon elegans* Rathke, 1837. Both their European distribution and morphological similarity make them interesting candidate species for developing population and cytogenetic analyses, complementing phylogenetic studies in order to clarify the evolution of the genus *Palaemon*. Furthermore, given the commercial relevance of *P. serratus*, this thesis aims to contribute to the effective management and traceability of this natural resource. Firstly, we applied cytogenetic analyses based on conventional staining, banding techniques and fluorescence *in situ* hybridisation to identify and characterise these two prawn species. The results obtained from this study revealed a high degree of diversity in chromosome number, karyotype and sex determination system, ranging from the putative absence of heteromorphic sex chromosomes to the multiple chromosome system $X_1X_1X_2X_2/X_1X_2Y$. Afterwards, we carried out a phylogeographic analysis of *P. serratus* to test intraspecific genetic diversity and geographic structure. A pronounced genetic differentiation was detected between Mediterranean and Atlantic populations and an uncommon phylogeographic boundary was unveiled. Finally, we developed a species-specific PCR-assay as a traceability tool for authentication of *P. serratus* and other four economically important prawn species.

Resumen

En la presente tesis doctoral se han realizado diversos estudios genéticos en dos especies europeas de camarón pertenecientes al género *Palaemon* Weber, 1795 (Crustacea, Decapoda): el camarón común *P. serratus* (Pennant, 1777) y el camarón de roca *P. elegans* Rathke, 1837. Su distribución europea y su similitud morfológica, las convierten en interesantes candidatas para desarrollar análisis de poblaciones y citogenéticos que complementen los estudios filogenéticos existentes y ayuden a clarificar la evolución del género *Palaemon*. Además, debido a la importancia comercial de *P. serratus*, esta tesis pretende contribuir a la gestión y conservación efectiva de este recurso natural, así como a su trazabilidad. En primer lugar, se emplearon técnicas citogenéticas basadas en tinciones convencionales, técnicas de bandeo e hibridaciones *in situ* fluorescente para identificar y caracterizar estas dos especies de camarón. Los resultados obtenidos revelaron disparidad en el número cromosómico diploide, cariotipo y sistema de determinación sexual de las dos especies estudiadas, variando desde la supuesta ausencia de cromosomas sexuales heteromórficos hasta el sistema múltiple de determinación sexual $X_1X_1X_2X_2/X_1X_2Y$. En segundo lugar, se realizó un análisis filogeográfico en *P. serratus* para evaluar la diversidad genética intraespecífica así como su estructura poblacional. Se detectó una pronunciada diferenciación genética entre las poblaciones atlántica y mediterránea que revelaron la existencia de una barrera filogeográfica poco frecuente. Finalmente, se desarrolló un método especie-específico basado en la PCR como herramienta de trazabilidad alimentaria para la autentificación de *P. serratus* y otras cuatro especies de langostinos y gambas de interés comercial.

Resumo

Na presente tese de doutoramento realizáronse diversos estudos xenéticos en dúas especies europeas de camarón pertencentes ao xénero *Palaemon* Weber, 1795 (Crustacea, Decapoda): o camarón común *P. serratus* (Pennant, 1777) e o camarón de roca *P. elegans* Rathke, 1837. A súa distribución europea e a súa similitude morfolóxica, converten estas dúas especies en interesantes candidatas para desenvolver análises citoxenéticas e de poboacións que complementen os estudos filoxenéticos existentes e que axuden a clarificar a evolución do xénero *Palaemon*. Ademais, debido á importancia comercial de *P. serratus*, esta tese pretende contribuir á xestión e conservación efectiva deste recurso natural, así como á súa trazabilidade. En primeiro lugar, empregáronse técnicas citoxenéticas baseadas en tincións convencionais, técnicas de bandeo e hibridacións *in situ* fluorescentes para identificar e caracterizar as devanditas especies de camarón. Os resultados obtidos revelaron disparidade no número cromosómico diploide, cariotipo e sistema de determinación sexual das dúas especies estudadas, variando dende a suposta ausencia de cromosomas sexuais heteromórficos ata o sistema múltiple de determinación sexual $X_1X_1X_2X_2/X_1X_2Y$. En segundo lugar, realizouse unha análise filoxeográfica en *P. serratus* para avaliar a diversidade xenética intraespecífica así como a súa estrutura poboacional. Detectouse unha pronunciada diferenciación xenética entre as poboacións atlántica e mediterránea que revelaron entre elas unha barreira filoxeográfica pouco frecuente. Finalmente, desenvolveuse un método especie-específico baseado na PCR como ferramenta de trazabilidade alimentaria para a autentificación de *P. serratus* e outras catro especies de lagostinos e gambas de interese comercial.

EXTENDED ABSTRACT

Resumen extendido

En la presente tesis doctoral se han realizado diversos estudios genéticos en dos especies europeas de camarón pertenecientes al género *Palaemon* Weber, 1795 (Crustacea: Decapoda): el camarón común *P. serratus* (Pennant, 1777) y el camarón de roca *P. elegans* Rathke, 1837. Actualmente se considera que este género comprende 87 especies de acuerdo con la reciente sinonimia de *Palaemonetes* Heller, 1869, *Coutierella* Sollaud, 1914 y *Exopalaemon* Holthuis, 1950 con *Palaemon*, y teniendo en cuenta varias sinonimias a nivel de especie y tres especies nuevamente descritas. Sin embargo en los últimos años, la filogenia del género ha estado bajo revisión continua y en la actualidad no está completamente resuelta. Esto se debe en parte a que la discriminación morfológica entre congéneres es particularmente difícil debido a que los criterios taxonómicos de identificación se basan en diferencias muy sutiles, como por ejemplo, en la denticulación del rostro o en la morfología del flagelo antenular, lo que deriva a menudo en una identificación errónea de las especies. Las dos especies de camarón seleccionadas en este estudio ilustran un caso de extremada semejanza morfológica dentro del género. *Palaemon serratus* y *P. elegans* comparten además gran parte de su nicho ecológico y rango de distribución. En concreto, se distribuyen desde el Mar del Norte hasta Mauritania y Namibia respectivamente, incluyendo el Mar Mediterráneo y el Mar Negro. Desde los años 1950s, *P. elegans* también se encuentra en el Mar Caspio y en el Mar de Aral debido a introducciones humanas y más recientemente, desde el año 2000, se ha detectado su presencia en el Mar Báltico, dónde está remplazando a su congénere nativo *P. adspersus*.

Las características que comparten estas dos especies las convierten en apropiadas candidatas para desarrollar análisis de poblaciones y estudios de citogenética comparada que complementen los estudios filogenéticos y filogeográficos existentes y ayuden a clarificar la evolución del género *Palaemon*. A su vez, la elevada demanda comercial de *P. serratus* en diversas regiones europeas entre las que destaca el litoral de Galicia (España), ha dado lugar a una intensa explotación pesquera de este recurso natural. Es por ello que esta tesis pretende contribuir a la gestión y conservación efectiva de esta especie de crustáceo decápodo, por una parte mediante la identificación de posibles unidades o stocks genéticos a lo largo de su rango de distribución y por otra parte, a través del diseño de una herramienta molecular de autentificación dirigida sobre todo a empresas de alimentación, para su empleo en puntos de control de calidad o en auditorías.

Los objetivos concretos de esta tesis fueron 1) caracterizar los cariotipos de *P. serratus* y *P. elegans* identificando sus cromosomas sexuales, y con los resultados obtenidos, realizar un estudio de citogenética comparada; 2) evaluar la variabilidad genética intraespecífica de *P. serratus* así como su estructura poblacional a lo largo de su rango de distribución; y 3) desarrollar un método molecular de trazabilidad alimentaria que permita autentificar la presencia de *P. serratus* en productos frescos y congelados, garantizando así su calidad y combatiendo el fraude comercial. Además, en este último objetivo se incluyeron otras especies de crustáceos (langostinos y gambas) de gran importancia económica.

Dichos objetivos se enmarcan dentro de las áreas de citogenética, genética de poblaciones e identificación genética de especies o *DNA barcoding*. Consecuentemente, la metodología empleada a lo largo de la tesis varía en cierto modo en cada uno de los capítulos que la conforman y puede resumirse como sigue: en el CAPÍTULO 1.1 titulado "*Karyological analysis of the shrimp Palaemon serratus (Decapoda: Palaemonidae)*" y en el CAPÍTULO 1.2 "*Comparative cytogenetic analysis of marine Palaemon species reveals a* $X_1X_1X_2X_2/X_1X_2Y$ *sex chromosome system in Palaemon elegans*", se utilizaron técnicas citogenéticas de extensión cromosómica, técnicas de bandeo cromosómico e hibridación *in situ* fluorescente (FISH) de los genes ribosomales mayores (18S-5.8S-28S) y de diversas secuencias teloméricas, así como el empleo de técnicas de microscopía en campo claro y fluorescente. Asimismo, se desarrollaron las sondas mediante reacción en cadena de la polimerasa (PCR), clonación y marcaje fluorescente de las secuencias de DNA. En el CAPÍTULO 2 que lleva por título "*Genetic differentiation between Mediterranean and Atlantic populations of the common prawn Palaemon serratus (Crustacea: Palaemonidae) reveals uncommon phylogeographic break*" la metodología utilizada fue diferente. Se extrajo DNA de muestras de camarón procedentes de diferentes localidades atlánticas y mediterráneas. Posteriormente, se amplificaron mediante PCR dos fragmentos de DNA mitocondrial (el gen que codifica la subunidad 1 de la citocromo *c* oxidasa, Cox1 y el gen que codifica la subunidad mayor del ribosoma mitocondrial, 16S rRNA) y un fragmento de origen nuclear (el gen que codifica la enzima Enolasa implicada en la glucólisis). Los productos de PCR resultantes fueron secuenciados mediante el método Sanger y las secuencias obtenidas fueron analizadas aplicando herramientas bioinformáticas. Por último, en el CAPÍTULO 3 titulado "*Molecular authentication of five economically important prawn species by species-specific PCR-assay*" se siguió una metodología similar a la del capítulo anterior, pero en esta ocasión el marcador molecular elegido fue un pequeño fragmento del gen mitocondrial citocromo *b* oxidasa (cyt *b*) a partir del cual se diseñaron cebadores

especie-específicos. Las secuencias obtenidas en esta tesis se depositaron en las bases de datos GenBank/EMBL.

A continuación se resumen los principales resultados obtenidos en cada uno de los capítulos de esta tesis:

En el CAPÍTULO 1.1 "*Karyological analysis of the shrimp Palaemon serratus (Decapoda: Palaemonidae)*" se caracterizó por primera vez el camarón común europeo *P. serratus* a nivel citogenético. Se observó que esta especie cuenta con una dotación diploide de 2n=56 cromosomas, el número cromosómico más bajo descrito en la familia Palaemonidae hasta la fecha. El cariotipo de *P. serratus* consistió en 2 grandes pares de cromosomas metacéntricos, 6 pares de cromosomas subtelocéntricos y 20 pequeños pares de cromosomas telocéntricos. La tinción con plata (Ag-NOR) y la hibridación *in situ* fluorescente de la sonda de los genes ribosomales mayores, localizaron las regiones organizadoras nucleolares (NORs) en los telómeros de cuatro pequeños cromosomas telocéntricos. La tinción con DAPI reveló bloques ricos en AT en las regiones centroméricas/pericentroméricas de todos los cromosomas además de bandas intercalares en los dos pares metacéntricos grandes.

En el CAPÍTULO 1.2 "*Comparative cytogenetic analysis of marine Palaemon species reveals a $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in Palaemon elegans*" se profundizó en el análisis citogenético de *P. serratus* y se extendió el estudio a su congénere europeo *P. elegans*. Se observó que el número cromosómico diploide de *P. elegans* es 2n=89 cromosomas en machos y 2n=90 cromosomas en hembras. El cariotipo consistió en 43 pares cromosómicos autosómicos: 5 metacéntricos/submetacéntricos, 4 subtelocéntricos/telocéntricos y 34 cuya morfología fue difícilmente distinguible dado su pequeño tamaño. El cariotipo de las hembras incluyó además dos pares de cromosomas telocéntricos más grandes; mientras que el complemento cromosómico de machos presentó un cromosoma metacéntrico grande y dos cromosomas telocéntricos. Así, el cromosoma metacéntrico de mayor tamaño y exclusivo de machos (Y) pone de manifiesto que los machos constituyen el sexo heterogamético en esta especie. El comportamiento de los cromosomas durante la primera división meiótica permitió definir sin lugar a duda el sistema de determinación del sexo de la especie. En diacinésis, cada brazo del cromosoma Y metacéntrico se asociaba terminalmente con cada uno de los cromosomas telocéntricos, formando un trivalente (X_1X_2Y). En hembras, los cromosomas sexuales telocéntricos apareaban dos a dos dando lugar a dos bivalentes ($X_1X_1X_2X_2$). Consecuentemente, se comprobó la existencia en *P. elegans*

del sistema múltiple de determinación del sexo $X_1X_1X_2X_2/X_1X_2Y$. El análisis de extensiones meióticas de *P. serratus* mostró 28 bivalentes en ambos sexos y no se detectó la existencia de ningún par cromosómico heteromórfico entre machos y hembras. Con el fin de clarificar la posible existencia de cromosomas sexuales en esta especie se realizó la técnica de bandeo C cromosómico. Dicha técnica reveló que, cuando estaba presente, la heterocromatina constitutiva tenía una distribución predominantemente telomérica; aunque sí se detectaron bandas heterocromáticas débiles en posición intersticial en los grandes cromosomas metacéntricos. No se observaron diferencias entre el patrón de bandas C de machos y hembras de *P. serratus*. En los cromosomas sexuales de *P. elegans* no se detectó la presencia de bandas C. Considerando este resultado, y teniendo en cuenta el gran tamaño del cromosoma Y así como la existencia de recombinación en meiosis de los cromosomas sexuales de macho, se postuló que el sistema múltiple de determinación sexual quizá sea el resultado de una evolución reciente.

Siguiendo con los resultados derivados del bandeo C, en ambas especies de *Palaemon* se observó la presencia de grandes bloques heterocromáticos en los telómeros de 4 pequeños cromosomas, señales coincidentes con los sitios de hibridación de la sonda de los genes ribosomales mayores. Dada las diferencias identificadas entre los cariotipos de *P. serratus* y *P. elegans*, la existencia de dos *loci* ribosomales quizá constituya un carácter plesiomórfico dentro del género *Palaemon*. La tinción con DAPI mostró bloques ricos en AT en las regiones centroméricas/pericentroméricas de todos los cromosomas de *P. serratus* y *P. elegans*. Además en este capítulo, se detectó en ambas especies de camarón la presencia del motivo telomérico ancestral de artrópodos TTAGG en los telómeros de todos los cromosomas. No se encontraron señales intersticiales en ninguno de los complementos. En base a los resultados citogenéticos obtenidos para *P. serratus* y *P. elegans* y la revisión bibliográfica de los datos disponibles en la familia Palaemonidae, parece probable que un elevado número cromosómico represente una condición ancestral del género *Palaemon*, mientras que el reducido número cromosómico característico de *P. serratus* constituya un carácter derivado. De acuerdo con esto, parece plausible que diversas fusiones cromosómicas constituyan el principal mecanismo responsable del origen del cariotipo de *P. serratus*, lo cual ha sido sugerido también para otras familias de decápodos como Astacidae y Parastacidae.

En el CAPÍTULO 2, *"Genetic differentiation between Mediterranean and Atlantic populations of the common prawn Palaemon serratus (Crustacea: Palaemonidae) reveals uncommon phylogeographic break"*, se realizó un estudio biogeográfico de *P. serratus* con muestras tomadas en localidades situadas a lo largo de la costa

europea. Con este fin, se secuenciaron y analizaron dos genes mitocondriales (Cox1 y 16S rDNA) y uno nuclear (Enolasa). Se construyeron redes de máxima parsimonia estadística para cada uno de los genes, se calculó la diversidad haplotípica y la diversidad nucleotídica y se realizó un análisis molecular de la varianza (AMOVA). Los resultados obtenidos revelaron diferenciación genética entre las localidades mediterráneas, el Estuario del Guadalquivir y las localidades atlánticas. Los individuos de las dos primeras áreas están relacionados entre sí formando parte de la población o linaje mediterráneo. El segundo linaje, la población atlántica contiene los individuos de las localidades atlánticas al norte del Golfo de Cádiz. Por tanto, la barrera filogeográfica que limita el flujo génico entre las poblaciones atlántica y mediterránea de *P. serratus* se encuentra en algún lugar al este del Golfo de Cádiz. Aunque la diferenciación intraespecífica atlántico-mediterránea ha sido detectada en diversas especies marinas, la barrera que se describe en este trabajo es muy poco frecuente en la literatura. Se ha postulado que el Frente Almería-Orán es la principal frontera hidrográfica que da lugar a diferenciación entre las poblaciones atlánticas y mediterráneas en muchas especies y de hecho se ha demostrado su efecto en la estructura genética de *P. elegans*.

Los análisis realizados en este estudio (CAPÍTULO 2) mostraron que la población atlántica es genéticamente muy diversa, exhibiendo 18 haplotipos diferentes para Cox1 y todas las localidades analizadas presentan diversidades haplotípicas y nucleotídicas muy altas, así como elevado flujo génico entre ellas. Del mismo modo, el análisis de diversidad haplotípica de la población mediterránea sugiere que también esta población representa un sistema estable con valores de flujo génico relativamente altos. Sin embargo, dentro de este linaje, el flujo génico de las localidades situadas en el Mar Mediterráneo con respecto a los individuos del Estuario de Guadalquivir es restringido y, como consecuencia, estos últimos forman un clado bastante homogéneo con haplotipos endémicos. A la vista de los resultados, parece probable la ocurrencia de una colonización reciente desde el este del Mar Mediterráneo al Golfo de Cádiz originando un efecto fundador que explicaría la baja variabilidad génica en esa localidad.

En el Capítulo 3 "*Molecular authentication of five economically important prawn species by species-specific PCR-assay*", se desarrolló un método de trazabilidad alimentaria basado en la PCR para autentificar la presencia de *P. serratus* además de otras cuatro especies de crustáceos de elevado interés comercial; en concreto: el langostino tigre (*Penaeus indicus*), el langostino jumbo (*Penaeus monodon*), el gambón argentino (*Pleoticus muelleri*) y la gamba rosada (*Aristeus antennatus*). Pese a su importancia comercial, *Aristeus antennatus* y *Palaemon serratus* se analizaron

aquí por primera vez desde una perspectiva de trazabilidad alimentaria. En este estudio, a partir de un fragmento de DNA mitocondrial (cyt b) de 181pb se desarrollaron cebadores especie-específicos. En el caso de *P. serratus* la autentificación se llevó a cabo en dos ciclos de amplificación, primero con el par de cebadores crustF/Se-cytbR (resultado positivo) y a continuación con los cebadores crustF/Mon-cytbR que no da lugar a amplificación por ser específico de *P. monodon*. En los demás casos, un resultado de amplificación positivo de la muestra con el correspondiente par de cebadores específicos verifica la identidad de la especie para la que fue diseñado. La especificidad de los cebadores fue testada en todas las especies incluidas en el análisis además de en el camarón blanco, *Litopennaeus vannamei*, que contribuye al 80% de la producción mundial de camarón. No se documentaron casos de amplificación cruzada. Esta metodología puede emplearse tanto en tejido fresco como en tejido congelado. Además, el tipo de marcador molecular elegido (mitocondrial) y el pequeño tamaño de los amplicones, convierte a este método en un buen candidato para su empleo en productos procesados donde el DNA está habitualmente dañado y fragmentado.

Las principales conclusiones de esta tesis se enumeran en los siguientes puntos:

- El número cromosómico diploide de machos y de hembras de *P. serratus* es de 2n=56 cromosomas. En ambos sexos, el cariotipo consistió en 2 grandes pares de cromosomas metacéntricos, 6 pares de cromosomas subtelocéntricos y 20 pequeños pares de cromosomas telocéntricos. El análisis comparativo entre las extensiones metafásicas de machos y de hembras de *P. serratus* indicó la supuesta ausencia de cromosomas sexuales heteromórficos.

- El número cromosómico diploide de *P. elegans* es de 2n=90 en hembras y de 2n=89 en machos. En ambos sexos, el cariotipo consistió en 43 pares cromosómicos autosómicos: 5 pares metacéntricos/submetacéntricos, 4 pares subtelocéntricos/telocéntricos, y 34 pares cuya morfología fue difícilmente reconocible debido a su pequeño tamaño. El complemento cromosómico de hembras incluyó además 4 grandes cromosomas telocéntricos ($X_1X_1X_2X_2$), mientras que el de machos reveló un gran cromosoma metacéntrico (Y) y dos cromosomas telocéntricos (X_1X_2).

- El análisis comparativo entre cromosomas metafásicos de ambos sexos de *P. elegans*, además de su comportamiento en meiosis, revelaron la presencia de un sistema de determinación sexual múltiple $X_1X_1X_2X_2/X_1X_2Y$

en esta especie. La recombinación en meiosis de los cromosomas sexuales masculinos formando el trivalente observado, en combinación con el hecho de que el cromosoma Y es eucromático y de gran tamaño, pueden ser indicativos de que este sistema de determinación sexual ha surgido recientemente en la evolución de la especie.

- La revisión de los números cromosómicos de los miembros de la familia Palaemonidae, además de ciertas evidencias aportadas en esta tesis, sugieren que el reducido número cromosómico de *P. serratus* constituye un carácter derivado dentro de la familia. La evolución cromosómica del género *Palaemon* podría estar relacionada con diversos eventos de fusión cromosómica, hipótesis que necesita futuras investigaciones.

- La técnica de bandeo-C reveló que la heterocromatina constitutiva tiene una distribución predominantemente telomérica en los cromosomas de ambas especies de *Palaemon*. En cuanto a los cromosomas sexuales, en *P. serratus* no se encontraron diferencias en el patrón de heterocromatina constitutiva entre sexos. En *P. elegans*, esta técnica reveló la ausencia de heterocromatina constitutiva en los cromosomas sexuales.

- De acuerdo con los resultados de hibridación *in situ* fluorescente, tanto en *P. serratus* como en *P. elegans*, se localizaron 4 señales (dos *loci*) de los genes ribosomales mayores en posición terminal, lo que podría constituir un carácter plesiomórfico del género. Además, en ambas especies, se detectó la presencia de la repetición TTAGG en los telómeros de todos los cromosomas, revelándose por primera vez la existencia de este motivo ancestral de artrópodos en la familia Palaemonidae.

- El análisis poblacional de *P. serratus* resultante de analizar dos genes mitocondriales y un gen nuclear (Cox1 mtDNA, 16S rRNA and Enolase nuDNA), mostró diferenciación genética entre las localidades del Mar Mediterráneo, el Estuario de Guadalquivir y el Océano Atlántico.

- La población mediterránea incluye los individuos del Estuario del Guadalquivir, indicando que la barrera geográfica que limita el flujo génico entre las poblaciones atlánticas y mediterráneas de *P. serratus* está localizada al oeste del Estrecho de Gibraltar, una barrera filogeográfica poco frecuente.

- En relación a la gestión de *P. serratus,* nuestros resultados revelaron la existencia de dos poblaciones dentro de esta especie que apuntan hacia la necesidad de establecer dos áreas prioritarias de gestión.

- Se desarrolló un método sencillo de detección especie-específico basado en la PCR destinado a autentificar la identidad de cinco especies de crustáceos económicamente importantes en productos de alimentación: el langostino tigre (*Penaeus indicus*), el langostino jumbo (*Penaeus monodon*), el gambón argentino (*Pleoticus muelleri*), la gamba rosada (*Aristeus antennatus*) y el camarón común (*Palaemon serratus*).

- Este método de trazabilidad alimentaria, que tiene como diana un pequeño fragmento del gen citocromo *b* oxidasa, asegura la detección del molde de DNA en productos frescos y congelados y proporciona un método simple, rápido y económicamente asequible para su aplicación en la industria de la alimentación así como para los laboratorios de control de calidad.

INTRODUCTION

Organisation of the thesis

In this PhD thesis, different genetic studies were carried out in two marine *Palaemon* species: the common littoral prawn *Palaemon serratus* (Pennant, 1777) and the rockpool prawn *Palaemon elegans* Rathke, 1837 (Crustacea, Decapoda). The European distribution and morphological similarity between these two prawn species make them interesting candidates to perform population and cytogenetic analyses. These assays will complement previous phylogenetic studies in order to shed light on the evolution of the genus *Palaemon*. Furthermore, given the commercial relevance of *P. serratus*, this PhD thesis aims to contribute to the effective management and traceability of this natural resource. In terms of methodology, several cytogenetic and molecular biology approaches were followed to answer questions related to evolutionary and population genetics, conservation or food traceability among other issues.

This thesis was funded by two different research projects by Xunta de Galicia (GRC2014/050) and Ministerio de Economía, Industria y Competitividad (CTM2014-53838-R), reflecting the economic and ecological importance of the selected species.

Given that the present thesis has been written as a compendium of research articles, each of them has its own introductory section. Therefore, in this general introduction, I will give an overview on the organisation of the thesis, providing some useful information that, due to editorial reasons, was not included in the articles themselves.

For the sake of clarity, the following RESEARCH ARTICLES were ordered by publishing date and topic.

In CHAPTER 1, we aimed to provide the first karyological data for *P. serratus* and *P. elegans* as well as identifying their sex chromosome system. Mitotic and meiotic karyotypes of these species were characterised based on conventional staining, banding techniques and fluorescence in situ hybridization (FISH) with ribosomal and telomeric probes.

In CHAPTER 2, we focused on *P. serratus* prawn to perform a phylogeographic analysis on individuals collected along European coastlines. We assessed intraspecific genetic diversity and population structure of this species using mitochondrial and nuclear molecular markers.

In CHAPTER 3, we developed a DNA-based technique to authenticate the presence of *P. serratus* in food products using a small sized mitochondrial marker. Also, we

extended our analysis to four economically important prawn species, in particular *Pleoticus muelleri*, *Penaeus monodon*, *Penaeus indicus* and *Aristeus antennatus.*

In the CORRIGENDUM, we report some errors observed after the publication of the research articles that are part of this thesis.

The GENERAL DISCUSSION addresses to examine the implications of the work developed in this thesis regarding further genetic studies in the genus *Palaemon*.

Finally, we summarise the main CONCLUSIONS inferred from the results of this thesis.

Introduction to *Palaemon* species

The genus *Palaemon* Weber, 1795 (Crustacea, Decapoda) is one of the most species-rich genus of the family Palaemonidae Rafinesque, 1815 (De Grave and Fransen 2011; De Grave and Ashelby 2013). It includes marine, estuarine and freshwater shrimps and prawns, as well as amphidromous representatives, distributed around the world (Carvalho et al. 2017). *Palaemon* currently consists of 87 species, according to the recent synonymization of the genera *Palaemonetes* Heller, 1869, *Coutierella* Sollaud, 1914 and *Exopalaemon* Holthuis, 1950 (De Grave and Ashelby 2013) with *Palaemon*, and taking into account species-level synonymies (Carvalho et al. 2014a, b; Tzomos and Koukouras 2015) and three species newly described *P. minos* Tzomos and Koukouras, 2015; *P. colossus* Tzomos and Koukouras, 2015 and *P. leucurus* Ashelby, 2018.

Eleven species of the genus inhabit in European waters, in particular, six species of *Palaemon*, namely, *P. adspersus* Rathke, 1837, *P. elegans* Rathke, 1837, *P. longirostris* H. Milne-Edwards, 1837, *P. serratus* (Pennant, 1777), *P. xiphias* Risso, 1816, and the recently introduced *P. macrodactylus* Rathbun, 1902; four species of formerly named *Palaemonetes*, namely, *P. antennarius* (H. Milne-Edwards, 1837), *P. turcorum* (Holthuis, 1961), *P. varians* (Leach, 1814) and *P. zariquieyi* (Sollaud, 1939) (González-Ortegón and Cuesta 2006); and the new species redescripted by Tzomos and Koukouras (2015), *Palaemon migratorius* (Heller, 1862).

Palaemon species occupy a broad spectrum of habitats with wide geographical distributions along the tropical and temperate regions. They present diverse reproductive strategies and morphological traits. The aforementioned variability might indicate close phylogenetic relationships among species which share the same character states. Alternatively, similarities between species might also be the result of convergent evolution due to similar selective pressures (Carvalho et al. 2017). This could be the case of the species under study, *P. serratus* and *P. elegans*, which share an extreme morphological similarity.

The life cycle of *Palaemon* prawns includes four main distinguishable stages: eggs, larvae, juvenile prawns and adults. *Palaemon* species are sexually dimorphic, with males generally being smaller than females.

The total size of adult individuals of *P. serratus* ranges between 25 and 90 mm. The rostrum is serrated with 6-8 dorsal teeth, and 5 teeth on the ventral margin. The abdomen shows a red coloured striped pattern. However, this coloration may be reduced or absent in specimens living in turbid waters (González-Ortegón and Cuesta

2006). *Palaemon elegans* grows up to 65 mm length. The rostrum, serrated as well, is strongly expanded ventrally, being extremely high at the level of the first ventral tooth, with 7-9 dorsal teeth and a single row of setae on ventral margin. The carapace and the abdomen are usually black with a striped pattern. The joints of the leg are defined by yellow bands and the palm of the chela of the second legs is blue. Nevertheless, as for *P. serratus*, such coloration may vary, depending on environmental conditions (González-Ortegón and Cuesta 2006).

From a morphological point of view, the number of dorsal teeth behind their ocular orbit at the serrated rostrum unequivocally differentiates *P. serratus* from *P. elegans* (two and three teeth, respectively; González-Ortegón personal communication, 2018). Although these species are morphologically similar, they differ in physiology, life history strategies and larval development (Fincham 1983; González-Ortegón et al. 2006; González-Ortegón and Gimenez 2014; Madeira et al. 2015).

Palaemon serratus and *P. elegans* have a wide geographical distribution, ranging from the North Sea to Mauritania and Namibia, respectively and including the Mediterranean and Black Sea (Holthuis 1955; d'Udekem d'Acoz 1999). Nowadays, *P. elegans* also inhabits the Aral and Caspian Sea, due to unintentional human introductions in the 1950s (Zenkevich 1963; Grabowski 2006). Similarly, this species was introduced into the Baltic Sea, and it is replacing the native species *P. adspersus* since 2000 (Reuschel et al. 2010).

They are both marine species, but whereas *P. serratus* inhabits estuaries in the reproductive season, *P. elegans* is common in rockpools, *Zostera*, *Posidonia* and *Cymodocea* meadows and it also appears in slightly brackish water close to river mouths (Cuesta et al. 2006). According to Madeira et al. (2015) they both present different vertical distributions in the intertidal zone: *P. elegans* can be found in intertidal pools in the middle to upper shore and *P. serratus* occupies pools from middle to lower shore and subtidal habitats. On the other hand, *P. serratus* is adapted to lower shore and subtidal conditions meanwhile *P. elegans* is adapted to higher shore conditions.

As invertebrates species, these prawns are relevant elements of marine ecosystems mechanisms, providing a link between trophic levels by feeding on algae, bryozoans, or small crustaceans, and in this case, being prey of relevant commercial fish species, for instance from the family Moronidae or Sparidae (Madeira et al. 2015).

Its broad ecological niche and the recent range expansion of *P. elegans* make this prawn key species within the European marine littoral fauna (Reuschel et al. 2010).

Both species are under commercial exploitation (Holthuis 1980), but only one of them, *P. serratus*, is highly important from an economic point of view in many European regions due to the size that it reaches and its valuable flavor. It has been commercially exploited using prawn trawls along the coast of Welsh, Ireland, England, France, Spain and Portugal from the early 1970s. Thus, the capture of this resource is an important traditional activity from social and economic perspectives. In fact, *P. serratus* fishery contributes more than ten million euros annually to the European economy (Kelly et al. 2008). In Galicia (Northwest Spain), during the last ten years, the volume of catches varied from 47,576 to 90,710 Kg per year, which signifies an approximate worth of 2 million euros per year (data obtained from https://www.pescadegalicia.gal/ on 20 Feb 2018, Xunta de Galicia).

State-of-the-art of genetic studies in study species

Palaemon serratus and *P. elegans* have been used, so far, as marine invertebrate models to investigate the effect of environmental stressors, such as trace metal contaminant, and they have proved to be suitable bioindicator species in ecotoxicology (e.g. Lorenzon et al. 2000; Sanders et al. 2005; Lozano et al. 2010; González-Ortegón et al. 2013; Oliveira et al. 2013). Furthermore, Erraud et al. (2017) lately suggested the interest of palaemonid prawns in ecogenotoxicology. In particular, these authors adapted the comet assay in *P. serratus* to asses the contamination impact on the sperm quality and to use this technique for biomonitoring issues.

Genetic studies on these species are really scarce. The few genetic studies on *P. serratus* and *P. elegans* are focused on population genetics and phylogenetic relationships of the genus. Cuesta et al. (2012) accomplished a molecular study based on the mitochondrial marker 16S rRNA in order to clarify the phylogeny of European *Palaemon* and *Palaemonetes,* including 20 representatives (*P. serratus* and *P. elegans* among them). The results of this study confirmed the paraphyly of these genera, as it had already been pointed out by Murphy and Austin (2003; 2005) based on Australian representatives. Such status was partially resolved by De Grave and Ashelby (2013), who inferred a systematic relationship in Palaemoninae from analyses based on the mitochondrial 16S rRNA and nuclear Histone (H3) genes, synonymizing the genera *Palaemonetes*, *Exopalaemon* and *Coutierella* with *Palaemon*. However, a recent phylogeny based on partial sequences of 16S rRNA, histone H3 and 18S rRNA from 60 species of *Palaemon* and 15 species from other Palaemonidae (Carvalho et al. 2017), provided new evidences. According to Carvalho et al. (2017) the genus, as redefined De Grave and Ashelby (2013), remains non-

monophyletic, and it can be divided into four clades: *Palaemon sensu stricto; ´Alaocaris´;* and the monospecific *P. mercedae* and *P. concinnus* clades. Their results also supported previous studies (Ashelby et al. 2012; Cuesta et al. 2012; Botello and Álvarez 2013) reporting that the colonization of American and European waters likely occurred multiple times.

In regard to population studies in *P. elegans*, a phylogeographic study of this species based on the mitochondrial genetic markers 16S rRNA and cytochrome *c* oxidase I (Cox1), revealed the existence of three haplogroups, one of them present in the Atlantic populations (type I), and two in the Mediterranean populations (type II and type III) (Reuschel et al. 2010). In light of the results obtained in this work, genetic differentiation between the Atlantic (type I) and Mediterranean population (type II) of *P. elegans* has been suggested due to the existence of an oceanographic barrier, the Almería-Orán front, that limits gene flow between the Atlantic Ocean and the Mediterranean Sea, and a process of speciation during the Mediterranean salinity crisis (Messinian crisis) in the Pliocene that would have given rise to a cryptic species (type III) within *P. elegans*. The authors also determined that this species was introduced from the Mediterranean Sea (type III) into the Baltic Sea by human action. Bilgin et al. (2015) studied different shrimp species by means of sequencing Cox1 marker and confirmed the existence of two different *P. elegans* haplogroups along the coast of Turkey, which supports the theory of the existence of a cryptic species within *P. elegans* proposed by Reuschel et al. (2010). Recently, Deli et al. (2017) examined the genetic structure and biogeographic patterns of both Mediterranean types (type II and type III), analysing all previously generated sequences of *P. elegans* from the Mediterranean (Reuschel et al. 2010; Bilgin et al. 2015) along with new sequences. The results showed that the haplogroup type III prevails in the western Mediterranean, while the type II is mostly restricted to the eastern Mediterranean. Their results also showed a marked latitudinal cline between these two types in the southeastern Mediterranean and along the Italian coast.

Despite the importance of *P. serratus* fishery, its stock status is unknown (Haig et al. 2014). Understanding the spatial structure of this economic and ecological important resource is crucial for implementing effective management strategies. In this regard, Bilgin et al. (2015) pointed out that a single Mediterranean specimen of *P. serratus* from the Turkish coast may be distinct from its Atlantic counterparts. This data, joined with those previously documented for its congeneric and sympatric species *P. elegans*, have motivated our phylogeographic analysis of *P. serratus* based on one nuclear and two mitochondrial genes, which is the first one that delineates a biogeographic barrier for this species. In this way, to delve into population genetics

of this prawn using nuclear markers, our research group also isolated and characterized of 20 polymorphic microsatellite *loci* in *P. serratus* (Perina et al. 2016). Sixteen of these microsatellites were also amplified in *P. elegans* and other two congeneric species, *P. adspersus* and *P. longirostris*.

Recently, *de novo* transcriptome assembly and annotation for *P. serratus* from adult individuals and from a pool of larvae, was performed by high throughput sequencing (Perina et al. 2017), providing a baseline for new and diverse molecular studies.

AIMS AND OBJECTIVES

Within the broad aim to delve into *Palaemon serratus* and *Palaemon elegans* genetic knowledge, the particular objectives of this thesis are:

- To characterise the karyotypes of two prawn species, the common prawn *Palaemon serratus* and the rockpool prawn *Palaemon elegans*, identifying their sex chromosome systems; to accomplish a cytogenetic comparative analysis and to elucidate the main mechanism of chromosome evolution within the genus.

- To assess genetic variability and genetic structure of *Palaemon serratus* throughout its geographical distribution range in order to understand the population genetics of this species, using both nuclear and mitochondrial molecular markers.

- To develop a DNA-based method to authenticate the presence of economically relevant prawn species, including *Palaemon serratus*, in fresh and frozen samples; providing a traceability tool to guarantee the quality of the product and for combating commercial fraud.

RESEARCH ARTICLES

CHAPTER 1.1

Karyological analysis of the shrimp *Palaemon serratus* (Decapoda: Palaemonidae)

Ana M. González-Tizón, Verónica Rojo, Elisabetta Menini, Zeltia Torrecilla, and Andrés Martínez-Lage (2013) Karyological analysis of the shrimp *Palaemon serratus* (Decapoda: Palaemonidae). Journal of Crustacean Biology 33: 843-848.

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KARYOLOGICAL ANALYSIS OF THE SHRIMP *PALAEMON SERRATUS* (DECAPODA: PALAEMONIDAE)

Ana M. González-Tizón [1,*], Verónica Rojo [1], Elisabetta Menini [2], Zeltia Torrecilla [1], and Andrés Martínez-Lage [1]

[1] Department of Cell and Molecular Biology, Faculty of Sciences, University of La Coruña, A. Fraga 10, 15008 La Coruña, Spain
[2] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Vía Brecce Bianche, 60131 Ancona, Italy

ABSTRACT

Analysis of metaphases of *Palaemon serratus* (Pennant, 1777) revealed a diploid number of $2n = 56$ chromosomes with a karyotype composed of two large metacentric pairs, six subtelocentric pairs and twenty telocentric pairs. Four Ag-NORs and 45S rDNA signals were observed at the telomeres of two small telocentric chromosome pairs. Chromomycin A3 fluorochrome staining revealed GC-rich regions associated with Ag-NORs, and DAPI showed positive bright bands on centromeric or intercalary positions of subtelocentric and telocentric chromosomes.

KEY WORDS: Ag-NOR, chromosomes, Crustacea, Decapoda, FISH, *Palaemon serratus*

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INTRODUCTION

Decapoda comprises 233 families containing 2725 genera and an estimated 17 635 species, including both fossil and extant species (for review, see De Grave et al., 2009). Many of these species have commercial importance and are extensively exploited and consumed in different countries around the world. There are numerous studies on their biology, ecology and molecular genetics, but the knowledge of their karyology is very scarce despite their high diversity. This is because mitotic indexes are low, chromosomes are usually small-sized and highly condensed, and chromosome numbers are large. These reasons make difficult to obtain a good quality karyotyping and chromosome banding. However, the knowledge of the karyotype provides very useful information to clarify species status, to identify loci of interest in aquaculture and to provide useful information for conservation programs and sustainable exploitation.

To our knowledge, in the last twenty years (since 1992) karyological data have only been reported in 39 species of decapods (belonging to 23 genera included in 10 families) (Table 1). Most of these studies only provide the chromosome number (existing high variability as numbers range from $2n = 32$ to $2n = 200$), while karyotypes were described in 19 out of these 39 species. Fluorochrome staining and location of nucleolar organizer regions (NORs) have been applied in six species, and molecular cytogenetics (fluorescence *in situ* hybridisation, FISH) in four species. In four species, a variable number of B (supernumerary) chromosomes was described (for review, see Coluccia et al., 2004; Deiana et al., 2007).

The present study was carried out in the shrimp *Palaemon serratus* (Pennant, 1777), which is distributed in the European waters from Denmark to Mauritania, and in the Mediterranean and Black Seas (González-Ortegón and Cuesta, 2006). This species is under commercial exploitation in many countries, reaching high values in market and being an important fishery resource. This shrimp species inhabits rock pools in the intertidal zone and also populates shallow sand bottoms and brown algae belts. *P. serratus* has a cylindrical body composed of carapace at the front and six abdominal segments. It has a large upturned rostrum in front of the eyes with distinctive dorsal and ventral teeth (González-Ortegón and Cuesta, 2006).

In order to increase the knowledge of crustacean cytogenetics and as karyotyping provides useful information in conservation genetics, in genetic breeding programmes, and to develop genetic maps (among other applications) we investigated the chromosome number and morphology, the location of the major ribosomal loci by FISH and silver staining (Ag-NORs), and the location of AT-GC rich regions by means of fluorochrome stainings with Chromomycin A3 (CA3) and 4′,6-diamidino-2 phenyl-indole (DAPI) by first time in the shrimp *P. serratus*.

MATERIALS AND METHODS

Biological Material and Chromosome Preparation

Specimens of *P. serratus* were collected with a fish trap from the Artabro Gulf (43°25′N, 8°20′W, La Coruña, Spain). Once in the laboratory animals were maintained in a fish tank and fed with fish pieces for 24 hours.

Adult shrimps were injected at the epimeral line with 0.005% colchicine solution (5 μl/g body weight) 4-5 hours before anesthetization by exposure

* Corresponding author; e-mail: hakuna@udc.es

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Table 1. Karyological studies in Decapoda from last twenty years. $2n$, diploid chromosome number; FISH 45S, location of major ribosomal loci by FISH; FISH 5S, location of minor ribosomal loci by FISH C, C-banding; Q, Quinacrine staining, DAPI, 4′,6-diamino-2 phenyl-indole staining; CA3, chromomycin A3 staining.

	$2n$	Karyotype	Banding, FISH	Reference	Remarks
Suborder Dendrobrachiata					
Family Penaeidae					
Peneaus monodon	88	8m + 10sm + 5st + 21t		Kumar and Lakra, 1996	
Peneaus indicus	88	27m + 13sm + 4st		Lakra et al., 1997	
Peneaus vannamei	88	4m + 10sm + 56st + 18t	Telomeric FISH	Morelli et al., 1998	
Peneaus californiensis	88	4m + 10sm + 56st + 18t		Campos-Ramos, 1997	
Peneaus semisulcatus	90	24m + 11sm + 1st + 9t		Alcivar-Warren et al., 2006	
Peneaus merguiensis	88	21m − sm + 23st − t		Campos-Ramos, 1997	
Peneaus sculentus	88			Hosseini et al., 2004	
Parapenaeopsis stylifera	74	1m + 18sm + 1st + 17t		Amini and Mansouri, 2010; Mansouri et al., 2011	
Trachypenaeus curviristris	70	21m + 5sm + 6st + 3t		Zhang et al., 2002b	
Metapeneaus ensis	78	20m + 5sm + 7st + 7t		Mukesh and Lakra, 2000	No figures, only idiogram
Metapeneaus affinis	88			Zhou et al., 1999	Only abstract available in English
Fenneropeneaus penicillatus	88			Zhang et al., 2002b	Only abstract available in English
Fenneropeneaus chinensis	88		FISH 5S	Zhang et al., 2002b; Huan et al., 2010	
Farfantepeneaus aztecus	88			Zhang et al., 2002b	
Farfantepeneaus duorarum	88			Zhang et al., 2002b	
Litopeneaus septiferus	90			Zhang et al., 2002b	
Litopeneaus stylirostris	88			Zhang et al., 2002b	
Xiphopeneaus kroyeri	78			Zhang et al., 2002b	
Marsupeneaus japonicus	86			Zhang et al., 2002b	
Family Sicyonidae					
Sicyonia ingentis	64			Zhang et al., 2002b	
Suborder Pleocyemata					
Family Atyidae					
Atyaephyra desmarestii	32	11m + 5sm		Anastasiadou and Leonardos, 2010	
Family Palaemonidae					
Macrobrachium rosenbergui	118			Lakra et al., 1997	
Macrobrachium rosenbergui	104	11m + 26sm + 4st + 11t		Qian et al., 2005	Only abstract available in English
Exopalaemon modestus	90	28m + 4sm + 6st + 7t		Jiang et al., 2008	Only abstract available in English
Palaemon serratus	56	2m + 6sm + 20t	FISH 45S, CA3, DAPI, Ag-NORs	This study	

Table 1. (Continued.)

	2*n*	Karyotype	Banding, FISH	Reference	Remarks
Family Nephropidae					
Nephrops norvegicus	131-140	m, sm, t (no number)	C-, Q-, Ag-NOR	Deiana et al., 1996	
Homarus americanus	136 (mode)	m, sm, t (no number)	C-, DAPI, CA3	Coluccia et al., 2001	
Family Astacidae					
Astacus astacus	176	52m + 35sm + 1t	FISH 45S, DAPI	Mlinarec et al., 2011	
Astacus leptodactylus	180		FISH 45S, DAPI	Mlinarec et al., 2011	
Astacus fluviatis	116			Lecher et al., 1995	
Austropotamobius torrentium	164			Pavlica et al., 2008	
Family Cambaridae					
Procambarus llamasi	120	All telocentrics		Indy et al., 2010	
Procambarus digueti	102	35M + 15m + 1st		Diupotex Chong et al., 1997	
Family Parastacidae					
Cherax destructor	188	70m + 42sm + 48st + 28t		Scalici et al., 2010	
Cherax quadricarinatus	200	33m + 25sm + 14st + 28t		Tan et al., 2004	
Family Palinuridae					
Palinurus elephas	138-150		Ag-NORs, FISH, 45S rDNA, CA3	Salvadori et al., 1995; Coluccia et al., 2006	
Palinurus mauritanicus	113-130			Coluccia et al., 2003	
Family Scyllaridae					
Scyllarus arctus	70			Salvadori et al., 1992	
Scyllarides latus	126			Salvadori et al., 1992	

to ethyl ether. After removing the carapaces, we collected thorax tissue, which we hypotonized in 0.56% KCl solution for 10 minutes and fixed three times in freshly prepared ethanol/glacial acetic acid fixative (3:1) at 4°C. Finally, a piece of about 3 mm of fixed tissue was placed into an microtube containing 45% acetic acid solution and minced with the aid of a microtube pestle. The cell suspension was then dropped onto slides heated to 43°C and air-dried. Metaphases were stained with 4% Giemsa in phosphate buffer pH 6.8.

Chromosome Staining and Fluorescence *in situ* Hybridisation

CA3 and DAPI staining were applied following Schweizer (1976, 1980), and Ag-NOR staining was performed as described by Howell and Black (1980).

Chromosomal location of 45S rDNA loci was carried out by FISH as described in González-Tizón et al. (2000), using the DNA probe pDm 238 from *Drosophila melanogaster* (Roiha et al., 1981) labelled by nick translation with digoxigenin-11-dUTP (Roche). Briefly, the slides were pretreated with DNAse-free RNAse (100 μg/ml in 2 × SSC) for 1 h at 37°C, incubated in pepsin (10% in 100 mM HCl) for 10 min at 37°C, post-fixed in formaldehyde (1% in PBS 50 mM) for 10 minutes, washed in 2 × SSC for 10 minutes and finally dehydrated in a graded ethanol series and air-dried.

A hundred ng of labelled probe were made up to 30 μl with hybridization buffer (50% formamide, 2 × SSC and 10% dextran sulphate), predenatured at 75°C for 15 minutes, chilled on ice, and placed on a slide under a sealed coverslip. Gradual denaturation and annealing of chromosomal DNA was done in a slide-thermal cycler as follows: 7 minutes at 75°C, 2 minutes at 55°C, 30 seconds at 50°C, 1 minute at 45°C, 2 minutes at 42°C, 5 minutes at 40°C, 5 minutes at 38°C, and 5 minutes at 37°C. Finally, we incubated the slides overnight in a moist chamber at 37°C. Post-hybridization washes consisted of two 5-minute incubations in 2 × SSC at 37°C and at room temperature, respectively, followed by a 5-minute incubation in 0.1 M Tris, 0.15 M NaCl, 0.05% Tween-20.

Signal detection was carried out with mouse anti-digoxigenin antibody, FITC-rabbit anti-mouse IgG (Sigma) and FITC-goat anti-rabbit IgG (Sigma-Aldrich). Chromosomes were counterstained with DAPI (50 ng/ml antifade) and visualized and photographed using a Nikon Microphot-FXA microscope equipped with a NIS-Elements D 3.10 software and a digital camera DS-Qi1Mc.

Results

The analysis of 62 metaphase plates obtained from four males and five females showed a modal number of 56 chromosomes (20 metaphases), which states a diploid number of $2n = 56$ chromosomes (Fig. 1a). The karyotype consisted of two clearly identifiable large metacentric pairs, six subtelocentric, and twenty telocentric pairs, all of them hardly distinguishable due to size similarities.

The Ag-NORs were located at the telomeres of four small telocentric chromosomes (Fig. 1b). From the analysis of 46 metaphases obtained from two females and two females, 4 metaphases showed one Ag-NOR (4.35%), 20 showed two Ag-NOR (43.48%), 15 showed three signals (32.61%), and 9 showed four Ag-NORs (19.56%). The signals revealed by FISH confirmed the positions of the ribosomal loci on the four small telocentrics (Fig. 1c). The CA3 positive bands were also visualized in the terminal regions on four small telocentrics (Fig. 1d). DAPI staining showed bright fluorescence at centromeres of the two large metacentrics when these chromosomes are highly condensed (Fig. 1e). However, when chromosomes are decondensed,

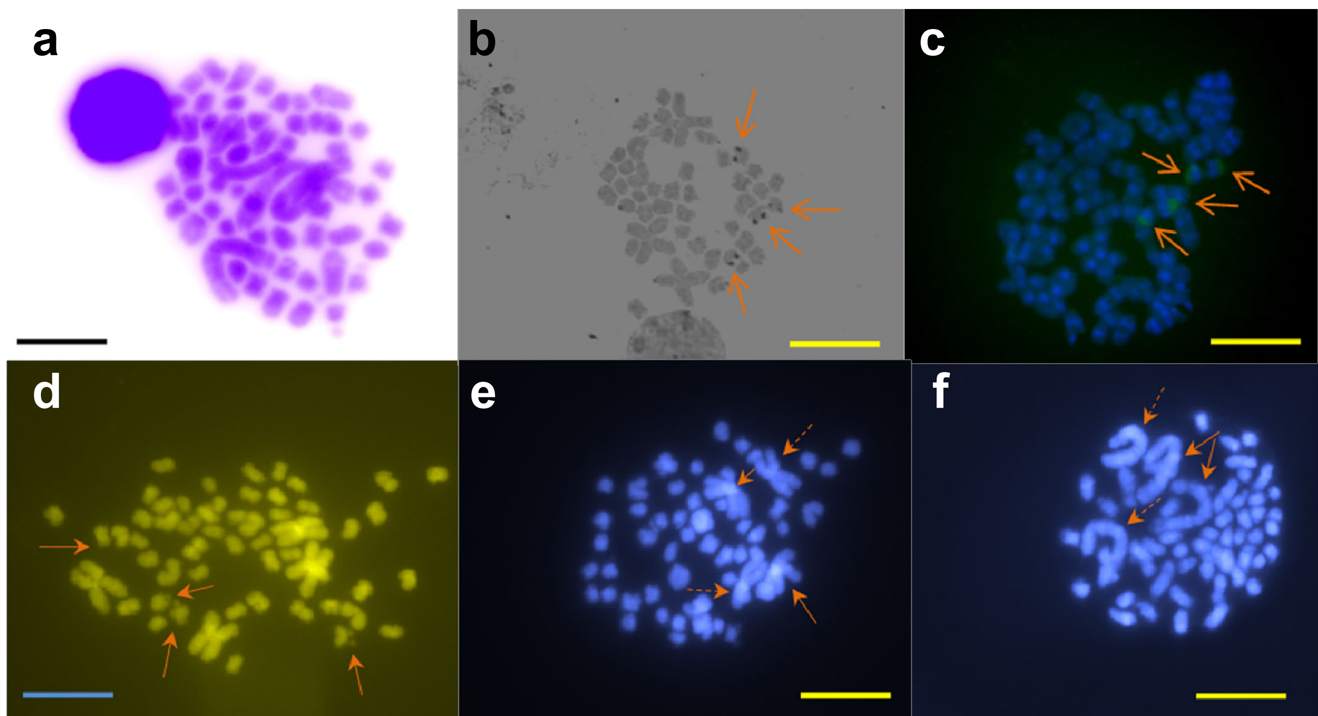

Fig. 1. A, Giemsa stained metaphase chromosomes of *Palaemon serratus* ($2n = 56$) showing two large metacentric pairs, six subtelocentric, and twenty telocentric pairs which are very similar in size; B, Ag-stained metaphase plate; arrows show the location of the Ag-NORs (which were active in the precedent interphase); C, chromosomal mapping of the 45S ribosomal using FISH; arrows point to hybridization signals at the ends of two small chromosome pairs; D, GC-rich regions revealed with Chromomycin A3 (arrows); E, AT-rich regions after DAPI staining; when chromosomes are highly condensed only the centromeric regions of chromosome pairs nos. 1 (solid arrows) and 2 (dotted arrows) show bright signals; F, AT-rich regions revealed with DAPI staining on low-condensed chromosomes; the centromeric signals appear less intense than on condensed chromosomes, but in contrast longitudinal banding is observed on chromosomes nos. 1 and 2 and on other chromosomes of the complement. Scale bar = 10 μm.

some intercalar bands can be visualized on chromosomes 1 and 2, as well as other DAPI positive bands on centromeric or intercalar positions of subtelocentric and telocentric chromosomes (Fig. 1f).

Discussion

The knowledge of the cytogenetics of Decapoda is very poor and generally restricted to the estimation of the chromosome number, and, in the last twenty years the use of different chromosome banding and FISH techniques did not have much progress in comparison with other animal taxa. Hence, the location of major ribosomal loci (45S rDNA) was described in *Astacus astacus* and *A. leptodactylus* (Mlinarec et al., 2011) and in *Palinurus elephas* (Coluccia et al., 2006), the location of minor ribosomal loci (5S rDNA) in *Fenneropenaeus chinensis* (Huan et al., 2010), and the location of telomeric sequences in *Penaeus vannamei* (Alcivar-Warren et al., 2006). Other banding techniques such as Ag-staining and fluorochrome staining were applied in *Nephrops norvegicus* (Deiana et al., 1996), *Homarus americanus* (Coluccia et al., 2001), *A. astacus* and *A. leptodactylus* (Mlinarec et al., 2011) and *P. elephas* (Salvadori et al., 1995; Coluccia et al., 2006). These six species belongs to the families Penaeidae (*P. vannamei*), Nephropidae (*N. norvegicus* and *H. americanus*), Astacidae (*A. astacus* and *A. leptodactylus*) and Palinuridae (*P. elephas*).

Among Palaemonidae, three species have been studied until now: *Macrobrachium rosenbergui* (Lakra et al., 1997, only data on chromosome number), *M. nipponense* (Qian et al., 2005, data on chromosome number and karyotype) and *Exopalaemon modestus* (Jiang et al., 2008, data on chromosome number and karyotype).

The present study contributes to increase the cytogenetic data in Palaemonidae and in Decapoda in general. The species we studied, *P. serratus*, shows a modal diploid chromosome number of $2n = 56$, the lowest number in Palaemonidae analyzed to date, as *E. modestus* has $2n = 90$, *M. nipponnense* $2n = 104$ and *M. rosenbergi* $2n = 118$. These data support the molecular taxonomy and phylogenetics of palaemonid shrimp (concerning to the genera *Macrobrachium* and *Palaemon*), which state that major lineages within Palaemonidae are paraphyletic (Cuesta et al., 2012).

The application of Ag-NOR banding and FISH of major ribosomal loci (45S rDNA) revealed that *P. serratus* presents two ribosomal loci (four signals) at terminal position on four small subtelocentric chromosomes. The number of signals in *P. serratus* is lower than that in *P. elephas* (multiple Ag-NORs and five rDNA loci) and *N. norvegicus* (5-8 Ag-positive regions) (Coluccia et al., 2006; Deiana et al., 1996, respectively). The staining with CA3 produced bright fluorescence signals which are probably coincident with FISH signals (as happens in many other species), showing a GC-richness of these regions. No variability in the number of signals after FISH was observed. Additionally, AT-rich areas appeared distributed on the centromeric regions of the two large metacentric chromosomes and at intercalary positions in a great number of subtelocentric and telocentric chromosomes.

In conclusion, this work provides information on the chromosome number, karyotype and chromosome banding in *P. serratus* and contributes to the increase of cytogenetic studies in Crustacea. Although further studies are required in this field, the cytogenetic analyses, altogether with molecular studies, are useful to investigate systematic relationships among members of Palaemonidae.

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CHAPTER 1.2

Comparative cytogenetic analysis of marine *Palaemon* species reveals a $X_1X_1X_2X_2$/ X_1X_2Y sex chromosome system in *Palaemon elegans*

Zeltia Torrecilla, Andrés Martínez-Lage, Alejandra Perina, Enrique González-Ortegón, and Ana M. González-Tizón (2017) Comparative cytogenetic analysis of marine *Palaemon* species reveals a $X_1X_1X_2X_2$/ X_1X_2Y sex chromosome system in *Palaemon elegans*. Frontiers in Zoology 14:47.

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Comparative cytogenetic analysis of marine *Palaemon* species reveals a $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in *Palaemon elegans*

Zeltia Torrecilla[1], Andrés Martínez-Lage[1], Alejandra Perina[1], Enrique González-Ortegón[2,3] and Ana M. González-Tizón[1*]

Abstract

Background: The maintenance of species and the promotion of speciation are closely related to chromosomal rearrangements throughout evolution. Decapoda represents the most species-rich order among crustaceans and, despite its ecological and economic importance, little is known about decapod karyology. We aim at cytogenetically characterizing two sympatric prawn species.

Results: Analysis of mitotic metaphases and meiotic diakinesis of the common prawn *Palaemon serratus* and the rockpool prawn *P. elegans*, revealed considerable differences between their karyotypes including chromosome numbers and sex determination systems. The cytogenetic data for *P. serratus* showed a diploid number of 56 and the putative absence of heteromorphic sex chromosomes. However, the diploid chromosome number in *P. elegans* was 90 for females and 89 for males. The karyotype of the females consisted of the three largest acrocentric pairs and 42 submetacentric and metacentric pairs, while the karyotype of the males comprised a clearly identifiable large metacentric chromosome and two acrocentric pairs as well as the smaller 42 pairs. These results highlight the presence of the $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system in *P. elegans*, which constitute the only sexual system for Decapoda reported cytogenetically using modern techniques. The origin of this sex chromosome system is discussed. We hypothesize that the chromosome evolution within the genus could involve several fusion events giving rise to a reduction on the chromosome number in *P. serratus*. In both species, the major ribosomal genes were located in two chromosome pairs and hybridization signals of the telomeric sequences $(TTAGGG)_n$ were visualized at the telomeres of all chromosomes. C-banding revealed that, when present, constitutive heterochromatin had a predominantly telomeric distribution and no centromeric constitutive heterochromatin was observed.

Conclusions: Although more comparative cytogenetic analyses are needed to clarify our hypotheses, the findings of this work indicate that the prawns of the genus *Palaemon* represent a promising model among Decapoda representatives to investigate the karyotype evolution and the patterns of sex chromosome differentiation.

Keywords: Comparative cytogenetics, Decapoda, FISH, Karyotype, *Palaemon elegans*, *Palaemon serratus*, Sex chromosomes

* Correspondence: hakuna@udc.es
[1]Grupo de Investigación en Biología Evolutiva (GIBE), Centro de Investigaciones Científicas Avanzadas (CICA), Departamento de Biología, Facultad de Ciencias, Universidade da Coruña, Campus A Zapateira, 15071 A Coruña, Spain
Full list of author information is available at the end of the article

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Background

Decapoda is the most species-rich order within Crustacea. This extremely diverse group plays a key role in the aquatic trophic relationships [1, 2] and many of these species have a significant commercial importance since they are exploited for human consumption in different countries around the world [3, 4]. However, despite the importance of this group, the limited knowledge of decapod crustacean karyology constitutes an obstacle to elucidate different modes of sex determination, the occurrence of chromosomal rearrangements along their evolution or clarify phylogenetic relationships between related species. To our knowledge, during the last 25 years karyological data have only been reported in 46 species of decapods belonging to 10 families (for a review, see [5]). This scarcity of studies is mostly caused by decapod chromosomes peculiarities, usually small-size, numerous and highly condensed [6].

The family Palaemonidae comprises 981 species [7] of which only 13 belonging to three genera (*Palaemon*, *Exopalaemon* and *Macrobrachium*) have been studied at the cytogenetic level. These species show a wide karyotypic diversity and remarkable differences in their diploid chromosome number (Table 1). The existence of sex chromosomes was never determined cytogenetically in any species of the genera of Palaemonidae family and only rarely in Decapoda.

The genus *Palaemon* Weber, 1795 (Crustacea: Decapoda) is a group of caridean prawns of the family Palaemonidae. Recently, phylogenetic and taxonomic revisions changed the status of the genus *Palaemon* [8–11] as well as the number of its species. The genus *Palaemon* currently comprises 86 species, two of which have been recently described (*Palaemon minos sp. nov*. and *Palaemon colossus sp. nov.*) [10].

Table 1 Chromosome numbers in the members of the family Palaemonidae

Species	Chromosome number	Reference
Palaemon serratus	2n = 56	[5]
Palaemon khori	2n = 96	[36]
Palaemon elegans	2n = 89♂/90♀	This study
Exopalaemon modestus	2n = 90	[34]
Exopalaemon carinicauda	2n = 90	[35]
Macrobrachium carcinus	2n = 94	[59]
Macrobrachium superbum	2n = 100	[60]
Macrobrachium siwalikensis	2n = 100	[61]
Macrobrachium nipponense	2n = 104	[62]
Macrobrachium idella	2n = 104	[63]
Macrobrachium scabriculum	2n = 104	[64]
Macrobrachium lamarrei	2n = 118	[65]
Macrobrachium rosenbergii	2n = 118	[65]
Macrobrachium villosimanus	2n = 124	[66]

The selected species, the common prawn *P. serratus* and the rockpool prawn *P. elegans*, have a wide geographical distribution from the North Sea to Mauritania and Namibia, respectively, including the Mediterranean and Black Seas [12, 13]. These species differ in physiology, life history strategies and larval development [14–16]. They are both marine prawns, but whereas *P. serratus* inhabits estuaries in the reproductive season, *P. elegans* is common in tidal rockpools, *Zostera*, *Posidonia* and *Cymodocea* meadows and it also can be found in slightly brackish water close to river mouths [17].

Whilst the species are morphologically similar, it is unknown whether they share chromosome number and morphology. The karyotype of *P. serratus* was recently described. In our previous study, the karyotype of *P. serratus* was described [5].

Here, we aim at: (i) extending the previous knowledge on the cytogenetics of *P. serratus*; (ii) providing the first karyological data for *P. elegans* and compare them with what is known about *P. serratus* and (iii) identifying their sex chromosome systems. For this purpose we have studied the mitotic and meiotic chromosomes of both species and applied conventional staining and banding techniques, fluorescence in situ hybridization (FISH) with 18S–5.8S-28S rDNA and telomeric $(TTAGGG)_n$, $(TTAGG)_n$ and $(TAACC)_n$ probes.

Methods

Biological material and chromosome preparation

Specimens of *P. serratus* and *P. elegans* used in this study were collected from the Artabro Gulf (43° 25′N, 8° 20′W) in the northwest of Spain. Animals were captured with a fish trap and carried alive to the laboratory. Animals were kept at 18 °C in an aerated aquarium and fed with frozen brine shrimp for 24 h. Individuals were sorted into species [13] and the sex was determined by the presence (in males) or absence (in females) of the masculine appendix on the endopodite of the second pleopod [18]. Metaphase chromosome spreads were obtained according to previously described protocol [5]. Briefly, adult shrimps were injected at the epimeral line with 0.005% colchicine solution (5 μl/g body weight) 3–5 h before anesthetization by exposure to ethyl ether. Cefalothorax content (including gonad, circulatory tissue, digestive tissue and muscular tissue) was removed from each individual and then immersed into a hypotonic solution of 0.56% KCl for 10 min at room temperature. The tissue was then fixed four times in freshly prepared ethanol/glacial acetic acid (3:1) for 20 min each time at 4 °C, followed by overnight incubation in a fresh fixative at 4 °C. The following day a piece of about 3 mm of the heterogeneous fixed material was

dissolved in 45% acetic acid and a cell suspension was obtained. Then, 4–5 drops of this suspension were pipetted onto pre-heated slides at 43 °C and air-dried.

Chromosome staining and fluorescence in situ hybridization

The slides were stained with anti-fade medium Vectashield (Vector Laboratories) containing 1.5 μL/mL 4′, 6-diamidino-2-phenylindole (DAPI). C-banding was performed on metaphase plates following Sumner [19].

To locate the position and number of the 18S–5.8S-28S rDNA sites we used the DNA probe pDm 238 from *Drosophila melanogaster* [20] labeled with FITC by using Prime-It Fluor fluorescence labeling kit (Stratagene) following the manufacturer's instructions.

Chromosome mapping of the telomeric sequences was carried out using a (TTAGGG)n Cy3-labeled pan-telomeric probe (Cambio) according to the instructions of the manufacturer; a PCR generated pentanucleotide (TTAGG)n repeat according to Ijdo et al. [21] labeled with rhodamine-dUTP and the $(TAACC)_2$ probe was synthesized and directly 5′ labeled with Cy3 (Isogen Life Science).

In situ hybridization was performed as described in González-Tizón et al. [22] with minor pre-hybridization and post-hybridization modifications. The slides were pretreated with DNAse-free RNAse (100 μg/mL in 2 x SSC) for 30 min at 37 °C, washed in 2 x SSC for 5 min and dehydrated in a graded ethanol series. Post-hybridization washes consisted of two 5-min incubations in 2 × SSC at 37 °C and at room temperature, respectively, followed by a 5-min incubation wash in 0.1 M Tris, 0.15 M NaCl and 0.05% Tween-20 at room temperature. Chromosomes were counterstained with 40 μL of anti-fade medium Vectashield containing 1.5 μL/mL DAPI.

Images were captured using a Nikon Microphot-FXA epifluorescence microscope equipped with a Nikon DS-Qi1Mc digital camera and processed with the NIS-Elements D 3.10 software.

The cytogenetic analyses described above were performed on *P. serratus* and *P. elegans* with the exception of the 45S rDNA chromosomal location in *P. serratus*, characterized in a previous work [5].

Results

Karyotypes, heterochromatin distribution and Fluorochrome staining

Mitotic and meiotic metaphases were obtained from 18 *P. elegans* specimens (8 females and 10 males) and 10 *P. serratus* specimens (6 females and 4 males). At least 15 metaphases per individual were observed, specifically 126 in *P. elegans* females, 153 in *P. elegans* males, 92 in *P. serratus* females and 31 in *P. serratus* males.

The diploid chromosome number in *P. elegans* was 90 for females and 89 for males (Fig. 1a, b; Table 1). The karyotype consisted of 43 autosomal chromosome pairs: 5 metacentric/submetacentric, 4 subtelocentric/telocentric, and 34 hardly distinguishable due to size similarities (Fig. 2). The karyotype of the females also included two large telocentric sex chromosome pairs (Fig. 2a), while that of the males included one clearly identifiable large metacentric chromosome and two telocentric chromosomes (Fig. 2b). Thus, male heterogamety is evidenced by a metacentric chromosome present only in the male karyotype (Y chromosome) which is the largest element of the complement. During meiotic diakinesis, each arm of the large metacentric Y is terminally associated with one acrocentric chromosome (X_1 and X_2) forming a trivalent (X_1X_2Y, Fig. 1d). Therefore, in diakinetic plates males exhibited 43 autosomal bivalents and one sex trivalent while females showed 45 undistinguished bivalents (Fig.1d, c).

In *P. serratus*, the karyotype was identical to that previously described (2n = 56) [5]. At meiotic diakinesis 28 bivalents in both sexes were observed (Fig. 1e).

Fluorochrome staining with DAPI revealed bright centromeric/pericentromeric AT-rich blocks on all chromosomes in *P. elegans* and *P. serratus* (Fig. 1) whereas interstitial bands were observed on the four largest chromosomes of *P. serratus*. In *P. elegans* chromosomes DAPI-bands were noticed in some terminal regions, always weaker than those found at the centromeres. We also detected large telomeric DAPI faint segments in a few chromosomes.

C-banding revealed that, when present, constitutive heterochromatin had a predominantly telomeric distribution in both species of *Palaemon* (Fig. 3a, b). Furthermore, no centromeric constitutive heterochromatin was observed. A large heterochromatic block was also found in the telomeres of four small size chromosomes in both species. In *P. elegans* the two X chromosomes and the Y chromosome were C-negative (Fig. 3a). In *P. serratus*, small weak bands of heterochromatin were also localized in interstitial positions of the large metacentric chromosomes. Slides containing C-banded chromosomes were previously stained with DAPI (Fig. 3c, d).

Chromosomal mapping of the 18S–5.8S-28S rDNA genes

In situ hybridization of the 18S–5.8S-28S rDNA genes on meiotic chromosomes of both sexes of *P. elegans* revealed four sites of probe hybridization (Fig. 3e, f). The rDNA probe mapped the free telomeres of two bivalents paired at one end (dumbbell-shape bivalents). Both 18S–5.8S-28S rDNA-bearing chromosome pairs were heteromorphic showing different hybridization intensity of the homologous chromosomes. FISH signals coincided with the heterochromatic blocks observed.

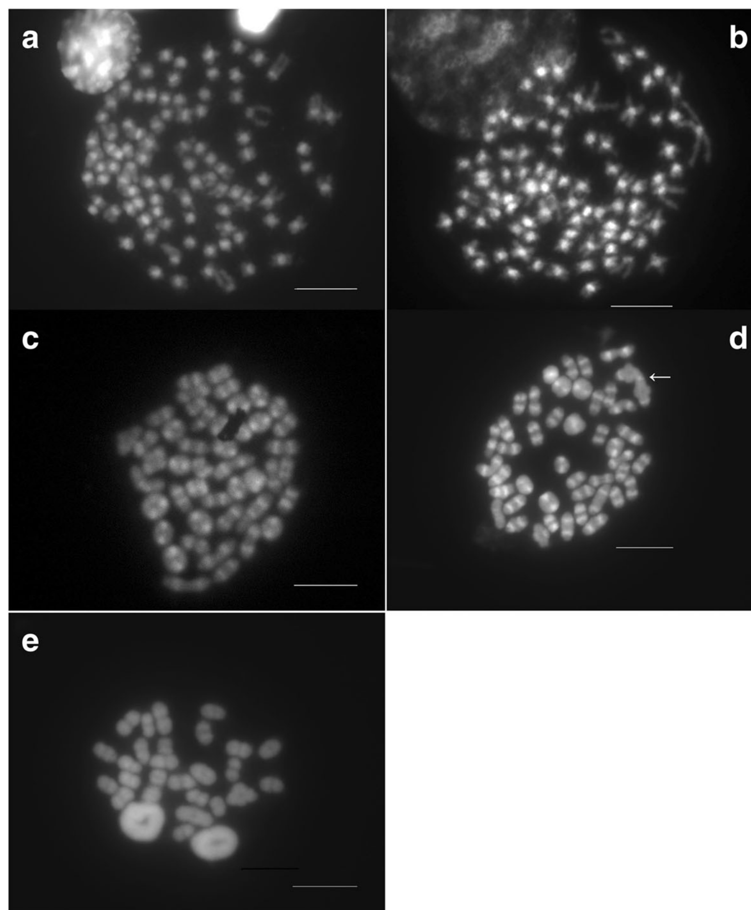

Fig. 1 Metaphase plates of *P. elegans* (**a**) female and (**b**) male. Meiotic diakinesis of *P. elegans* (**c**) female and (**d**) male; the arrow shows the sex trivalent. (**e**) Meiotic diakinesis of *P. serratus* male. The bar equals 10 μm

Chromosomal location of the telomeric probes

In situ hybridization of the $(TTAGGG)_n$, $(TTAGG)_n$ and the $(TAACC)_n$ telomeric sequences were made in *P. serratus* and *P. elegans*. No hybridization signals were detected with the $(TTAGGG)_n$ or the $(TAACC)_n$ probes while FISH with the $(TTAGG)_n$ pentanucleotide repeat produced discrete fluorescence signals at the telomeres of all chromosomes in *P. serratus* and in all the diakinetic bivalents in *P. elegans* (Fig. 3g, h).

Discussion

Chromosome number and karyotypes

The diploid chromosome number obtained in this study for *P. elegans* falls within the range of the published chromosome numbers in other members of the family Palaemonidae, with *P. serratus* displaying the lowest number in the family (2n = 56).

The lack of cytogenetic studies in other members of the genus *Palaemon* hinders the definition of clear trends in karyotype evolution in these species. However, some evidence supports the hypothesis that the chromosome evolution within the genus could involve several fusion events giving rise to a reduction on the chromosome number in *P. serratus*: i) We observed interstitial DAPI-bright bands on the large metacentric chromosomes of *P. serratus*, being DAPI-positive bands that are characteristic of centromeric regions in both *Palaemon* species, as observed in other families of decapods such as Astacidae [23, 24], Cambaridae [25], Nephropidae [26], Scyllaridae [27] and Palinuridae [28]. ii) The presence of interstitial C-bands on these chromosomes may represent a chromosome fusion event. In general, decapod species on which this technique has been performed to date showed positive C-bands at the centromeres of almost all chromosomes (e.g. [29–32]), with the only exception of *P. serratus* and *P. elegans* wherein heterochromatin is located, mainly, in the telomeres. Macgregor and Sessions [29] postulated that the heterochromatin expansion is originated in the centromeres and then is dispersed towards the telomeres. Hence, according to this theory, dispersed distributions of heterochromatin (interstitial or telomeric) have an older phylogenetic status. Iii) Recent

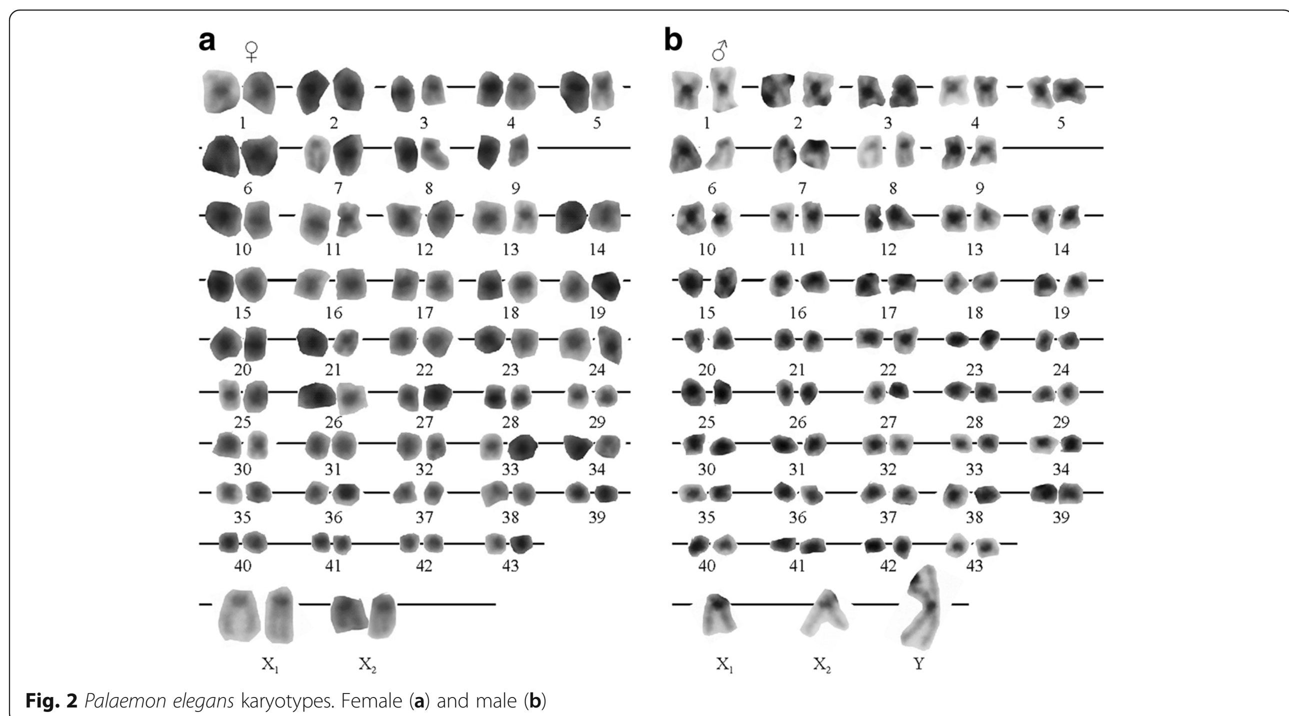

Fig. 2 *Palaemon elegans* karyotypes. Female (**a**) and male (**b**)

molecular phylogenetic studies have suggested that genus *Exopalaemon* should be included within *Palaemon* [8, 33]. Among *Exopalaemon*, karyological analysis of *E. modestus* and *E. carinicauda*, have shown a diploid chromosome number of 90 [34, 35]. More recently, the determination of the *Palaemon khori* karyotype was performed showing 2n = 96 [36].

In the light of our results, the phylogeny and the chromosome numbers found in the family Palaemonidae (Table 1), it seems likely that the high chromosome number detected represents the ancestral condition in this lineage whereas the reduced chromosome number of 2n = 56 observed in *P. serratus* constitutes a derived character. According to that, it seems plausible that the fusions constitute the main mechanism responsible for the origin of the *P. serratus* karyotype, which was also suggested for Astacidae and Parastacidae among Decapoda [24]. Further cytogenetic studies are still necessary in order to determine the mechanisms underlying the karyotype evolution in this group of species.

Ribosomal loci

As previously reported in *P. serratus* [5], *P. elegans* revealed four sites of 18S–5.8S-28S rDNA probe hybridization corresponding to two loci. Given the divergence observed between both karyotypes, this may constitute a plesiomorphic condition for genus *Palaemon*. In all cases, the ribosomal clusters were located in terminal positions on two small chromosome pairs. In addition, conspicuous heterochromatin blocks were located in the major ribosomal genes sites, closely related to large telomeric DAPI faint segments, highlighting the rDNA GC-richness as reported for a wide variety of organisms (e.g. [37] and references therein).

Moreover, in *P. elegans* both rDNA-bearing chromosome pairs showed heteromorphism in size of the 18S–5.8S-28S rDNA locus between homologous as observed in males of some species of the Astacidae [23, 24]. Mlinarec et al. [24] have speculated from these findings that the heteromorphic chromosome pair could represent male sex chromosomes suggesting the presence of an XX-XY sex determination system, even though the karyological characterization of females is a pending issue. Conversely, our results show that in *P. elegans* the heteromorphic rDNA-bearing chromosome pairs correspond to autosomes, which have been reported for many animal groups (e.g. [38–41]).

Telomeric repeats

This study shows for the first time the presence of the TTAGG repeat, known as the ancestral motif of arthropod telomeres, in the family Palaemonidae [42]. Since the presence of this repeat has not been demonstrated in most decapod families, it is interesting to confirm the constant presence of this motif within Decapoda, particularly when some animal groups have lost the TTAGG repeat during their evolution such as the crustacean species *Asellus aquaticus* (Isopoda) [43].

FISH with the (TTAGGG)n probe found in all vertebrates [44] and the (TAACC)n probe identified in the

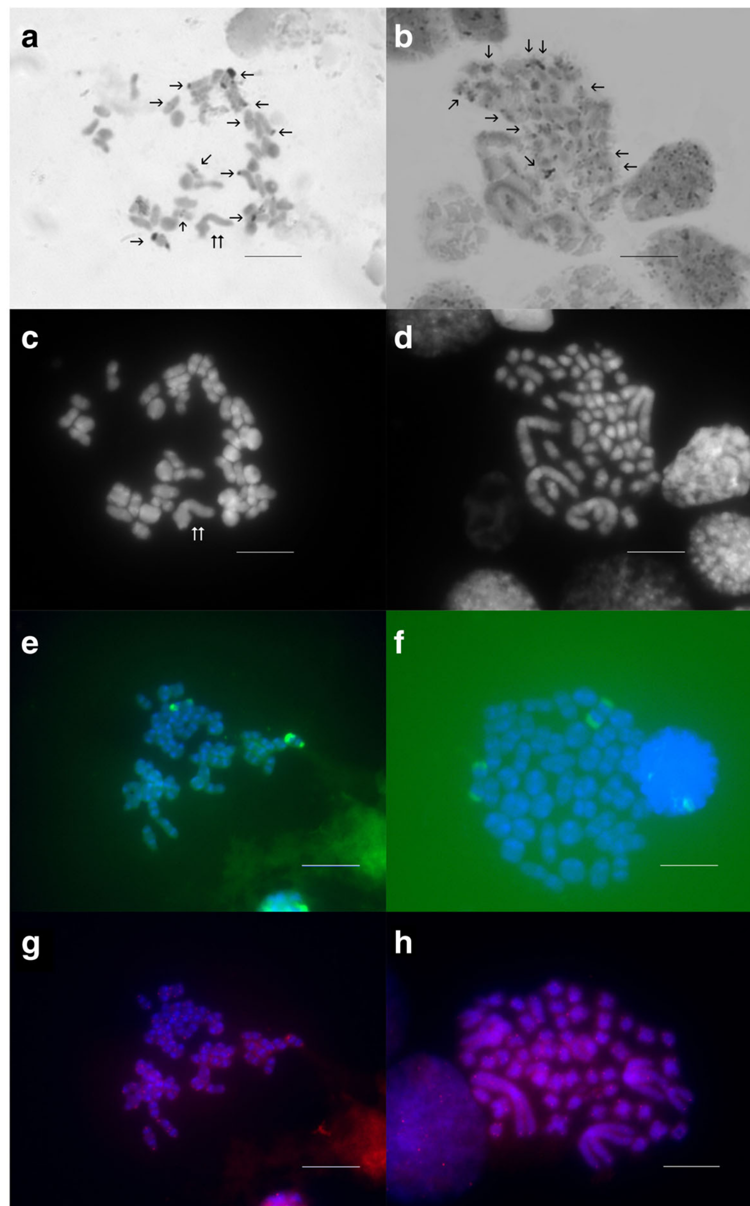

Fig. 3 C-banded plates of (**a**) meiotic diakinesis of *P. elegans* and (**b**) mitotic metaphase of *P. serratus* males. Single arrows show C-band blocks, double arrow shows the sex trivalent (**c**, **d**) The same meiotic diakinesis of *P. elegans* and mitotic metaphase of *P. serratus* males, stained with DAPI. Chromosomal localization of the 18S–5.8S-28S rDNA genes of (**e**) *P. elegans* male and (**f**) female. Chromosomal localization of the (TTAGG)n telomeric sequences in (**g**) *P. elegans* male and (**h**) *P. serratus* male. Asterisks in *a* and *c* indicate the sex trivalent. The bar equals 10 μm

shrimp *Penaeus vannamei* [45] gave no hybridization signals. On the contrary, in both *P. serratus* and *P. elegans*, the hybridization signals of the (TTAGG)n probe were located at the telomeres of all chromosomes. Nonetheless, no interstitial telomeric signals were found as evidence of structural reorganizations occurring throughout chromosomal evolution. However, the fusion sites of ancestral chromosomes do not always preserve the telomeric sequences, and when retained these non-functional repeats could undergo a progressive degeneration or reduction [46], that could impede their detection by FISH.

Sex chromosomes

The comparative analysis between the karyotypes of both sexes of *P. elegans* in addition to their meiotic behaviour showed a heteromorphism between males and females, which is compatible with the presence of an $X_1X_1X_2X_2$/X_1X_2Y sex chromosome system, in which the Y chromosome would correspond to the large metacentric

chromosome exclusive to males, and the X_1 and X_2 chromosomes would correspond to two of the largest acrocentric chromosomes of the complement. According to this system females of *P. elegans* have 2n = 90 (86 + $X_1X_1X_2X_2$) whereas males have 2n = 89 (86 + X_1X_2Y).

Interestingly, the C-banding technique revealed a lack of constitutive heterochromatin in the sex chromosomes, not even in the Y chromosome which also turned out to be remarkably large.

Typically, during the evolution of sex chromosomes from autosomes, the reduction of recombination between the sex-determining regions is the first step to produce simple sex chromosome systems (XY or ZW). Then, the differential accumulation of repetitive sequences and deleterious mutations favour the heteromorphism between the X and Y (or Z and W), either in size, morphology or through banding techniques [47], and the recombination is kept in the pseudoautosomal regions of the sex chromosomes. In regard to the multiple sex chromosome systems, the initial stage of differentiation seems to be associated with chromosomal rearrangements between the chromosomes bearing sex-determining genes and an autosome (e.g. [48–50]). Consequently, due to rearrangements, even newly evolved sex chromosomes can be heteromorphic [51] and not necessarily involve heterochromatin increase [49]. These considerations may explain the existence of meiotic recombination between the *P. elegans* X and Y chromosomes, the lack of heterochromatin in them and the size of the euchromatic Y chromosome; indicating the possibility that the multiple sex chromosome system in this prawn species is a result of recent evolution. In light of this possibility, and bearing in mind male and female karyotypes and their meiotic behaviour, the initial step of sex chromosome differentiation in this species could be a centric fusion between two nonhomologous acrocentric chromosomes, forming the large metacentric neo-Y and leading to two acrocentric chromosomes without homologous in males (neo-X_1 and X_2 chromosomes). Accordingly, during meiosis, the recently formed neo-Y would pair with the neo- X_1 at one end and with the neo-X_2 at the other end, which would lead to the formation of a trivalent such as we observed.

In neither this nor our previous report [5], did we identify sex chromosomes in *P. serratus*. Also, we did not find differences in the constitutive heterochromatin pattern between sexes. Even so, the results demonstrated that the sex chromosome systems of both congeneric species are different since mitotic and meiotic metaphases displayed the same chromosome number in both *P. serratus* males and females, making a multiple sex determination system impossible in that species. In this regard, future studies involving comparative genomic hybridization would be helpful in investigating the putative absence of heteromorphic sex chromosomes in detail in the aforementioned species.

A review of the literature suggests that the multiple sex chromosome system $X_1X_1X_2X_2/X_1X_2Y$ found in *P. elegans* may be unprecedented among decapods with the exception of *Cervimunida princeps* [52]. However, without additional studies using current techniques, the *C. princeps* sex determination system formulated in 1959 is questionable considering that it was based on male chromosome number (2n = 109) and the presence of three univalents at meiotic metaphase I, observations that could correspond for instance to an XX/XY_1Y_2 system.

The present data show the first karyotype with distinguishable heteromorphic sex chromosomes within the family Palaemonidae, where a ZZ/ZW sex chromosome system had been suggested for *Macrobrachium rosenbergii*, in which it is believed that the female is the heterogametic sex on the basis of molecular studies [53]. In fact, the ZZ/ZW sex-determining mechanism was never determined cytogenetically in any member of Decapoda although its existence has also been inferred in the crayfish species *Cherax quadricarinatus* (infraorder Astacidea) [54] and some penaeid shrimps (for a review, see [55, 56]). In contrast, male crabs (infraorder Brachiura) are reported to be the heterogametic sex based on their karyotype, with an XX/XY sex chromosome system and even an XX/XO system being observed (see the reviews [6, 57]). Notwithstanding, due to the inherent limitations of the techniques used at the time, we should be cautious as to the reliability of these studies. Recently, the ZZ/ZW sex determination system was proposed for the Chinese mitten crab *Eriocheir sinensis* (infraorder Brachiura) based on QTL mapping and confirmed by triploid induction experiments [58].

Our results on *Palaemon* sex determination systems and our bibliographic review reveal a large variability within Decapoda. They also show the difficulty of identifying sex chromosomes in this order using cytogenetic methods. The absence of heterochromatic blocks in the sex chromosomes in *P. elegans* could be a widespread characteristic in decapods. Besides, the high chromosome number and their small and homogenous size complicate the identification of sex chromosome pairs, especially if the meiotic stage, where the homologous are connected and the chromatin more condensed, is not analyzed.

Conclusions

This and our previous study [5] show that the congeners *P. serratus* and *P. elegans* present a high degree of diversity in their chromosome number, karyotype and sex determination system, ranging from the putative absence of heteromorphic sex chromosomes to the multiple chromosome system ($X_1X_1X_2X_2/X_1X_2Y$). Such variability, even

between species so closely related, makes this genus a promising model among Decapoda to investigate not only the karyotype evolution but also the patterns of sex chromosome differentiation.

In this perspective, future comparative cytogenetic analyses comprising other *Palaemon* species are needed to clarify the hypothesis developed in this work where fusions events would constitute the main mechanism of karyotype evolution in the genus. Likewise, the sex determination system in *P. serratus* and the existence of additional sex chromosome systems in the genus that shed light on the genus sex chromosome evolution are interesting aspects to be elucidated in further studies.

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Availability of data and materials
Not applicable.

Authors' contributions
AGT and AML designed the study and helped to draft the manuscript; ZT, AP and AML collected samples; EGO identified specimens; ZT performed the experiments and wrote the manuscript. All authors revised the manuscript critically and approved the final manuscript.

Ethics approval and consent to participate
No approval by an ethical committee was required to achieve the goals of the present study because experimental work was accomplished with an unregulated marine invertebrate.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Author details
[1]Grupo de Investigación en Biología Evolutiva (GIBE), Centro de Investigaciones Científicas Avanzadas (CICA), Departamento de Biología, Facultad de Ciencias, Universidade da Coruña, Campus A Zapateira, 15071 A Coruña, Spain. [2]Instituto de Ciencias Marinas de Andalucía (ICMAN, CSIC), Campus Universitario Río San Pedro, 11510 Puerto Real, Cádiz, Spain. [3]International Campus of Excellence of the Sea (CEI-MAR), Edificio Hospital Real, 11003 Cádiz, Spain.

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CHAPTER 2

Genetic differentiation between Mediterranean and Atlantic populations of the common prawn *Palaemon serratus* (Crustacea: Palaemonidae) reveals uncommon phylogeographic break

Ronja Weiss, Zeltia Torrecilla, Enrique González-Ortegón, Ana M. González-Tizón, Andrés Martínez-Lage and Christoph D. Schubart (2017) Genetic differentiation between Mediterranean and Atlantic populations of the common prawn *Palaemon serratus* (Crustacea: Palaemonidae) reveals uncommon phylogeographic break. Journal of the marine Biological Association of the United Kingdom 1-10.

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Genetic differentiation between Mediterranean and Atlantic populations of the common prawn *Palaemon serratus* (Crustacea: Palaemonidae) reveals uncommon phylogeographic break

RONJA WEISS[1], ZELTIA TORRECILLA[2], ENRIQUE GONZÁLEZ-ORTEGÓN[3,4], ANA M. GONZÁLEZ-TIZÓN[2], ANDRÉS MARTÍNEZ-LAGE[2] AND CHRISTOPH D. SCHUBART[1]

[1]Zoologie & Evolutionsbiologie, Universität Regensburg, D-93040 Regensburg, Germany, [2]El Departamento de Biología Celular y Molecular, CICA, Universidade da Coruña, E-15008 A Coruña, Spain, [3]Instituto Español de Oceanografía, Centro Oceanográfico de Cádiz, Puerto Pesquero, Muelle de Levante s/n, E-11006 Cádiz, Spain, [4]Instituto de Ciencias Marinas de Andalucía (CSIC), Campus Universitario Río San Pedro, 11519 Puerto Real, Cádiz, Spain

The Atlantic–Mediterranean transition zone between the Alborán Sea and the Gulf of Cádiz constitutes the most prominent marine geographic barrier in European waters and includes known phylogeographic breaks such as the Strait of Gibraltar and the Almería-Oran Front. A genetic shift in this area has been previously documented for the European littoral shrimp Palaemon elegans. *Here we carried out a phylogeographic analysis with the congeneric and sympatric species* Palaemon serratus *to test for similar intraspecific genetic differentiation and geographic structure. This littoral prawn is distributed in the Northeastern Atlantic Ocean, the Mediterranean Sea and the Black Sea. We compared DNA sequences from the mitochondrial genes* Cox1 *and to a lesser extent from 16S rRNA of several Atlantic and Mediterranean populations. Furthermore, sequences from the nuclear gene Enolase were included for corroborating differences between Mediterranean and Atlantic individuals. A pronounced genetic differentiation was detected between the Mediterranean and Atlantic populations, amounting to 10.14% in* Cox1 *and 2.0% in 16S, indicating the occurrence of two independent evolutionary lineages. Interestingly, specimens from the Atlantic Gulf of Cadiz cluster together with the Mediterranean individuals, indicating that a biogeographic barrier appears to be located west of the Strait of Gibraltar.*

Keywords: Decapoda Caridea, Mediterranean Sea, Atlantic Ocean, Gulf of Cadiz, phylogeography, mtDNA *Cox1*

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INTRODUCTION

Recent molecular biodiversity studies increasingly reveal local genetic differentiation in biphasic marine species with a larval dispersal phase (e.g. Mathews, 2006; Galarza *et al.*, 2009; Ragionieri *et al.*, 2009; Fratini *et al.*, 2016). Geographic barriers are the main historical factor determining the biogeography of European marine species, as documented for caridean shrimps (Reuschel *et al.*, 2010; González-Ortegón *et al.*, 2016). The most prominent geographic barrier in European marine waters is the one between the Atlantic Ocean and the Mediterranean Sea. It is located somewhere in between the Alborán Sea and the Gulf of Cádiz, and either the Strait of Gibraltar or the Almería-Oran Front have been postulated to act as the main barrier, as recently revised and discussed (Patarnello *et al.*, 2007; García-Merchán *et al.*, 2012). In recent geological history, the shallow Strait of Gibraltar isolated the Mediterranean Sea repeatedly from the Atlantic, caused by sea level regressions, sometimes even resulting in major desiccations (Hsü *et al.*, 1977). These repeated isolations led to a very high level of endemism in the Mediterranean Sea (Hofrichter, 2002), and many marine species show a phylogeographic break among Atlantic and Mediterranean populations (Zane *et al.*, 2000; Patarnello *et al.*, 2007; Luttikhuizen *et al.*, 2008; Deli *et al.*, 2016). The Messinian Salinity Crisis in the late Miocene (around 5.5 Ma) was probably the most dramatic isolation event (Krijgsman *et al.*, 1999) and ended with the Zanclean Flood, i.e. when Atlantic waters re-flooded the Mediterranean Basin, leading to a re-colonization of the Mediterranean Sea with Atlantic species (García-Castellanos *et al.*, 2009). Even today, the waters between the Strait of Gibraltar and the Almería-Oran Front seem to act as a barrier to gene flow for many marine species, determining the genetic structure and diversity of many European coastal water species.

The shrimp genus *Palaemon* Weber, 1795 (Crustacea: Decapoda: Caridea) belongs to the large family Palaemonidae Rafinesque, 1815 and includes many important

Corresponding author:
C.D. Schubart
Email: Christoph.Schubart@ur.de

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representatives from coastal waters all over the world. Recently, important systematic and taxonomic changes were postulated for this family, summarized in the review by De Grave & Ashelby (2013), with the consequence that the genera *Palaemonetes* Heller, 1869, *Exopalaemon* Holthuis, 1950 and *Coutierella* Sollaud, 1914 were synonymized and re-arranged within the genus *Palaemon* that now contains 83 species (for the most recent phylogeny see Carvalho *et al.*, 2016). Morphological distinction within this genus is partly difficult, because only small differences serve as identification criteria, as for example the upper antennular flagellum or the denticulation of the rostrum (González-Ortegón & Cuesta, 2006). This often results in wrongly classified species within the genus *Palaemon*. For example, *Palaemon garciacidi* Zariquiey Álvarez, 1968 turned out to be a synonym of *Palaemon longirostris* Milne-Edwards, 1837 (Cuesta *et al.*, 2012; Cartaxana, 2015). Also the existence of cryptic species is discussed for this genus, since Reuschel *et al.* (2010) gave evidence for differentiation along the Atlantic–Mediterranean barrier within *Palaemon elegans* Rathke, 1837 and the possible existence of a cryptic species within the Mediterranean Sea. Thus, it seems worthwhile to use genetic techniques to study phylogeographic relationships between Atlantic–Mediterranean populations of other coastal species of *Palaemon* and to help redefine the morphological classification in this taxonomic group. It is of particular interest to focus on species living in both the Atlantic Ocean and the Mediterranean Sea, to determine if similar differentiation patterns may be revealed as in *P. elegans*. In Europe, six marine and/or estuarine species (including the recently introduced *P. macrodactylus* Rathbun, 1902 (see González-Ortegón *et al.*, 2007; Ashelby *et al.*, 2013)) and four fresh to brackish water species of *Palaemon* can be found (Cuesta *et al.*, 2012). The former ones include the widespread and commercially exploited species *Palaemon serratus* (Pennant, 1777), also known as the common prawn. It could make an interesting study object regarding phylogeographic analyses, as a recent published record indicated that a single Mediterranean specimen from the Turkish coast may be distinct from its Atlantic counterparts (Bilgin *et al.*, 2014).

Palaemon serratus lives in rocky crevices along the European and North African coastlines in shallow waters up to a depth of 40 m. Its distribution area ranges from Scotland and Denmark to Mauritania and it is common in the Atlantic Ocean, with occasional records in the Mediterranean Sea, and the Black Sea (Lagardère, 1971; d'Udekem d'Acoz, 1999). Adult individuals have a total length of 90–110 mm and the cephalothorax and abdomen are transparent with a dark reddish-brown striped pattern which can vary widely between different regions or habitats (Carlisle, 1955; González-Ortegón & Cuesta, 2006). Larval development is linked to initial larval body mass and female body size and can be modified by environmental conditions experienced by the larvae (Reeve, 1969; González-Ortegón & Giménez, 2014).

There is an important commercial fishery on *P. serratus*, especially around the British Isles, France and northern Spain, and the high commercial value could possibly lead to overfishing, as already expressed by Fahy *et al.* (2006). Even though the latter study only evaluated the fisheries around Ireland, the same problems could also occur elsewhere. Currently, baseline monitoring data are gathered in the UK and Ireland to ascertain the stock status and population trends and to identify possible problems caused by the fishery exploitation (Haig *et al.*, 2014). Although this species was proposed as suitable for cultivation (Reeve, 1968; Rodríguez, 1981), further studies about its ecology and husbandry conditions are necessary to take this step. For this endeavour, basic knowledge on the natural genetic diversity and the phylogeography of this species will be critically important.

This study thus focused on the genetic differentiation among various populations of *P. serratus* along European coastlines. DNA sequences for the mitochondrial gene *Cox1* were analysed. To confirm the obtained results additional sequences of the more conserved mitochondrial *16S* rRNA and the nuclear gene *Enolase* were included. *Cox1* and *16S* rRNA have been shown to be suitable marker genes for DNA barcoding in crustaceans (Schubart *et al.*, 2000; Lefébure *et al.*, 2006; Costa *et al.*, 2007) and also within the Palaemonidae (Reuschel *et al.*, 2010; Cartaxana, 2015). Special attention is paid to the possible divergence among Atlantic and Mediterranean populations, as previously reported for the closely related prawn *P. elegans* (see Reuschel *et al.*, 2010).

MATERIALS AND METHODS

Specimens of *Palaemon serratus* used for the analysis originated from the authors' collections and were stored in 70–95% ethanol. Most individuals were sampled by hand with the aid of a dip net. Sampling localities, coordinates and number of sequences for each available population of *P. serratus* are shown in Figure 1 and Table 1. For some populations, only the locality was given and the coordinates were estimated. DNA-extractions were performed following either the Puregene Method (from Gentra Systems: Minneapolis, MN55447, USA) or the Realpure – Spin Kit 250 Extract (from Durviz – Gentaur: Brussels, Belgium).

Two different mitochondrial DNA gene fragments were amplified by means of PCR reactions: the mitochondrial gene *Cox1* encoding subunit 1 of the cytochrome c oxidase gene that is especially suitable for intraspecific comparisons and used as the biological barcoding gene (e.g. Costa *et al.*, 2007) (N = 67 sequences). In addition, one individual of each of five Atlantic populations and four individuals of Mediterranean populations were analysed for variation of the more conserved *16S* rRNA gene, transcribed to the structural rRNA of the large subunit *16S* of mitochondrial ribosome. The only available *16S*-sequence in GenBank (JQ042291) was downloaded and added to the analysis, allowing comparison of a total of 10 sequences. In addition, the nuclear gene *Enolase*, better suited for interspecific comparisons (e.g. Ip *et al.*, 2015), was amplified for some of the Mediterranean and Atlantic populations (N = 26 sequences).

For both mitochondrial genes, different primer combinations were used. For *Cox1*, a few long fragments of 1276 basepairs (bp) were amplified with primers COL6 or COL6a and COH1b. This was not possible for the Mediterranean individuals, so a shorter fragment was amplified using the primers COL1Pe and COH1b (630 bp). For the amplification of the *16S* rRNA gene, the forward primer 16L29 and the reverse primer 1472 were used (~580 bp). The nuclear gene *Enolase* was amplified using the primer combination ENEA1 and ENES1 (409 bp). All primer sequences and the corresponding references are listed in Table 2.

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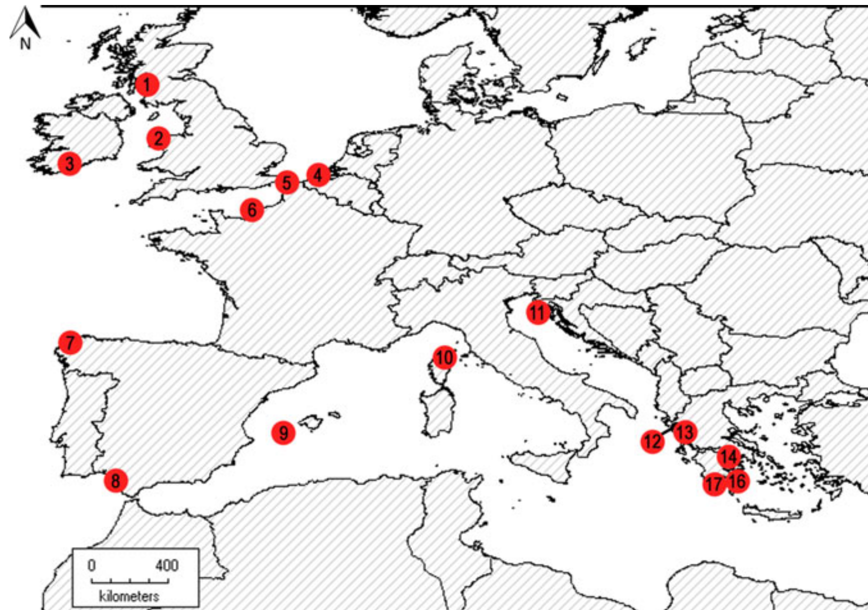

Fig. 1. Sampling localities of *Palaemon serratus* (Pennant, 1777). The locality numbers correspond to the code numbers in Table 1.

PCR reactions were run in a total volume of 25 µl with different protocols optimized for the respective Taq polymerase (Fermentas Taq, Promega GoTaq or Roche Taq). The temperature profile of the PCR for all three genes consisted of an initial denaturation step at 94°C for 4 min, followed by 35–40 cycles and a final elongation step at 72°C for 5–10 min. The cycle program included denaturation at 95°C for 45 s, annealing at 52°C for the primer combination COL6 or COL6a/COH1b, 48°C for all other mitochondrial gene combinations, and 50°C for *Enolase* for 45–60 s, and elongation at 72°C for 60 (short fragments) or 90 (long fragments) seconds. In a few difficult cases, the annealing temperature was decreased to 45°C to obtain useable PCR products.

Most of the long fragments of the *Cox1* gene were sequenced in both directions, while the short fragments were only sequenced with COH1b. The *16S* rRNA fragments were sequenced with 16L29 and the *Enolase* fragments with ENEA1. Sanger sequencing took place at the Laboratory of Molecular Biology, SAI (Servicios de Apoyo a la Investigación; University of A Coruña, Spain) or was outsourced to Macrogen Europe (the Netherlands). Sequence chromatograms were proofread using Chromas Lite version 2.1.1 (Technelysium Pty Ltd) and edited manually, if necessary. All sequences were aligned with BioEdit version 7.2.0 (Hall, 1999) using the ClustalW algorithm (Thompson *et al.*, 1994) and, if necessary, adjusted manually. Five different alignments were created, three with the *Cox1* sequences, depending on the primer combinations and sequence length, and one in each case for the *16S* and the *Enolase* datasets. No ambiguities were encountered during the alignment

Table 1. Coordinates and number of individuals of the studied populations of *Palaemon serratus* from west to east.

						Sequences		
Code (Fig. 1)	Population	Latitude	Longitude	Date	N	*Cox1*	*16S*	*Enolase*
3	Cork (Ireland, A)	51.9	−8.483333	August 2012	1	–	1	–
7	Artabro Gulf (Spain, A)	43.366667	−8.466667	October 2012	15	12	1	–
8	Guadalquivir Estuary (Spain, A)	36.783333	−6.366667	August 2012	21	14	1	10
1	Millport (UK: Scotland, A)	55.749917	−4.927883	November 2003	1	–	–	1
2	Anglesey (UK: Wales, A)	53.133333	−4.283333	January 2012	9	7	1	–
6	Saint-Jouin-Bruneval (France, A)	49.645283	0.1525	August 2012	10	7	–	5
9	Cala Nova (Spain: Ibiza, M)	39.007595	1.581657	October 2011	2	2	–	–
9	Cala Llenya (Spain: Ibiza, M)	39.015299	1.587782	October 2009	11	10	1	1
5	Calais (France, A)	50.966667	1.80	September 2012	1	–	1	–
4	Duinbergen (Belgium, A)	51.35	3.25	October 2012	1	1	–	–
10	Marine de Farinole (France, M)	42.729274	9.339269	June 2003	1	1	1	1
11	Pula (Croatia, M)	44.860903	13.81284	September 2004	1	1	1	1
12	Acheron Estuary (Greece, M)	39.236111	20.479722	July 1993	2	2	–	2
13	Ligia (Greece, M)	39.154657	20.563746	September 2003	1	1	1	–
17	Githion (Greece, M)	36.791944	22.596389	July 1986	3	3	–	3
14	Nafplio (Greece, M)	37.547222	22.818056	December 2013	1	1	–	1
16	Limin Ieraka (Greece, M)	36.785833	23.0825	July 1986	1	1	–	1

A, Atlantic Ocean; M, Mediterranean Sea.

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Table 2. DNA primer sequences and corresponding references.

Primer	Gene	Sequence 5′ – 3′	Reference
COL6	*Cox1*	TYT CHA CAA AYC ATA AAG AYA TYG G	Schubart (2009)
COL6a	*Cox1*	TCW ACA AAT CAT AAA GAY ATT GG	Schubart (2009)
COL1Pe	*Cox1*	TAC YTC RTT CTT TGA TCC TGC	New
COL1b	*Cox1*	CCW GCT GGD GGW GGD GAY CC	Schubart (2009)
COH1b	*Cox1*	TGT ATA RGC RTC TGG RTA RTC	Schubart (2009)
16L29	*16S*	YGC CTG TTT ATC AAA AAC AT	Schubart *et al.* (2001)
1472	*16S*	AGA TAG AAA CCA ACC TGG	Crandall & Fitzpatrick (1996)
ENEA1	*Enolase*	CAG CAA TCA ATG TCA TCA AYG GWG G	Tsang *et al.* (2014)
ENES1	*Enolase*	ACT TGG TCA AAT GGR TCY TCA AT	Tsang *et al.* (2014)

process. Before building the parsimony network, the alignments were cropped manually to the same length and short sequences were eliminated. All new DNA sequences (all haplotypes in case of *Cox1*) were submitted to the European Nucleotide Archive (ENA as part of GenBank) and have been assigned accession numbers LT717247 to LT717310. Alignments were converted from FASTA to PHYLIP format with FaBox version 1.41 (URL 1). Finally, five networks were constructed with the software TCS version 1.21 (Clement *et al.*, 2000) based on the single alignments. In the three *Cox1* networks, the maximum connection steps had to be specified manually (65–130 steps) in order to allow the haplogroups to be connected, while in the *Enolase* network a 95% and in the *16S* network a 94% connection limit between the different genotypes were used. Nucleotide and haplotype diversities were calculated with the software DnaSP version 5.10.01 (Librado & Rozas, 2009) for six different populations (only populations with more than two individuals were included). A permutation test was performed with 10,000 replicates. Also a mismatch analysis of sequences was carried out with DnaSP version 5.0 for the *Cox1* and for the *16S* alignments containing Mediterranean individuals. A constant population size was chosen as the model for expected values. Finally, an analysis of molecular variance (AMOVA) was carried out for two of the populations of the Mediterranean (with more than eight individuals) and the population of Guadalquivir using the software DnaSP version 5.0.

RESULTS

For many of the long fragments of *Cox1*, clean reads were only obtained for one direction. Therefore, sequences were subdivided into three length groups of which three different alignments and three different parsimony networks were constructed. The first alignment consists of 17 consensus sequences of forward and reverse reads of the long primer combination, resulting in 1199 bp. The second alignment contains only the sequences read with the forward primers COL6 or COL6a (26 sequences, 811 bp) and the third alignment the ones read with the reverse primer COH1b (58 sequences, 611 bp). Since all three parsimony networks show similar distribution patterns, only the one with most individuals, i.e. the one sequenced with the primer COH1b, is shown (Figure 2). The other two can be found in the Supplementary material (Appendices 1–2). For the graphic presentation of the networks, the two localities of Ibiza (Cala Llenya and Cala Nova) were summarized as one population, since they are geographically very close to each other and there were no important differences in their sequences. The same approach was used for the different sites in Greece.

The maximum parsimony network based on the alignment of the COH1b sequences (Figure 2) shows a clearcut dissociation between the Mediterranean populations (including the Guadalquivir Estuary) and the more western Atlantic populations. Sequences from the Guadalquivir Estuary are recovered as four haplotypes, of which haplotype A with 10 individuals holds a central position. Interestingly, the sequences of Guadalquivir Estuary do not share haplotypes with the Mediterranean populations, despite being closely related. Consequently, the Mediterranean populations have their own central haplotype B found in 12 individuals. The Atlantic haplotype closest to the ones of the Guadalquivir Estuary is separated by 62 mutation steps (equivalent to 10.14% of the COH1b alignment length) and belongs to the population of Anglesey. In contrast to the Mediterranean populations and the one of the Guadalquivir Estuary, all the Atlantic populations are mixed and scattered with no recognizable central haplotype. Haplotype and nucleotide diversities were only calculated for the six populations with more than two individuals listed in Table 3. These values can only be compared conditionally, because the number of sequences of each population differs. The population of the Guadalquivir Estuary shows the lowest haplotype diversity with 0.49451, followed by the individuals of Greece, Ibiza and then Anglesey, while Saint-Jouin-Bruneval and Artabro Gulf with 1.0 each have the highest values. The highest nucleotide diversity is also found in the population of Saint-Jouin-Bruneval, followed by Anglesey and the population of Artabro Gulf and then Ibiza and Greece. The lowest nucleotide diversity was found in the population of the Guadalquivir Estuary with 0.0009.

For the AMOVA, we also used the COH1b-alignment of the *Cox1* gene, since it contains most sequences. Eight individuals of the different sites in Greece, 12 individuals of the two populations of Ibiza and 14 individuals of the population of Guadalquivir Estuary were compared. Between Ibiza and Greece, a Φ_{ST} value of 0.04562 ($P = 0.1$) indicates the high level of panmixia between these populations of the Mediterranean Sea. In contrast, the Φ_{ST} values of the pairwise differences between Ibiza and the Guadalquivir Estuary (0.62084) and Greece and the Guadalquivir Estuary (0.68065) are highly significant ($P < 0.01$). They demonstrate that there is restricted gene flow across the Strait of Gibraltar, despite the genetic similarity between the Guadalquivir and Mediterranean populations.

To confirm the results of the *Cox1* gene, a small network was constructed with 10 sequences of the *16S* mtDNA gene

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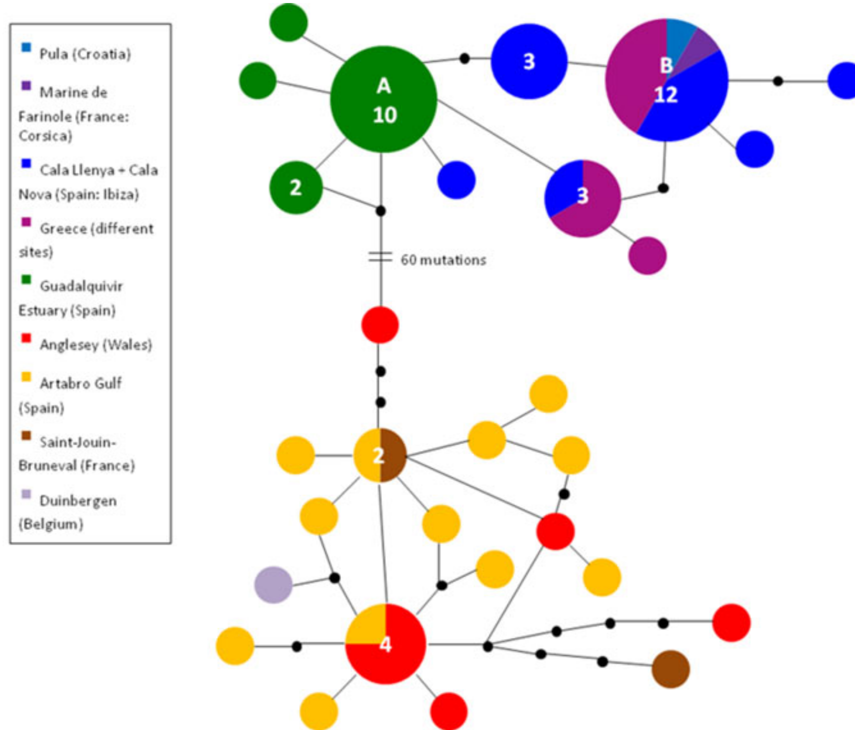

Fig. 2. Maximum parsimony network of *Cox1* mtDNA (COH1b alignment) of *Palaemon serratus*, constructed with TCS version 1.21 and a connection limit of 65 steps, based on an alignment of 58 sequences (611 base pairs). The numbers within the circles correspond to the number of individuals represented by that haplotype (circles without a number represent single individuals). Black spots represent missing haplotypes.

Table 3. Estimates of haplotype and nucleotide diversities of *Palaemon serratus*, based on the COH1b alignment of the *Cox1* gene with 611 base pairs. Calculated with DnaSP version 5.10.01. Only populations with more than two individuals were included. Permutation test with 10,000 replicates.

Population	Number of sequences	Number of haplotypes	Haplotype diversity	Nucleotide diversity
Saint-Jouin-Bruneval (F)	7	7	1	0.00834
Guadalquivir Est. (Spain)	14	4	0.49451	0.0009
Artabro Gulf (Spain)	12	12	1	0.00491
Anglesey (Wales)	7	5	0.85714	0.0053
Greece	8	3	0.60714	0.00216
Ibiza (Spain)	12	6	0.80303	0.00288

based on an alignment of 499 basepairs (Figure 3). It consists of four different haplotypes. One of these haplotypes represents three Mediterranean individuals, while the fourth Mediterranean individual only differs in one position. Two individuals of the Gulf of Cádiz population directly west of the Strait of Gibraltar (Cádiz and Guadalquivir Estuary) are also separated from the larger Mediterranean haplotype by one mutational step. In contrast, the fourth haplotype is separated from all others by at least 10 mutational steps (equivalent to 2.0%) and is found in four individuals of the other Atlantic populations (one of each location from Artabro Gulf, Calais, Cork and Anglesey). Therefore, the *16S* network confirms the structure of the *Cox1* network.

The mismatch analyses for both the COH1b sequences of *Cox1* mtDNA and *16S* rRNA show a bimodal distribution, i.e. two separate peaks, in the frequency of pairwise differences among haplotypes (Figure 4). This reflects the fact that the haplotypes are either very close to each other (within Atlantic and Mediterranean) or more distantly related (between Atlantic and Mediterranean). In *Cox1* mtDNA, haplotypes are either separated by not more than nine or between 62–71 mutational steps, whereas in *16S* mtDNA there are either up to two or between 10 and 11 mutational steps. In both cases, the observed frequency (dashed line) did not correspond to the expected values for a constant population size (solid line).

An independent nuclear marker was applied as third evidence and to test for possible ongoing speciation: The nuclear gene *Enolase* was sequenced from 26 individuals, resulting in an alignment with a length of 409 bp. It includes only two parsimony-informative sites that consistently separate two genotypes. The corresponding maximum parsimony network can be found in Figure 5. Ten individuals originating from Croatia, Greece, Ibiza (Spain) and Corsica (France) and 10 from the population of the Guadalquivir Estuary share one genotype, whereas the second genotype is found in five individuals of the Atlantic coast of France and one specimen from Scotland. Therefore, a clean split is confirmed between the Mediterranean plus the Gulf of Cádiz sequences and the more northern Atlantic populations.

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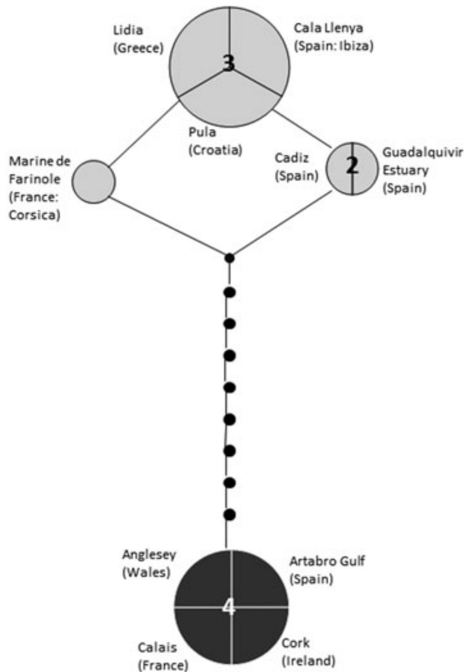

Fig. 3. Maximum parsimony network of *16S* mtDNA of *Palaemon serratus*, constructed with TCS version 1.21 and a connection limit of 94%, based on an alignment of 10 sequences (499 base pairs). The number within the circles correspond to the number of individuals represented by that haplotype (circles without a number represent single individuals). Black spots represent missing haplotypes.

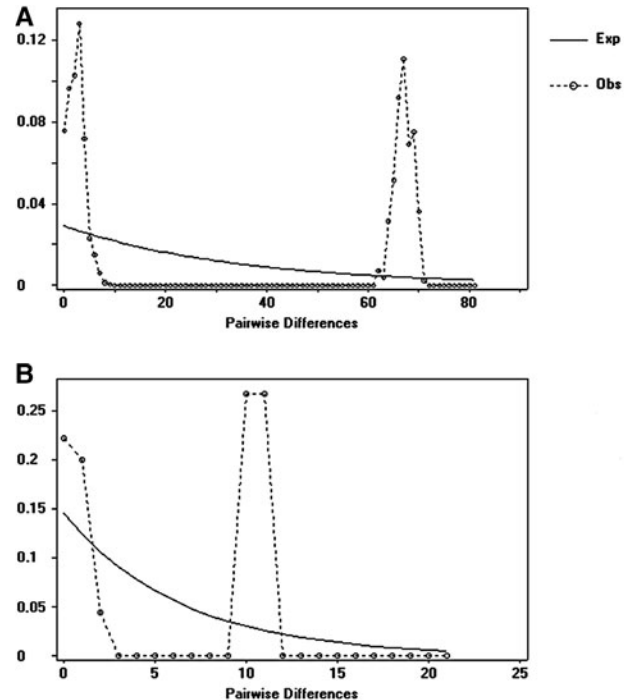

Fig. 4. Mismatch distribution of pairwise differences in two mitochondrial genes of *Palaemon serratus.* Calculated with DnaSP version 5.10.01. The dashed lines show the observed, the solid lines the expected frequencies within a constant population size. (A) *Cox1* mtDNA (COH1b alignment with 58 sequences, 611 base pairs). (B) *16S* mtDNA (10 sequences, 499 base pairs).

DISCUSSION

Results of three analysed genes (*Cox1* mtDNA, *16S* rRNA and *Enolase* nuDNA) show genetic differentiation among the populations of *Palaemon serratus* in the Mediterranean Sea, the Gulf of Cádiz and the Atlantic Ocean. The individuals of the first two areas are closely related and will henceforth be termed the Mediterranean lineage (ML) which thus includes the populations from the Guadalquivir Estuary and the Mediterranean populations. A sequence from GenBank (JQ042291) corresponding to the *16S* mtDNA of an individual from Cádiz confirms that populations from the Gulf of Cádiz are more closely related to Mediterranean populations than to other Atlantic ones. The second lineage, henceforth called the Atlantic lineage (AL), contains all the other Atlantic populations included in this study. The *16S* network consists of three different haplotypes in the ML and only one haplotype in the AL, while all three *Cox1* networks have a smaller number of haplotypes in the ML than in the AL. This can be due to the fact that only one individual per population was used for the *16S* analysis, so that most likely not all occurring haplotypes are represented in the network. In the *Enolase* alignment, only two different genotypes can be found, corresponding to ML and AL and separating these in the same fashion as the mitochondrial genes.

The *Cox1* haplotypes of the populations of the Mediterranean are linked tightly, and while the Greek individuals show a moderate haplotype diversity ($h_d = 0.60714$), the individuals of the populations of Ibiza are quite heterogeneous ($h_d = 0.80303$). This suggests that the Mediterranean stocks represent a healthy and stable system, with relatively high gene flow among the sampled populations. Nevertheless, this should be tested with more individuals and more populations, since only two Mediterranean populations were used for the analysis of haplotype and nucleotide diversities in this study.

The specimens of the Guadalquivir Estuary form a very homogeneous clade, with only a few haplotypes ($h_d = 0.49451$) being very close to each other ($n_d = 0.0009$). This can be best explained by a founder effect, i.e. a new population founded by only a few individuals and therefore with low genetic variety. The proximity of some *Cox1* haplotypes from Ibiza to the Guadalquivir haplotype *A* suggests a relatively recent colonization from the western Mediterranean. Another possible explanation would be reduced gene flow to and from the Guadalquivir population, because of its geographically marginal position, compared with a population that is located in the centre of the range of a genetic lineage. In any case, current gene flow of the population from the Guadalquivir Estuary with the Mediterranean populations seems limited.

The fact that the population of the Guadalquivir Estuary nevertheless belongs to the ML raises the question why it groups together with Mediterranean populations instead of Atlantic ones. Most other studies of species with a genetic separation of the Atlantic and the Mediterranean populations reveal the opposite scenario: They show an extension of the Atlantic genotypes into the Alborán Sea which is the westernmost part of the Mediterranean Sea. This is for example the case in the closely related prawn *Palaemon elegans* (see Reuschel *et al.*, 2010) as well as in many other marine species, e.g. the scallops *Pecten jacobeus* and *P. maximus* (see Ríos *et al.*, 2002) or the euphausiid *Meganyctiphanes norvegica* (see Zane *et al.*, 2000). This extension of Atlantic

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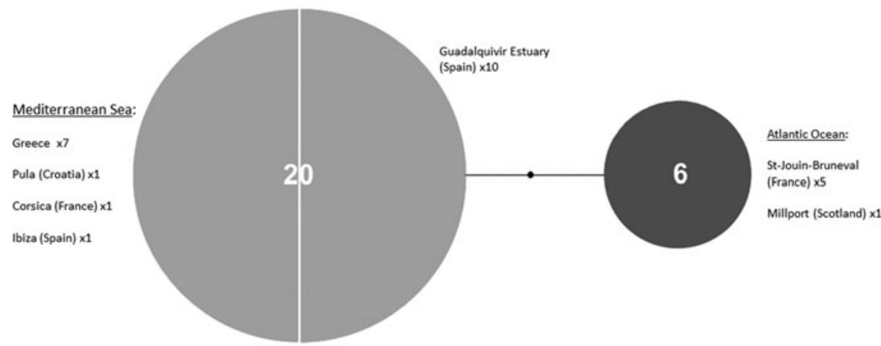

Fig. 5. Maximum parsimony network of *Enolase* nuDNA of *Palaemon serratus*, constructed with TCS version 1.21 and a connection limit of 95%, based on an alignment of 26 sequences (409 base pairs). The numbers within the circles correspond to the number of individuals represented by that genotype. Black spot represents missing genotype.

genotypes into the Mediterranean Sea has been explained by the intrusion of Atlantic waters into the Alborán Sea, where they circulate as two gyres, so that the Almería-Oran Front often represents the real hydrographic boundary of Atlantic and Mediterranean surface waters (Tintoré *et al.*, 1988), rather than the Strait of Gibraltar itself. However, in a recent study on several crustacean species, the role of the Strait of Gibraltar as a gene flow barrier compared with the one of the Almería-Oran Front has been emphasized (García-Merchán *et al.*, 2012).

Palaemon serratus is not the only species in which populations of the Guadalquivir Estuary differ genetically from other Atlantic populations, as this was also reported in the mysid *Neomysis integer* (see Remerie *et al.*, 2009). It remains unknown, when Mediterranean *P. serratus* may have migrated from the Mediterranean Sea through the Strait of Gibraltar and settled in the Gulf of Cádiz. Furthermore, we do not know if they replaced 'typical' Atlantic *P. serratus* which theoretically should be better adapted to the local conditions, like temperature and tides, in the Gulf of Cádiz. Alternatively, it is conceivable that the Gulf of Cádiz population may have diverged from Atlantic stocks independently and gave rise to the Mediterranean populations with the flooding of the Mediterranean Basin. Hence, more detailed studies about the dispersal and ecology of *P. serratus*, especially in the area west and east of the Strait of Gibraltar, are necessary to reconstruct how the extension of Mediterranean haplotypes into the Atlantic Ocean took place. A human introduction can be excluded, because the haplotypes from the Guadalquivir Estuary of both mitochondrial genes have so far not been sampled in the Mediterranean Sea and thus appear to be endemic. It remains to be solved, where the contact zone with the other Atlantic populations is, and if there is genetic mixing. One possible explanation for endemic genotypes could be the fact that the population of the Guadalquivir Estuary belongs to the most important nursery area in the Gulf of Cádiz, favouring the reproduction and/or the settlement of local individuals (González-Ortegón *et al.*, 2015) and probably a more estuarine genotype. Further investigations of different populations along the coastline of Andalusia to Portugal are necessary to define the exact border between AL and the haplotypes of the Gulf of Cádiz, and to determine what induces the biogeographic break within the Atlantic Ocean.

The populations of AL are genetically very diverse with 18 different haplotypes in the COH1b network (Figure 2), and all tested populations show very high haplotype and nucleotide diversities. According to the coalescent theory of Kingman (1982), high genetic diversity leads to the conclusion that the corresponding species is presumably of a great age (the higher the diversification the longer the required time span to develop this diversity). This provides evidence that the Atlantic stock of *Palaemon serratus* represents a very stable and healthy system, which has probably existed for a long time. The maximum parsimony networks as well as the haplotype diversities indicate high gene flow among the Atlantic populations, with the exception of the ones from the Gulf of Cádiz. It would be useful to confirm these assumptions with more individuals per population and more populations to cover the whole distribution area of *P. serratus* and to quantify gene flow statistically, as no populations east of Belgium and south of Spain are represented in this study.

Our results suggest the existence of a potential cryptic species or at least ongoing speciation within *P. serratus*, i.e. a morphological indistinct lineage separated by genetic differences at species level (Belfiore *et al.*, 2003). The separation of the ML and the AL amounts to 2.0% in the *16S* gene and 10.14% in the *Cox1* gene and is thus comparable to recognized species from other crustacean genera with an Atlantic–Mediterranean separation, such as in *Carcinus* (see Geller *et al.*, 1997; Roman & Palumbi, 2004; Ragionieri & Schubart, 2013) or *Brachynotus* (see Schubart *et al.*, 2001). Furthermore, the independently evolving nuclear gene *Enolase* confirms the same differentiation pattern. So far, there are no morphological characters known that differ in Atlantic and Mediterranean specimens of *P. serratus*, but this needs to be verified in a separate study. It would also be important to know if hybridization of Atlantic and Mediterranean individuals is possible. Considering the fact that in this study only a few populations were represented by more than 10 individuals, statistical possibilities are limited and make further studies with larger sample sizes necessary. No type locality for *Palaemon serratus* was indicated by Pennant (1777), but even though a few Mediterranean individuals were included in his first study characterizing the species, most of the individuals studied were from the Atlantic Ocean, i.e. from the English Channel, Belgium and the Netherlands (De Man, 1915). For that reason, and because Pennant published his results in British Zoology, it appears logical that the species name *Palaemon serratus* would remain with the Atlantic

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individuals, if two separate species were to be recognized in the future.

Overall, this study reveals some interesting phylogeographic aspects, although the results have limited conclusive significance, due to the restricted number of populations and the fact that some analysed populations consisted of very few individuals and therefore could not be used for statistical analyses. Hence, more research is needed regarding the population genetics and taxonomic status of *P. serratus*, including morphological and morphometric aspects, to understand the remarkable case of an Atlantic population with haplotypes that are more closely related to Mediterranean ones than to the other Atlantic ones, and to decide whether a cryptic species is involved.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit https://doi.org/10.1017/S0025315417000492

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Correspondence should be addressed to:
C. D. Schubart
Zoologie & Evolutionsbiologie,
Universität Regensburg,
D-93040 Regensburg, Germany.
email: Christoph.Schubart@ur.de

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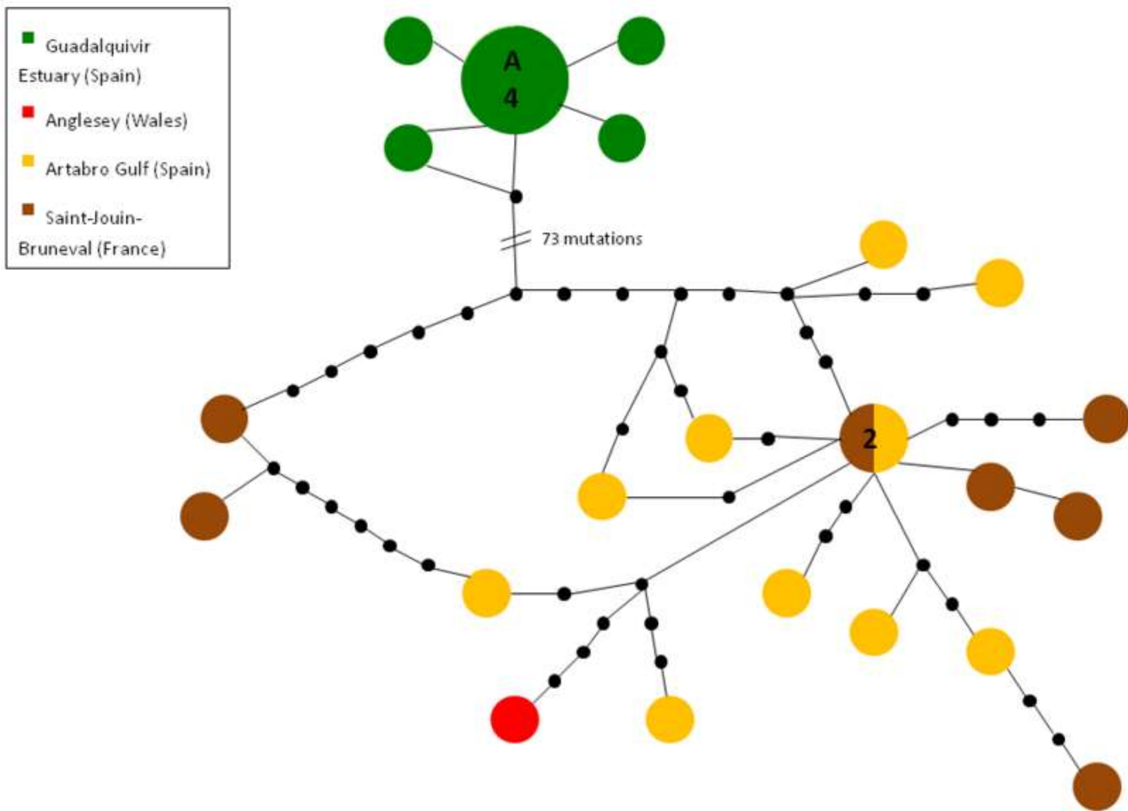
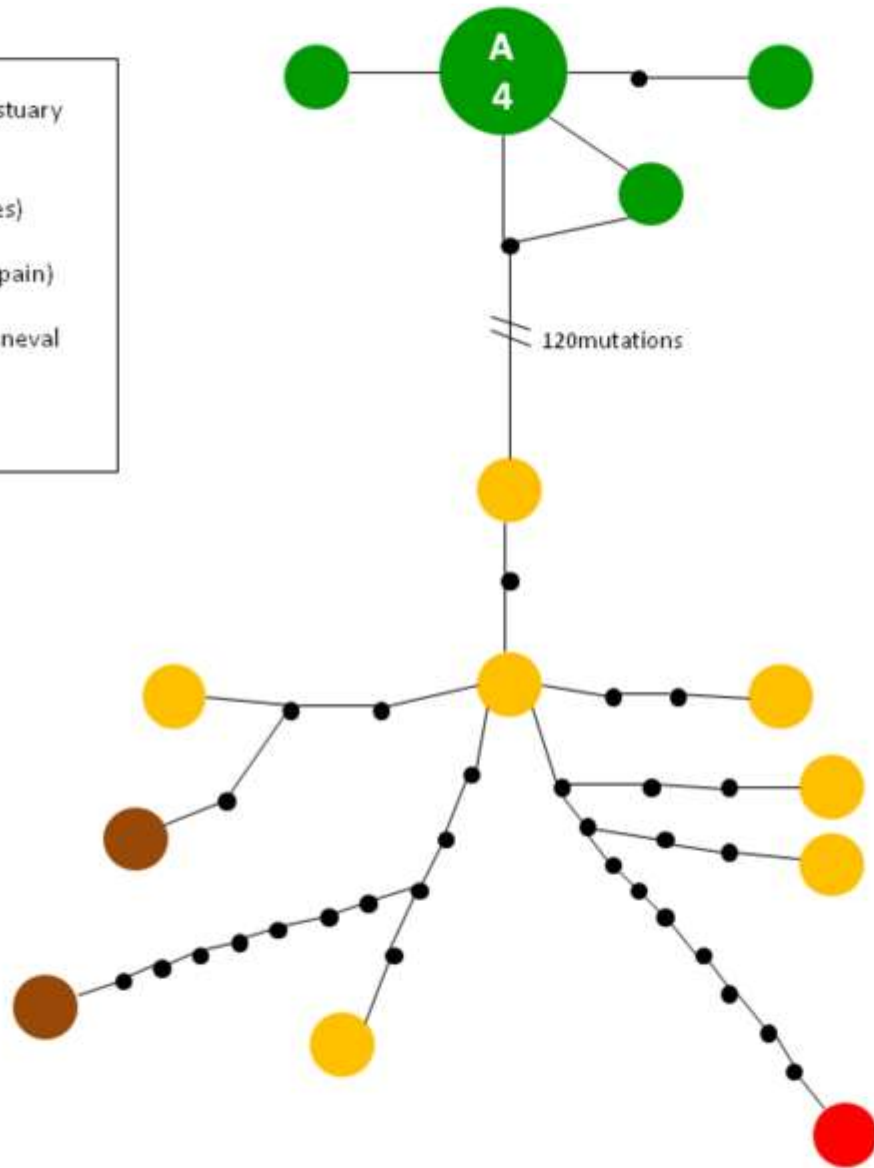


Supplementary material
Appendix 1



Supplementary material
Appendix 2

CHAPTER 3

Molecular authentication of five economically important prawn species by species-specific PCR-assay

Zeltia Torrecilla, Inés González-Castellano, Andrés Martínez-Lage, and Ana M. González-Tizón (2018) Molecular authentication of five economically important prawn species by species-specific PCR-assay.

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Molecular authentication of five economically important prawn species by species-specific PCR-assay

Zeltia Torrecilla, Inés González-Castellano, Andrés Martínez-Lage, Ana M. González Tizón*

Grupo de Investigación en Biología Evolutiva (GIBE), Centro de Investigaciones Científicas Avanzadas (CICA), Departamento de Biología, Facultad de Ciencias, Universidade da Coruña, Campus A Zapateira, 15071 A Coruña, Spain

*Corresponding autor: hakuna@udc.es (AM. González-Tizón) Tel.: +34 881012045

Abstract

Seafood traceability is crucial to avoid commercial fraud by species substitution, to certify product quality and to guarantee consumer health safety. Under European legislation, commercial and scientific names among other features have to be clearly indicated on the label. Prawn species are particularly susceptible of mislabelling due to the morphological similarities between them or the frequent absence of their carapaces in many seafoodstuffs. Thus, molecular tools are necessary to authenticate the species status. A species-specific PCR-assay was developed for authentication of five commercially relevant species: *Penaeus indicus, Penaeus monodon, Pleoticus muelleri*, *Aristeus antennatus* and *Palaemon serratus*. This method targeting a small-sized (<181 bp) mtDNA cytochrome b sequence ensures a template detection in fresh and frozen seafood products. Accuracy of the assay relies on the use of new species-specific primers. A reliable authentication is possible by this simple, fast and cost-effective PCR approach.

Keywords: prawn; shrimp; seafood; authentication; cytochrome b; species-specific primers

INTRODUCTION

Seafood is the most traded food commodity, even surpassing well-known agricultural products like wheat or sugar [1]. Crustaceans belonging to the order Decapoda comprise a wide variety of prawns, shrimps, lobsters, crayfish, and crabs of commercial interest. Among them, marine shrimps and prawns represent economically important worldwide resources [2].

In seafood products traceability is critical to guarantee quality and avoid health safety hazards such as allergies. Traceability also promotes conservation and sustainability of fisheries [3].

Though the European Union (EU) and USA are in the midst of the top seafood consumers per capita [4], the EU has currently superior seafood traceability regulations and requirements compared with other countries [3]. In the EU, the Council Reg. n. 1379/2013 [5], regulates the accuracy of labelling, repealing the previous provisions [for review see 6]. Under this

normative, fresh or processed crustaceans should be clearly labelled with commercial and scientific names, fishing gear category, net weight, food operator, identification mark, list of ingredients, production method, catch area and storage condition.

Mislabelling in seafood is often caused by unintentional or deliberate adulterations generally through the replacement of high-value species by lower priced ones [7]. Traditional species authentication for traceability purposes in prawns becomes complicated or even impossible due to morphological similarities between species. This is especially problematic when external anatomical parts are removed in commercialised products [8]. Therefore, reliable molecular tools are indispensable to authenticate species status, assuring the labelling compliance. It is necessary to point out that legislation is only useful when surveillance through audit, spot check or trace-back is adequately and regularly exercised by authorities and companies [9, 10].

Molecular tools relying on proteins or DNA analyses have been considered as suitable approaches for seafood species authentication. As DNA is more stable than proteins and it can resist processing, including high temperatures, protein-based methods have been overcome with the introduction of DNA tools [11, 7]. DNA-based methods, standing out polymerase chain reaction (PCR) assays, are the most widespread strategy for discriminating between closely related species [12].

Shrimps and prawns are one of the most globally demanded seafood group, accounting for 7.8 million tonnes in 2013, over 50% of crustacean market [13]. The highly commercialized Indian white shrimp (*Penaeus indicus)*, the black tiger prawn (*Penaeus monodon)*, the Argentine red shrimp (*Pleoticus muelleri),* the blue and red shrimp (*Aristeus antennatus)* the and common prawn (*Palaemon serratus)* were subjected to the present authentication study. In order to illustrate the great market demand of these species, stats data from the available sources collected in Food and Agriculture Organization of the United Nations (FAO) [13] are supplied here for each species. *Penaeus indicus* and *P. monodon* are subjects of an intense aquaculture farming activity, mainly in Indo-West Pacific countries, reaching 3,449 tonnes and 634,521 tonnes in 2014, respectively *Pleoticus muelleri* fishery takes place exclusively in Argentina, with 150,000 tonnes traded in 2016. *Aristeus antennatus* is fished along the Mediterranean coasts, with a global catch of 1,782 tonnes reported in 2014. *Palaemon serratus* fishery is conducted in some European communities, where 411 tonnes were traded in 2011. Although the amount of traded tonnes of *Aristeus antennatus* and *P. serratus* is lesser than the other three species, they reach high market prices, over 120 €/kg and 140 €/kg, respectively.

Mitochondrial DNA (mtDNA) markers have been applied as tracing tools for discriminating different prawn species. In particular, with regard to aforementioned species, Pascoal et al. [8] used cytochrome b (cyt b) in *Penaeus monodon* and *Pleoticus muelleri*; Pascoal et al. [14] targeted a 16S rDNA sequence in *Penaeus monodon* and *P. indicus;* and Fernandes et al. [7] used cytochrome c oxidase subunit I (COI) in *Penaeus indicus* and *P. monodon*. Nevertheless, species-specific approach was never accomplished in these species. Likewise, *A. antennatus* and *Palaemon serratus* are here analysed for the first time from a tracing perspective.

The aim of this work was the development of a species-specific PCR-assay to authenticate *Penaeus indicus, Penaeus monodon, Pleoticus muelleri*, *Aristeus antennatus* and *Palaemon serratus*, in fresh and frozen samples. A small-sized cyt b mtDNA sequence was chosen as a target using newly designed species-specific primers providing a feasible authentication methodology in the field of seafood inspection.

MATERIAL AND METHODS

Sample collection and DNA extraction

Frozen samples of *Penaeus indicus, P. monodon* and *Pleoticus muelleri* were collected from a local market. Fresh individuals of *A. antennatus* and *Palaemon serratus* were collected by the authors in several samplings in Mediterranean and Atlantic localities, respectively. All samples were stored in 70- 95% ethanol. Ten individuals from each species were analysed. Morphological

external traits were used for the all samples identification by a marine biologist expert in shrimp and prawn taxonomy from the Instituto de Ciencias Marinas de Andalucía (ICMAN, CSIC). Genomic DNA was extracted from about 25 mg of muscle abdominal tissue using NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal) following the manufacturer's instructions. DNA concentration and purity was determined with a NanoDrop 1000 Spectrophotometrer (ThermoScientific, Wilmington, DE, USA).

Primer design, PCR amplifications and electrophoretic analysis

Preliminarily, primers crustF (5´-GCTAATGGAGCGTCTTTCTTCTT-3´) and crustR (5´-TGGCTCCCCAGAATGATATTTG-3´) previously reported by Pascoal et al. [8] were used to amplify an internal 181 bp fragment of the mtDNA cyt b gene in the target species samples. PCR reactions were carried out in a total volume of 25 µL containing 25 ng of genomic DNA, 0.625 U of *Taq* DNA polymerase, 1× PCR buffer (Roche Diagnostics), 200 µM of each dNTP (Roche Diagnostics) and 0.2 µM of each primer. Amplification conditions were as described in Pascoal et al. [8]. PCR products were purified using ExoSap-IT PCR cleanup reagent (Affymetrix, Santa Clara, CA, USA), and all products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction v3.1 kit (Applied Biosystems, Paisley, UK) on an Applied Biosystem 3710 automated sequencer.

Identity of *Penaeus indicus, P. monodon* and *Pleoticus muelleri* was additionally confirmed by BLASTn (http://blast.ncbi.nlm.nih.gov), as cyt b sequences of these species were available in public databases.

Forward and reverse sequences were manually checked, edited with BioEdit v.7.2.5 software [15] and then aligned by the use of the Clustal W software [16] in order to identify nucleotide differences among species and to design species-specific primers. The novel cyt b primer pairs for each species are listed in Table 1.

PCR amplifications were performed in a 25 µL final volume containing 12.5 µL of NZYTaq II 2x Green Master Mix (NZYTech, Lisbon, Portugal), 0.5 µM of each primer and 25 ng of DNA template. Amplification profile was as follows: 94°C for 1 min 30 s, followed by 35 cycles of 94°C for 20 s, 51.5°C or 65°C for 20 s, 72°C for 30 s and a terminal extension step of 72°C for 15 min. Species-specific annealing temperature is detailed in Table 1. PCR products were run on 2% TAE agarose gels stained with Greensafe premium (Nzytech, Lisbon, Portugal) using a 50 bp DNA ladder VI as a molecular-weight size marker (Nzytech, Lisbon, Portugal). Then, amplicons were visualised under UV illumination in a Gel Doc XR system (BioRad Laboratories, Hercules, CA, USA).

All PCR assays were performed on a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA).

RESULTS AND DISCUSSION

Seafood has become an important key driver in European food industry [17], therefore authentication of commercial prawn species is crucial to avoid fraud by species substitution. Moreover, procedures as freezing can cause chemical and physical damages to the DNA, such as its degradation or fragmentation [18], complicating DNA testing issues. Consequently, molecular identification and authentication

Table 1 Oligonucleotide primers for cyt b species-specific PCR-assay

Species	Primer name	Oligonucleotides primers	Tm	Size (bp)	References
Penaeus indicus	crustF	5´-GCTAATGGAGCGTCTTTCTTCTT-3´	61	147	Pascoal et al. (2008)
	In-cytbR	5´-CACATATCCTAAGAAAGCT-3´			This study
Penaeus monodon	crustF	5´-GCTAATGGAGCGTCTTTCTTCTT-3´	65	149	Pascoal et al. (2008)
	Mon-cytbR	5´-AATACATACCCTAAGAAGGCAGTC-3´			This study
Pleoticus muelleri	crustF	5´-GCTAATGGAGCGTCTTTCTTCTT-3´	65	125	Pascoal et al. (2008)
	Mue-cytbR	5´-GCTATTACTAGCAGAAGGATTAC-3´			This study
Aristeus antennatus	crustF	5´-GCTAATGGAGCGTCTTTCTTCTT-3´	51.5	120	Pascoal et al. (2008)
	Ant-cytbR	5´-TACTAATAATAGAATCACAACTCCG-3´			This study
Palaemon serratus	crustF	5´-GCTAATGGAGCGTCTTTCTTCTT-3´	51.5	178	Pascoal et al. (2008)
	SeMon-cytbR	5´-CTCCCCAGAATGATATTTGTA-3´			This study

methods for food tracing have to assure the detection of the target DNA.

In the current study, a new simple, fast and cost-effective DNA-based technique was developed for the accurate authentication of five highly commercial value species: *Penaeus indicus, P. monodon, Pleoticus muelleri, A. antennatus* and *Palaemon serratus.* No molecular authenticating tool was previously reported for *A. antennatus* and *P. serratus* despite of their importance.

DNA amplification with primers crustF/crustR [8] allowed us to obtain cyt b sequences in target species (Fig. 1). Haplotype sequences are available in GenBank/EMBL under accession numbers: LT971317 to LT971325 and LT971329 to LT971331.

Novel reverse species-specific primers (Fig. 1) were designed and coupled with crustF, successfully amplifying in all individuals of each target species. The new primer pairs amplified fragments of 147 bp, 149 bp, 125 bp, 120 bp and 178 bp for *Penaeus indicus, P. monodon, Pleoticus muelleri*, *A. antennatus* and *Palaemon serratus* species, respectively (Table 1). The results of the amplification of the five-cited above species are shown in the Fig. 2. In this study the presence of a given species is not verified by bands size on electrophoresis gels, but through positive amplification with its respective primer pair.

Primers specificity was checked in all studied species. No cross-reactivity was found among them, except for *Palaemon serratus* crustF/Se-cytbR primer pair, which gave amplification in *P. serratus* and *Penaeus monodon* individuals*.* Afterwards *P. serratus* authentication was achieved by PCR product sequencing or by a second cross-reactivity-free PCR-assay using *Penaeus monodon* specific crustF/Mon-cytbR primer pair. A subsequent negative amplification reveals that the sample actually corresponds to *Palaemon serratus*. Targeting a small DNA region may result in the need for accomplish two PCR reactions, as in Pascoal et al. [14].

Mitochondrial DNA has been used successfully for food species identification due to its higher abundance respect to nuclear DNA in cells, rapid evolution, and greater sequence diversity compared to nuclear DNA [10]. In this field, mtDNA markers COI [e.g. 19], 16S [e.g.14], cyt b [e.g. 20] and 12S [e.g. 21] have been widely applied as well as the less extended ND4 [e.g. 22]. Regarding the identification of prawn and

Figure 1 Alignment and location of cyt b crust primers and novel species-specific primers designed in this study. Note: grey areas represent forward and reverse primers.

shrimp species in foodstuffs, there are many works using several mtDNA markers [see revision in 2]. Among these markers, cyt b has shown sufficient interspecific divergence to enable the design of specific primers in penaeid shrimps (Pascoal et al., 2008). Analysis of cyt b gene stands out in DNA-based methods in seafood authentication approaches [12]. Here, authentication of *Penaeus indicus, P. monodon, Pleoticus muelleri, A. antennatus* and *Palaemon serratus* was assured due to the use of species-specific cyt b designed primers. We reduced the cyt b target amplicon size under 180 bp in all ofstudied species. It has been reported that targeting short DNA fragment facilitates DNA amplification even in highly degraded samples caused by food industry [23, 24, 25] This fact makes our method a potential candidate to be implemented for authenticate the presence of these prawns in processed products in further studies.

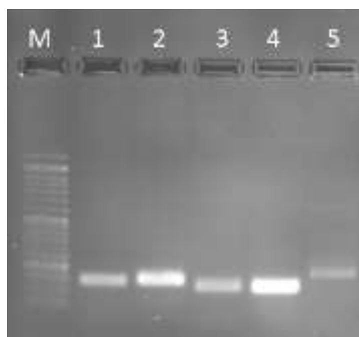

Figure 2 Size of cyt b PCR fragment for the five prawn species. Note: lane M represents DNA marker and lanes 1-5 represent *Penaeus indicus*, *Penaeus monodon*, *Pleoticus muellleri*, A*risteus antennatus* and *Palaemon serratus* respectively.

The PCR-based assay described in this study represents a suitable authentication tracing tool. PCR methods provide sensitivity, specificity, accuracy and reproducibility at once they do not require expensive equipment and highly qualified staff [26]. In order to contribute to the traceability effort, our protocol should be considered together with other valuable PCR-based seafood tracing methods previously reported, for instance PCR-RFLP [8], PCR-sequencing [19], RT-PCR [27], HRM [7], PCR-ELISA [28], FINS [29], SSCP [30], and RT-NASBA [31]. For seafood authenticity purposes, PCR using species-specific primers approaches, such the method described in this work, have been previously developed for several species [32 and references therein]. These species-specific methodologies are affordable because they neither require expensive fluorescent-labelled reagents or endonucleases nor sequencing after PCR. The use of the Master Mix cited here implies also a decrease of assay costs and time, as the addition of a dyed loading buffer is not needed in electrophoresis step. In this sense, a faster DNA extraction procedure recently described could be implemented for fresh, ethanol stored tissues or processed samples [33]. This method is appropriated for crustaceans, specifically *A. antennatus* was one of the species included in that study.

The approach described in this work represents a useful tool for authentication of relevant prawn species that complements previously developed methods. This new assay offers a necessary feasible tool for administration and seafood industry to verify labelling compliance.

CONCLUSIONS

A new PCR-assay using species-specific primers allows the detection and authentication of *Penaeus indicus, P. monodon, Pleoticus muelleri, A. antennatus* and *Palaemon serratus* fresh and frozen samples. The proposed method is simple, rapid and affordable without losing accuracy, reliability and sensitivity. We provide a useful tool for food industry and control laboratories for routine analysis of these commercially relevant prawn species to unmask mislabelling and/or fraudulent situations and to assess authenticity in seafood products.

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CORRIGENDUM

Since the publication of the scientific articles that constitute the present thesis, we have noticed some errors. Although in general do not alter the main conclusions of the articles we consider should be corrected. Therefore, we list them below. The typographical errors are not recorded, unless they were misleading.

Corrections to CHAPTER 1.1: **Karyological analysis of the shrimp *Palaemon serratus* (Decapoda: Palaemonidae).**

In this research article we have used the term 'shrimp' to refer to *Palaemon serratus,* we have also indiscriminately interchanged 'shrimp' and 'prawn' in CHAPTER 1.2, and from there on out we used 'prawn'. In scientific literature there is no clear distinction between both terms and, although the term 'shrimp' is sometimes applied to smaller species while 'prawn' is more often used for larger forms, their usage is often confused or even reversed depending on the country (FAO, 2018). Finally, we considered use the word 'prawn' since the naturalist Thomas Pennant categorised *P. serratus* as 'the common prawn' in 1777.

Corrections to CHAPTER 1.2: **Comparative cytogenetic analysis of marine *Palaemon* species reveals a $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in *Palaemon elegans*.**

During the revision of the proofs of this article, a paragraph in the 'Abstract' section was no modified: '*The karyotype of the females consisted of the three largest acrocentric pairs and (…)*' should be read as follows:

The karyotype consisted of 43 autosomal chromosome pairs: 5 metacentric/submetacentric, 4 subtelocentric/telocentric, and 34 hardly distinguishable due to the small size. The karyotype of the females also included two large telocentric sex chromosome pairs, while that of the males included one clearly identifiable large metacentric chromosome and two telocentric chromosomes.

In the 'Abstract' section the telomeric sequence was incorrectly written. The correct sequence is $(TTAGG)_n$.

GENERAL DISCUSSION

Contributions of this thesis to cytogenetic data in *Palaemon*

Cytogenetic studies not only provide basic information on the number, size and morphology of chromosomes, but also allow to determine the ploidy level (Suda et al. 2006) and to identify chromosomal rearrangements (Gross et al. 2010). From a practical point of view, this genetic approach is extremely useful in aquaculture. Some of the most important applications of the cytogenetic in aquaculture include: 1) verifying the success of chromosome set manipulation processes, such as polyploidy induction (de Almeida et al. 2011), gynogenesis and androgenesis, sex control and intra- and interspecific hybridizations (Dai and Han, 2018), 2) assessing the level of cytogenetic stability among polyploids (Benabdelmouna and Ledu 2015), and 3) assigning linkage groups to physical chromosomes, to integrate physical and recombination genetic maps (Doudrick et al. 1995).

The economic importance of numerous crustacean species (i.e., the common prawn *Palaemon serratus*) and the prospect that in the near future these species can be farmed in fisheries and aquaculture have brought about a greater interest in some aspects of their cytogenetics. Hence, the cytogenetic data reported in CHAPTER 1.1 and 1.2, provide the necessary basis to implement possible genetic improvement technologies in this species.

On the other hand, the phylogeny of *Palaemon* has been under continual revision and it has not been completely clarified (Carvalho et al. 2017). In this context, karyological studies based on conventional staining and banding techniques have proven useful for inferring phylogenetic relationships and delimiting subspecies and species boundaries in many animal groups (e.g. Hillis 1991; Volleth and Heller 1994; Odierna et al. 1996; Mazurok et al. 2001; Shibusawa et al. 2004; Targueta et al. 2010). A recent cytogenetic study has shown the special value of chromosomal markers as a taxonomic character to detect invasive and cryptic species in marine mussels (García-Souto et al. 2017).

Unfortunately, the vast majority of crustacean species have not been subject of cytogenetic analyses (less than 2% of the Crustacea), having been considered particularly challenging for this type of analysis (Mlinarec et al. 2011). During the last years, the number of studies developed in this field has not increased significantly. Most of these studies only focus on estimating the chromosome number and applying conventional staining for cytogenetic characterisation, but do not perform FISH assays. Niijama (1959) and many authors thereafter, have attributed the lack of information to the fact that these species have high diploid chromosome numbers, usually small and of similar size and highly condensed. Only three *Palaemon* species

(*P. carinicauda*, *P. modestus* and *P. khori*), and from Asian regions, have been studied cytogenetically until now (Jiang et al. 2008; Li et al. 2012; Hassan et al. 2015). *Palaemon serratus* was the first European species of the genus to be cytogenetically characterised (CHAPTER 1.1). In order to start unravelling the chromosome evolution of the genus, in CHAPTER 1.2 we extended the cytogenetic analysis to other European representative, *P. elegans*. Overall, we applied classical and molecular cytogenetic methods based on conventional staining, banding techniques and fluorescence *in situ* hybridization in these species; and it allowed us to perform a detailed comparative cytogenetic analysis between them.

We revealed the diploid chromosome number of *P. serratus* (2n=56), the lowest known diploid chromosome number in the family Palaemonidae (CHAPTER 1.1). The karyotype consisted of 56 chromosome pairs: 20 telocentric, 6 subtelocentric, and 2 large metacentric. Interestingly, the karyotype of *P. elegans* was composed of 2n=90 chromosomes in females and 2n=89 chromosomes in males (CHAPTER 1.2). The diploid chromosome number of *P. elegans* is similar to those reported to *P. carinicauda*, *P. modestus* and *P. khori* (2n=90, 2n=90 and 2n=96, respectively), but it is strikingly higher than that of the other European species, *P. serratus* (2n=56). We hypothesized that the main mechanism of karyotype evolution of the genus would involve several fusion events giving rise to a reduction on the chromosome number in *P. serratus* (see CHAPTER 1.2 for details).

Ribosomal DNAs (rDNAs) are important components of eukaryotic chromosome complements and comprise conserved regions. They have been, and still are, the main chromosome marker of choice (Sochorová et al. 2017). The number of rDNA loci has been widely used to characterise animal and plant species (for a review, see García et al. 2014; Sochorová et al. 2017). In both *P. serratus and P. elegans*, the major ribosomal genes were observed at the telomeres of two chromosome pairs (four sites of probe hybridization). Considering the whole online animal rDNA database (www.animalrdnadatabase.com), this number reflects the average number of 45S per diploid chromosome set (3.8). Indeed, the median is two sites (single locus/1C) for 45S, indicating that most karyotypes tend to maintain loci numbers moderately low, despite large variation (1–54 sites) (Sochorová et al. 2017). Likewise, the distal and pericentromeric chromosomal positions are the most prevalent (> 75% karyotypes) for 45S loci (Sochorová et al. 2017). Telomeric sequences $(TTAGG)_n$ were detected at the telomeres of all chromosomes in the two studied species. Therefore, we demonstrated the presence of the TTAGG repeat, known as the ancestral motif of arthropod telomeres, for the first time in the family Palaemonidae.

The comparative analysis between the chromosome complements of both sexes of *P. elegans* showed considerable differences. The karyotype consisted of 43 autosomal chromosome pairs: 5 metacentric/submetacentric, 4 subtelocentric/telocentric, and 34 hardly distinguishable due to their reduced size. Female karyotype also included two large telocentric sex chromosome pairs, while that of the males included one clearly identifiable large metacentric chromosome and two large telocentric chromosomes. Thus, male heterogamety is evidenced by a metacentric chromosome present only in the male karyotype (Y chromosome). Analysis of meiotic diakinesis shed light on sex chromosome behaviour in *P. elegans*. During meiosis, each arm of the large metacentric Y is terminally associated with one telocentric chromosome (X_1 and X_2) forming a trivalent (X_1X_2Y). Thus, we confirmed the presence of the $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system in this species. This is the first sex chromosome system discovered in *Palaemon* and the only sexual system reported for Decapoda so far using modern cytogenetic techniques. Some considerations discussed in CHAPTER 1.2 led us to hypothesise that the multiple sex chromosome system in this prawn species is a result of recent evolution (see CHAPTER 1.2 for details). Remarkably, the C-banding technique revealed a lack of constitutive heterochromatin in the sex chromosomes, not even in the Y chromosome. It is also noteworthy that C-banding pattern did not evidence the existence of heteromorphic or heterochromatic chromosomes in *P. serratus*.

The hypotheses developed in CHAPTER 1.2 stress the need for future studies including cytogenetic and molecular approaches to better understand the driving forces and molecular pathways underlying the evolutionary dynamics of karyotypes, sex chromosomes, and sex-determining mechanisms of *Palaemon* in a comparative framework. For this purpose, we intend to select other congeneric species closely related to the members included in the present work. Based on a recent phylogenetic analysis (Carvalho et al. 2017), the ideal candidates would be the European species *P. adspersus* (the type species of the genus), *P. longirostris* and *P. xiphias*; and the American species *P. floridanus* and *P. northropi*.

Recently, a new databank from the first transcriptome assembly and annotation of *Palaemon serratus* (Perina et al. 2017) have been reported, opening new scenarios to investigate some essential questions (as *What is the sex determination mechanism in P. serratus?*) that remain unanswered. Chandler et al. (2017) have proposed the functionally conserved sex regulators, *Dmrts*, as target genes to guide studies into sex determination in Decapoda. So far, several *Dmrts* homologues have been identified in seven species comprising *Penaeidae*, *Palinuridae*, *Palaemonidae* and

Portunidae (Chandler et al. 2017). Future studies focusing on gene expression should address the validation of these sex-specific genes.

Contributions of this thesis to the management of *Palaemon serratus* populations

Population boundaries are difficult to define within marine ecosystems (Taylor et al. 2000); albeit, to achieve effective management of wildlife populations, it is necessary to delimitate discrete, countable, and reasonable units (Coder 1996; Evans and Teilmann 2009). Such units are separate groups of individuals within the species itself that form genetic units of evolution (Abdul-Muneer 2014) and thus will respond uniquely and independently to fishing pressure (Ovenden et al. 2015). These units, or biological stocks, are critical for the management and conservation of wild fisheries. In that sense, species affected by fishing should be managed to minimize the loss of genetic diversity, which is a key measure of resilience and abundance (Ovenden et al. 2015). Besides the genetic diversity, it is crucial to determine genetic differences among populations considering that it provides information about gene flow among them and about their evolutionary history (Allendorf et al. 2013). Proper management of the species reduces not only the depletion of stocks in terms of numbers but also minimizes their genetic deterioration (Romana-Eguia et al. 2015).

With the rapid growth of molecular biology, the use of genetic markers as tools to determine patterns of gene flow and, therefore, to define biological stocks, have been widespread (Watts et al. 2009). In this regard, because of its intrinsic properties (high mutation rate, absence of recombination…), mitochondrial DNA (mtDNA) has been widely used in studies of population genetics, species identification and molecular phylogenetics (Brown et al. 1979; Avise et al. 1987; Moritz et al. 1987; Hebert et al. 2003; Galtier et al. 2009). Such is its discriminatory power, that the mitochondrial fragment cytochrome *c* oxidase 1 (Cox1) was selected as the standardised tool for molecular taxonomy and identification (Ratnasingham & Hebert 2007). Nevertheless, the combination of mitochondrial and nuclear markers is considered the best option to evaluate the genetic status of populations. Actually, with respect to marine organisms, the most commonly used molecular markers are mitochondrial and microsatellite (nuclear) markers (Cuellar-Pinzón et al. 2016).

Reuschel et al. (2010), analysing the DNA-sequences of two mitochondrial genes (16S rRNA and Cox1), revealed a genetic differentiation between Atlantic and Mediterranean populations of *P. elegans*. Due to the ecological and economic relevance of the sympatric species *P. serratus*, it was necessary to test the occurrence of a similar population structure in this species that allows us to implement effective management strategies. In fact, Haig et al. (2014) pointed out in their review of the *P. serratus* fishery in UK and Ireland that *P. serratus* fishery is not

currently subject to any particular management regime other than a closed season between May and August in Ireland to protect stocks from overexploitation.

In CHAPTER 2, we provided the first phylogeographic analysis of *P. serratus* along European coastlines, based on one nuclear (Enolase) and two mitochondrial genes (Cox1 and 16S rRNA). In this study, a pronounced genetic differentiation was detected between the Mediterranean and the Atlantic localities. Besides *P. elegans* (Reuschel et al. 2010) and *P. serratus*, this Atlantic-Mediterranean division has been recorded for many marine species in general and numerous crustacean in particular such as the pelagic crustacean *Meganyctiphanes norvegica* (Zane et al. 2000), the shrimp *Crangon crangon* (Luttikhuizen et al. 2008), the spiny lobster *Palinurus elephas* (Palero et al. 2011), or the Norway lobster *Nephrops norvegicus* (Gallagher et al. 2018). Nevertheless, in many of these studies the phylogeographic break responsible for the species genetic subdivision is still unclear. Certainly, as noted in their review Patarnello et al. (2007) `*despite a wealth of historical and oceanographic data, the Atlantic-Mediterranean transition remains controversial at the biological level as there are discordant results regarding the biogeographical separation between the Atlantic and Mediterranean biota*`. In the case of *P. serratus*, a biogeographic barrier appears to be located west of the Strait of Gibraltar, given that sampling sites from the Gulf of Cádiz were more closely related to the Mediterranean population than to the other Atlantic localities. This was also reported in the mysid *Neomys integer* (Remerie et al. 2009). Interestingly, the opposite scenario was postulated by Reuschel et al. (2010) for the counterpart *P. elegans*, where the Almería-Orán Front is the hydrographic boundary between the Atlantic and Mediterranean populations. According to Tintore et al. (1988), the Almería-Orán Front is a semi-permanent dynamic oceanographic structure and it has been described as the main barrier restricting genetic flow along the Atlantic–Mediterranean transition area (e.g. Galarza et al. 2009). This is for instance the case of the krill *Meganyctiphanes norvegica* (Zane et al. 2000), the scallops *Pecten jacobeus* and *P. maximus* (Ríos et al. 2002), the cuttlefish *Sepia officinalis* (Pérez-Losada et al. 2002; 2007) or the European sea bass *Dicentrarchus labrax* (Lemaire et al. 2005). However, a recent study on seven crustacean species showed that the Almería-Orán Front has not effect on their genetic structure (García-Merchán et al. 2012). This statement is also in agreement with a phylogeography study on the red shrimp *Aristeus antennatus* (Fernández et al. 2011); indicating that the role of this front cannot be generalized and that other discontinuities, such as the Gibraltar Strait or the Ibiza Channel, can reduce the gene flow between the two basins (García-Merchán et al. 2012). In line with that, the boundary located somewhere west of the Strait of Gibraltar for *P. serratus* populations should be taken into account to

investigate genetic differentiation in other coastal species with a larval dispersal phase.

Concerning the management of the common prawn *P. serratus*, our results suggested the existence of two clearly differentiated populations within the species that indicate the need to establish two priority management areas. These areas should be taken into consideration when establishing specific policies for the sustainable regulation of regional fisheries. Genetic stocks should be also considered for the development of breeding and restocking programs for this highly commercial prawn species.

Now we know that genetic differentiation occurs in this species, further studies are indispensable to better define genetic stocks of this natural resource and to delimit the biogeographic barrier between the Atlantic and Mediterranean populations. In this regard, the analysis of polymorphic molecular markers such as microsatellites loci could reveal fine-scale genetic structure even the level of genetic differentiation among populations is low (Wright and Bentzen 1995).

Contributions of this thesis to seafood traceability

Traceability is destined to enable tracking a product along the production and distribution chain, from the raw materials to the end-consumer, and it makes use of the labelling as a tool for achieving this aim (D`Amico et al. 2016).

Given that seafood is the most traded food commodity worldwide, even surpassing well-known agricultural products (Asche, 2014), the EU has implemented a legal framework that regulates the accuracy of its labelling (EU 1379/2013) (D`Amico et al. 2016). Under the aforementioned normative, fresh or processed seafood should be clearly labelled with commercial and scientific names, fishing gear category, net weight, food operator, identification mark, list of ingredients, production method, catch area and storage condition. Labelling compliance is crucial to guarantee the product quality and minimize food safety risks (Leal et al. 2015) and also to promote the sustainable exploitation and the conservation of exploited stocks (Bernatchez et al. 2017). Thus, species identification and authenticity control are major concerns to the seafood industry. Traditionally, the identification of species in seafood products was based on observed morphological traits. However, these features are particularly difficult to use in species identification among seafood species in general, and in prawns belonging to the order Decapoda in particular, because of their phenotypic similarities and to the fact that during processing, the external carapace is often removed (Ortea et al. 2012). This explains why they are often regarded as adulteration targets (Fernandes et al. 2017). In 2014, a report by the Ocean Conservation Group, Oceana, revealed that 15% of prawns are mislabelled in terms of either the method of production or species (Wilwet et al. 2018).

Molecular authentication methods provide useful tools to verify species status and labelling information (Bernatchez et al. 2017). Actually, molecular tools relying on biomarkers targeting proteins or, more frequently, DNA, have been proposed as suitable strategies for prawn species identification or authentication (Griffiths et al. 2014; Fernandes et al. 2017). When talking about DNA-based approaches for food analysis purposes, the polymerase chain reaction (PCR) plays a crucial role (Böhme et al. 2015). PCR-based assays provide sensitivity, specificity, accuracy and reproducibility at once, and they do not require expensive equipment and highly qualified staff (Xue et al. 2017). Taking all of these advantages into account, species-specific PCR-assays are faster, simpler and more affordable than other PCR-based authentication methods as PCR-RFLP (Pascoal et al. 2008) or PCR-sequencing (see CHAPTER 3).

For seafood authenticity purposes, PCR-based methods using species-specific primers have been previously developed in several seafood species (Böhme et al. 2015 and references therein).

In order to provide new species authentication tools for traceability studies according to existing regulations, in CHAPTER 3 we developed a species-specific PCR-assay to authenticate the presence of common prawn *P. serratus* in fresh and frozen seafood samples. In this work, we also extended this procedure to other four economically important prawn species, in particular the Indian white shrimp (*Penaeus indicus*), the black tiger prawn (*Penaeus monodon*), the Argentine red shrimp (*Pleoticus muelleri*) and the blue and red shrimp (*Aristeus antennatus*). *Palaemon serratus* and *A. antennatus* were included here for the first time from this type of analysis. A small-sized cyt b mtDNA sequence was the target for species-specific primers design providing a sensitive authentication methodology to be applied in the field of seafood inspection.

CONCLUSIONS

Main conclusions of Chapter I: Karyological characterization of *Palaemon serratus* and *Palaemon elegans.*

I. The diploid chromosome number in males and females of *P. serratus* is 2n=56 chromosomes. In both sexes, the karyotype consists of 2 large metacentric, 6 subtelocentric and 20 telocentric chromosome pairs. Comparisons between *P. serratus* female and male karyotypes indicated a putative absence of heteromorphic sex chromosomes.

II. The diploid chromosome number of *P. elegans* is 2n=90 for females and 2n=89 for males. In both sexes, the karyotype consists of 43 autosomal chromosome pairs: 5 metacentric/submetacentric, 4 subtelocentric/telocentric, and 34 hardly distinguishable chromosomes due to their small size. The chromosome complement of females also includes 2 large telocentric chromosome pairs ($X_1X_1X_2X_2$), while that of the males includes one large metacentric (Y) and two telocentric chromosomes (X_1X_2).

III. Comparative analyses between the karyotypes of both sexes of *P. elegans* in addition to their meiotic behavior, revealed the presence of an $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system. The existence in males of meiotic recombination between the sex chromosomes, as well as the large euchromatic Y chromosome, suggests the possibility that the multiple sex chromosome system is a result of recent evolution.

IV. The reduced chromosome number of *P. serratus* constitutes a derived character in Palaemonidae. Fusion events could be the mechanism responsible for the origin of this karyotype, but this hypothesis needs further investigation.

V. C-banding revealed that constitutive heterochromatin has a predominantly telomeric distribution in both species of *Palaemon*. In *P. serratus* we did not find differences in the constitutive heterochromatin pattern between sexes. In *P. elegans*, C-banding revealed the absence of constitutive heterochromatin in the sex chromosomes.

VI. According to fluorescent *in situ* hybridisation, the major ribosomal genes are located in terminal positions on two small chromosome pairs in both

species, which may constitute a plesiomorphic character in the genus. Also, the presence of the ancestral motif of arthropod telomeres, a TTAGG repeat, was revealed.

Main conclusions of Chapter II: Genetic differentiation between Mediterranean and Atlantic populations of the common prawn *Palaemon serratus* (Crustacea: Palaemonidae) reveals uncommon phylogeographic break.

VII. Results of three analysed genes (Cox1 mtDNA, 16S rRNA and Enolase nuDNA) showed the existence of genetic differentiation between *P. serratus* localities in the Mediterranean Sea, the Guadalquivir Estuary and the Atlantic Ocean.

VIII. The Mediterranean population includes all individuals from Guadalquivir Estuary, indicating that a biogeographic barrier that limits genetic flow between Atlantic and Mediterranean populations is located west of the Strait of Gibraltar, an uncommon phylogeographic break.

IX. Concerning the management of the common prawn *P. serratus*, our results revealed the existence of two differentiated populations within the species that indicate the need to establish two priority management areas.

Main conclusions of Chapter III: Molecular authentication of six economically important prawn species by species-specific PCR-assay

X. The species-specific PCR-assay developed in this work allows for detection and authentication of five prawn species (*Penaeus indicus*, *Penaeus monodon*, *Pleoticus muelleri*, *Aristeus antennatus* and *Palaemon serratus*).

XI. This method, targeting a small cytochrome *b* fragment, ensures template detection in fresh and frozen food products, and provides a simple, fast and cost-effective tool for food industry and control laboratories.

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