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Genetic analyses in the gooseneck barnacles (genus *Pollicipes*)

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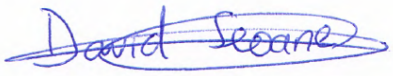
INFORMAN

Que el trabajo titulado "Genetic analyses in the gooseneck barnacles (genus *Pollicipes*)", presentado por D. David Seoane Miraz para optar al título de doctor en biología con mención internacional ha sido realizado bajo nuestra dirección en el Área de Genética del Departamento de Biología Celular y Molecular de la Universidade da Coruña.

A Coruña, 21 de septiembre de 2007



Fdo.: David Seoane Miraz

Fdo.: Ana M. González Tizón

Fdo.: Andrés Martínez Lage

To Fátima

To my parents

Acknowledgements

All big dreams in life begin as a small idea to materialize, a small challenge to conquer, a small goal to reach. But these dreams we long to see fulfilled are not achieved from one day to the next. They are a long process that requires learning, effort and dedication. They are unable to do by oneself and require the participation of people who are always by your side, although some of them believe they have not made notable contributions, the attainment of this dream would not be possible without all of them.

But I will call things by their name. As a child, I wanted to achieve a great dream: to become a good researcher in biology, specifically, a good geneticist. For this challenge, I needed to be diligent, hardworking and persevering. And, even after some years I have made some achievements as finishing my studies and complete this thesis, I am still far from my initial goal. During this long and committed journey, I realized that I could not have managed to achieve this goal alone. There have been many people who have been with me throughout this process, and their support and affection are essential to achieve closer to my dream.

Every path starts with a first step and reach the goal requires a strong will power. So, I want to thank everyone who believed in me during this journey, especially my partner, Fátima, for their unconditional support in all the times we shared, for encouraging me to improve and to be my biggest inspiration to keep going. To my parents, José Lino and María del Carmen, who encouraged me with their unshakable faith in me and are my greatest motivation. My grandparents, Martin and Victorina, thank you for always being so proud of my accomplishments and you are my example of self-improvement. And all the rest of my family, especially my cousins, Fran and Xana, for all the support they have given me during all the good times shared since ever and which have brought me to this day.

But to follow any path is always better to have the help of a guide, and in my case I have been fortunate to have two, my tutors, Ana Mª González Tizón and Andrés Martínez Lage, who I want to convey my deepest gratitude for preparing me to overcome the obstacles of the way, for putting them trust in me

and for can count on them at all times. But I have not travelled alone this road, and my partners from GIBE researching group, both those who have shared this entire race since its beginning as those that have been incorporated into this path, deserve my gratitude for helping me in every moment of difficulty and for having shared moments of happiness.

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And though this stage concludes, a new one opens before me which I hope it brought me closer to my dream…

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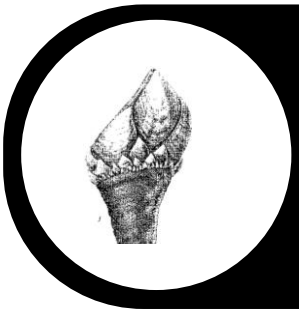


SUMMARIES

1.1. Resumo.

Os percebes do xénero *Pollicipes* son crustáceos mariños de vida sésil que habitan costas rochosas expostas a fortes mareas e ondas nas zonas costeiras occidentais de Europa, África e América. En base a diferenzas morfolóxicas do pedúnculo describíronse dous fenotipos: percebes de sol e de sombra. Os resultados obtidos permitiron reconstruír filoxenias nesta familia en base a marcadores mitocondriais (16S ADNr e COI) e nucleares (18S-28S ADNr e EF1α) que mostraron topoloxías discrepantes debido ás diferentes forzas evolutivas ás que se atopa sometido cada marcador. Os datos estruturais obtidos nas rexións ITS1, 5.8S e ITS2 permitiron identificar a orixe monofilética de certos taxones así como establecer os patróns de pregamento de ditas rexións no subfilo Crustacea. Os marcadores microsatélites optimizados permitiron comprobar a existencia de panmixis entre as poboacións dos percebes *P. pollicipes* nas costas atlánticas e elaborar un modelo loxístico para determinar a súa posible orixe xeográfica. Os estudios de expresión xénica permitiron validar a utilidade dos xenes de referencia albumina, HSP70, HSP90, actina, β-actina e histona H3 para este tipo de estudios e detectar diferenzas significativas entre certos xenes relacionados coa integridade e musculatura do pedúnculo entre os dous fenotipos do percebe.

1.2. Resumen.

Los percebes del género *Pollicipes* son crustáceos marinos de vida sésil que habitan costas rocosas expuestas a fuertes mareas y oleajes en las zonas costeras occidentales de Europa, África y América. En base a diferencias morfológicas del pedúnculo se han descrito dos fenotipos: percebes de sol y de sombra. Los resultados obtenidos permitieron reconstruir filogenias en esta familia en base a marcadores mitocondriales (16S ADNr y COI) y nucleares (18S-28S ADNr y EF1α) que mostraron topologías discrepantes debido a las diferentes fuerzas evolutivas a las que se encuentra sometido cada marcador. Los datos estructurales obtenidos en las regiones ITS1, 5.8S e ITS2 permitieron identificar el origen monofilético de ciertos taxones así como establecer los patrones de plegamiento de dichas regiones en el subfilo Crustacea. Los marcadores microsatélites optimizados permitieron comprobar la existencia de panmixis entre las poblaciones de los percebes *P. pollicipes* en las costas atlánticas y elaborar un modelo logístico para determinar su posible origen geográfico. Los estudios de expresión génica permitieron validar la utilidad de los genes de referencia albúmina, HSP70, HSP90, actina, β-actina e histona H3 para este tipo de estudios y detectar diferencias significativas entre ciertos genes relacionados con la integridad y musculatura del pedúnculo entre los dos fenotipos de percebe.

1.3. Abstract.

Barnacles from the genus *Pollicipes* are marine crustaceans of sessile lifestyle which inhabit rocky coasts exposed to string waves and tides in western coasts of Europe, Africa and America. Two phenotypes have been described based on morphological differences of the peduncle: sun barnacles and shadow barnacles. Obtained results let reconstruct phylogenies in this family using mitochondrial (16S rDNA and COI) and nuclear (18S-28S rDNA and EF1α) markers which showed discrepant topologies due to the different evolutionary forces which drive the molecular evolution of each marker. Structural data obtained from ITS1, 5.8S and ITS2 regions let identified the monophyletic origin of certain taxa, and establish the folding of these regions in subphylum Crustacea. Optimised microsatellite markers let corroborate the existence of panmixis between wild populations of *P. pollicipes* in Atlantic coasts and develop a logistic model to determine the geographical origin of these populations. Genic expression assays let validate the utility of the reference genes albumin, HSP70, HSP90, actin, β-actin and histone H3 in this kind of studies and detected significant differences between certain genes related to peduncular integrity and muscularity between both phenotypes.

1.4. Resumen extendido.

Los percebes del género *Pollicipes* son crustáceos marinos de vida sésil pertenecientes a la infraclase Cirripedia Burmeister, 1834; superorden Thoracica Darwin, 1854; orden Scalpelliformes Buckeridge and Newman, 2006. Las cuatro especies actuales pertenecientes a este género incluyen a *P. pollicipes* distribuido desde la costa norte de Francia (Bretaña) hasta las costas africanas de Dakar; *P. elegans* habita en las costas del océano Pacífico desde México hasta Chile; *P. polymerus* es frecuente en las regiones intermareales de Norteamérica desde Alaska hasta la península de Baja California; y *P. caboverdensis* se localiza en las costas que circunscriben el archipiélago de Cabo Verde. Estas cuatro especies habitan costas rocosas expuestas a fuertes oleajes y mareas donde el agua está constantemente agitada y oxigenada.

Morfológicamente se caracterizan por presentar un pedúnculo flexible revestido por una cutícula formada por pequeñas escamas calcificadas. Este pedúnculo está constituido por tres capas musculares con diferente orientación, el ovario y las glándulas cementantes. Sobre el pedúnculo se encuentra el capítulo constituido por placas de número y forma variables que presentan valor taxonómico para la identificación de especies y dentro del cual se encuentran protegidos los principales órganos vitales. La especie europea de percebe, *P. pollicipes*, presenta dos fenotipos en base a diferencias morfológicas de su pedúnculo: el fenotipo de sol, caracterizado por comprender a percebes más pequeños y robustos con pedúnculos musculosos; y el fenotipo de sombra, cuyos percebes son alargados y delgados y sus pedúnculos muestran un gran contenido en agua.

Los percebes del género *Pollicipes* son organismos hermafroditas con fecundación interna y su ciclo vital incluye seis fases de larvas nauplius y una final de larva cipris. En este último estadío larvario se fijan al pedúnculo de un adulto y sufren metamorfosis para convertirse en juveniles. El periodo reproductivo de *P. pollicipes* dura 210 días, desde marzo hasta septiembre, e incluye dos periodo de liberación de larvas, uno al final del invierno (marzo-abril) y otro durante el verano (julio-octubre).

En los mercados españoles, el percebe *P. pollicipes* es un importante recurso marisquero siendo Galicia el principal proveedor. Los precios que puede alcanzar en los mercados varían significativamente a lo largo del año y pueden llegar hasta los 254.50 €/kg en ciertas épocas. Esta gran demanda a la que se encuentra sometido ha causado la importación de percebe de menor calidad de otros países como Francia, Portugal, Marruecos e incluso Canadá y El Perú.

Los estudios genéticos previos realizados en organismos crustáceos han mostrado su origen parafilético, y constituiría un grupo monofilético junto con los insectos conocido como Pancrustacea o Tetraconata. Esta topología ha sido reconstruida por diferentes autores empleando diferentes marcadores mitocondriales y nucleares, y mediante el uso de caracteres morfológicos. A pesar de estos estudios filogenéticos, diferentes taxones de organismos se han ido incorporando a la filogenia de los crustáceos. En el caso de la familia Pollicipedidae, ha presentado un origen monofilético en las diferentes reconstrucciones filogenéticas que han sido realizadas por diferentes autores en base a marcadores moleculares nucleares y mitocondriales. A pesar del empleo de diferentes marcadores, las topologías filogenéticas obtenidas entre las cuatro especies que constituyen la familia Pollicipedidae no han sido concordantes.

La estructura secundaria de los ácidos nucleicos es necesaria para el correcto desarrollo de las funciones biológicas como la regulación de la expresión génica, los procesos de splicing o las interacciones macromoleculares. Esta estructura secundaria depende directamente de la estructura primaria, así mutaciones de la secuencia nucleotídica pueden causar alteraciones significativas en la estructura secundaria. Sin embargo, hay que tener en cuenta que algunos cambios compensados en ambas cadenas del ADN pueden no alterar la estructura secundaria. Los datos estructurales son útiles para redefinir y precisar las filogenias así como asistir en la identificación o reforzar nuevas especies. Una de las regiones más usada en estos estudios estructurales son los genes ribosomales mayores que aparecen repetidos en tándem en los genomas de los organismos cientos de veces. La estructura secundaria presentada por estos genes ha sido estudiada mediante técnicas

cristalográficas en eucariotas. En base a estos datos cristalográficos, la región del ADNr 5.8S ha mostrado dos regiones de emparejamiento con el ADNr 28S y tres hélices dobles, estando la primera de ellas en dirección 5'→3' dividida en dos subhélices. Los espaciadores internos transcritos 1 y 2 (ITS1 e ITS2) que flaquean el ADNr 5.8S presentan estructuras secundarias similares basadas en un anillo abiertos con un número variable de hélices prominentes. Existen muy pocos estudios estructurales en los organismos crustáceos, conociéndose solamente la estructura secundaria del ADNr 5.8S y el ITS2 en la especie *Artemia salina*.

Los marcadores microsatélites están ampliamente empleados en los estudios de análisis genético de poblaciones de organismos salvajes por ser muy útiles para determinar la diversidad genética, estructura poblacional, tamaño efectivo y posibles eventos de cuello de botella o tasas de migración. Estos marcadores se caracterizan por presentar de uno a seis nucleótidos, ser abundantes en los genomas nucleares, selectivamente neutrales, codominantes, polimórficos, presentar herencia mendeliana, estar distribuidos a lo largo del genoma y poder ser específicos de especie.

En el caso de los percebes *P. pollicipes*, como muchos organismos marinos, la estructura poblacional presentada por estos animales depende de las corrientes oceánicas. Esta estructura poblacional ha sido estudiada por varios autores que detectaron un alto grado de homogeneidad genética a lo largo de su rango de distribución. Estos análisis también incluyeron el estudio de los refugios glaciares que utilizó esta especie durante los eventos glaciares e interglaciares del Pleistoceno, y la expansión demográfica de la especie durante el último máximo glacial.

La variación fenotípica entre organismos de la misma especie puede ser debida a diferentes factores, siendo las diferencias en los niveles de expresión de los genes codificantes uno de los más importantes. La cuantificación de los niveles de expresión génica se lleva a cabo mediante el empleo de genes de referencia que muestren niveles de expresión estables en todas las condiciones estudiadas permitiendo estandarizar los niveles de expresión de los genes de interés. Estos experimentos se realizan mediante PCR en tiempo

real que permite detectar los niveles de los productos amplificados mediante el uso de reactivos fluorescentes.

Los objetivos planteados en esta tesis doctoral incluyen: 1) la inferencia de filogenias mediante la utilización de marcadores nucleares y mitocondriales que permitan establecer las relaciones evolutivas entre las especies de la familia Pollicipedidae; 2) la determinación de la estructura secundaria de las regiones del ADNr 5.8S y espaciadores internos transcritos 1 y 2 en crustáceos; 3) el desarrollo y optimización de marcadores microsatélites en la especie *P. pollicipes*, 4) la utilización de estos marcadores microsatélites para estudiar la estructura poblacional de esta especie de percebe; y 5) la diferenciación de los dos fenotipos descritos en *P. pollicipes* mediante diferencias en los niveles de expresión de ciertos genes relacionados con la musculatura e integridad del pedúnculo.

La metodología desarrollada para la reconstrucción de las filogenias de la familia Pollicipedidae ha implicado la extracción de ADN de las cuatro especies de percebe analizadas: *P. pollicipes*, *P. elegans*, *P. polymerus* y *P. cabverdensis*, así como de dos especies que actuarían como “outgroup”, *Chthamalus montagui* y *Lepas anatifera*. Se realizaron amplificaciones mediante PCR de tres marcadores moleculares, uno mitocondrial (ADNr 16S) y dos nucleares (ADNr 18S-28S y EF1α) mediantes cebadores diseñados por Crandall y Fitzpatrick (1996) para amplificar en estas especies, en el caso del ADNr 16S, o específicamente en este estudio para los dos genes nucleares. Secuencias de la región mitocondrial COI fueron tomadas de la base de datos del GenBank para todas las especies estudiadas. Una vez los productos de PCR fueron obtenidos, éstos fueron clonados y secuenciados. Las secuencias obtenidas fueron implementadas en diferentes programas informáticos para su procesado, concatenado y posterior uso para inferir filogenias de máxima verosimilitud que permitieran esclarecer las relaciones evolutivas entre las cuatro especies de la familia Pollicipedidae.

La simulación de los patrones de plegamiento de las regiones del ADNr 5.8S, y de las regiones ITS1 e ITS2 se llevó a cabo en 2,806 secuencias pertenecientes a 329 especies, procedentes del GenBank y ampliadas con las

obtenidas en esta tesis doctoral para los estudios filogenéticos. Los patrones de plegamiento de cada una de las tres regiones fueron simulados en un rango de temperatura que abarcó desde los 5ºC hasta los 37ºC, y se buscaron posibles cambios de base compensados y regiones de reconocimiento por factores de transcripción. Además, los datos estructurales obtenidos para la región del ADNr 5.8S fueron empleados en la reconstrucción de una filogenia "Neighbor-Joining".

El desarrollo y optimización de 16 marcadores microsatélites se llevó a cabo en ADN genómico perteneciente a 64 individuos procedentes de cuatro poblaciones dentro del rango de distribución de *P. pollicipes*: Safi en Marruecos, Guincho en Portugal, Islas Cíes en España y Brest en Francia. Los cebadores necesarios para amplificar estos marcadores fueron desarrollados a partir de secuencias EST procedentes del trabajo de Muesemann *et al.* (2010) y marcados con los compuestos fluorescentes FAM o HEX. Estos cebadores fueron empleados en reacciones de PCR para amplificar en los 64 individuos estudiados cada uno de los motivos microsatélite desarrollados y, posteriormente, estos productos de PCR fueron secuenciados y analizados bioinformáticamente.

El análisis de la estructura poblacional del percebe *P. pollicipes* fue llevado a cabo mediante los marcadores microsatélite descritos previamente en ADN genómico de individuos pertenecientes a nueve poblaciones, las cuales incluyen las cuatro anteriormente introducidas junto con Roncudo, Golfo Ártabro, Ortigueira, Andrín y Cabo de Ajo. La amplificación por PCR y secuenciación de fragmentos amplificados se realizó en las condiciones optimizadas previamente. El análisis bioinformático de los diferentes alelos descritos para estos marcadores incluyó la estima del número alélico, heteocigosidades observada y esperada, análisis del equilibrio Hardy-Weinberg, alelos privados, índices Fst, número de migrantes, frecuencia de alelos nulos y eventos de cuello de botella. Asimismo, se llevó a cabo una reducción de dimensiones mediante el análisis lineal de componentes principales (ACP) y no lineal mediante el algoritmo "non-centred minimum curvilinear embedding (ncMCE)". También se realizó un análisis de

agrupamiento bayesiano de las poblaciones e inferencia filogenética, así como el desarrollo de un modelo logístico en base a estos marcadores microsatélites.

Los estudios de diferenciación fenotípica en la especie *P. pollicipes* se realizaron mediante análisis estadístico de variables morfológicas mediante prueba-T y determinación de la variación de la expresión génica entre ambos fenotipos. Para ello, fue extraído ARN procedente de diferentes tejidos de individuos de ambos fenotipos: tejido capitular, músculo peduncular, y tejido cuticular en el caso de los percebes del fenotipo de sol, y tejido capitular, músculo peduncular, tejido cuticular, tejido de la región capitulo-pedúnculo y tejido de la región cutícula-pedúnculo en el caso del fenotipo de sombra. El ARN extraído de estos tejidos fue convertido en ADN complementario por reverso transcripción y empleado para comprobar la estabilidad de cinco genes de referencia necesarios para estandarizar los niveles de expresión de los genes de interés.

Los genes de referencia testados fueron amplificados por PCR en tiempo real utilizando cebadores desarrollados a partir de secuencias EST de Meusemann *et al.* (2010) para los genes albúmina, actina, β-actina, HSP70 y HSP90 y secuencias nucleotídicas de Pérez-Losada *et al.* (2004) para el gen de la histona H3. Se realizó una curva de calibrado para los genes de referencia en base a cuatro concentraciones de ADN complementario: 100, 20, 5 y 1 ng/µl. Las amplificaciones por PCR en tiempo real se realizaron mediante el kit comercial FastStart SYBR Green Master (Roche Diagnostic) por triplicado. Los genes de interés fueron amplificados mediante cebadores diseñados a partir de los unigenes ensamblados por Perina *et al.* (2014) y se emplearon los genes: proteína de unión al nucleótido guanina, unión de cutícula al epitelio basada en quitina, proteína cuticular 47Ee, proteína cuticular 11B y proteína cuticular RR-1. La amplificación de estos genes de interés se llevó a cabo en las mismas condiciones que las de los genes de referencia en todos los tejidos descritos previamente.

Los resultados mostrados por las inferencias filogenéticas realizadas en base a los marcadores mitocondriales y nucleares han presentado discrepancias tanto en los modelos evolutivos empleados como en las

topologías obtenidas con cada uno de los marcadores. Por una parte, el modelo de evolución molecular que más se ha repetido entre los marcadores empleados ha sido el “general time reversible model” presentado por los marcadores ADNr 16S, ADNr 18S-28S y EF1α, mientras que el gen COI ha mostrado el modelo de Tamura. Esto ha podido ser debido a las diferentes fuerzas evolutivas que regulan la evolución molecular de cada una de las regiones genéticas empleadas como marcadores.

Las topologías inferidas a partir de estos marcadores moleculares han diferenciado cada una de las cuatro especies de la familia Pollicipedidae incluidas en este análisis con valores de apoyo estadístico superiores al 89% en todas las filogenias reconstruidas. A pesar de que las filogenias inferidas con los diferentes marcadores han agrupado todas las secuencias de cada especie en la misma rama, las ramas que incluyen diferentes especies no han mostrado suficiente apoyo estadístico. Por otra parte, las topologías obtenidas no han coincidido con las descritas previamente para esta familia por otros autores en base a diferentes marcadores moleculares. Únicamente la topología presentada por la filogenia basada en el COI ha coincidido con la obtenida por Van Syoc *et al.* (2010) y Quinteiro *et al.* (2011) con este mismo marcador y por Quinteiro *et al.* (2011) empleando el ADNr 18S-28S como marcador.

El árbol filogenético obtenido mediante el concatenado de los cuatro marcadores empleados ha mostrado dos linajes evolutivos, uno constituido por *P. pollicipes* y *P. elegans*, y otro formado por *P. polymerus* y *P. caboverdensis*. A priori, este árbol filogenético debería mostrar los resultados más fiables ya que incluye diferentes regiones con diferentes modelos de evolución. Sin embargo, la topología mostrada en él no concuerda con los datos morfológicos mostrados por estas especies. En base a la forma, tamaño, número y color de las placas del capítulo de los percebes, las especies más similares serían *P. pollicipes* y *P. caboverdensis*. Este clado se encontraría próximo a *P. elegans*, y *P. polymerus* sería la especie más discrepante.

Las simulaciones de los patrones de plegamiento de las regiones 5.8S, ITS1 e ITS2 del ADN ribosomal han detectado un patrón general de plegamiento para la región del ADNr 5.8S en crustáceos. Este modelo general

de plegamiento consistió en dos regiones de emparejamiento con el ADNr 28S, y tres hélices, mostrando la primera hélice en dirección 5'→3' dos subhélices. Este patrón general de pegamiento predicho para esta región del ADN ribosómico en organismos crustáceos es coincidente con el patrón general detectado en eucariotas. No obstante, seis variantes estructurales fueron detectadas en los plegamientos de ciertas secuencias de las especies del subfilo Crustacea para esta región. Estas desviaciones del patrón de plegamiento en el ADNr 5.8S se relacionan con modificaciones del número de subhélices presentadas por cada doble hélice, así como la ausencia de alguna de las hélices. Por otra parte, los cambios de base compensados entre las secuencias de la región del ADNr 5.8S de una misma especie fueron detectados en los crustáceos *Acanthodiaptomus pacificus* y *Epilobocera sinuatifrons*.

Los datos estructurales obtenidos de esta región fueron los únicos empleados en la reconstrucción filogenética del subfilo Crustacea debido a la imposibilidad de alinear las secuencias pertenecientes a las regiones ITS1 e ITS2 por sus elevadas diferencias genéticas. Las cuatro filogenias inferidas en base a los datos estructurales de la región del ADNr 5.8S correspondieron a cada uno de las cuatro clases del subfilo Crustacea representadas en el conjunto de secuencias analizadas: Ostracoda, Branchipoda, Malacostraca y Maxillopoda. Estas reconstrucciones permitieron determinar el origen monofilético de los órdenes Anostraca y Diplostraca en la clase Branchiopoda, la familia Darwinulidae incluida en la clase Ostracoda, infraórdenes Brachyura, Anomura y Astacidea del orden Decapoda y familia Mysidae del orden Mysida en la clase Malacostraca, y finalmente, los órdenes Sessilia y Pedunculata pertenecientes a la clase Maxillopoda. Estos taxones identificados mediante datos estructurales concuerdan con los detectados por otros autores en las filogenias de Pancrustacea. Sin embargo, los datos estructurales no han permitido identificar especies o géneros debido a la presencia de secuencias de distintas familias en las mismas ramas de la filogenia.

Los patrones de plegamiento detectados para las regiones ITS1 e ITS2 han sido similares. Ambas regiones han mostrado un patrón general constituido por un anillo central abierto del cual se proyectan un número variable de dobles

hélices según la especie y que puede variar entre dos y 11 hélices en el caso del ITS1, y entre dos y 12 en el caso del ITS2. La estructura secundaria mostrada por la especie *P. pollicipes*, objeto de estudio de esta tesis doctoral, para la región ITS1 ha presentado seis dobles hélices a lo largo del anillo central, mientras que para la región ITS2 ha mostrado cinco dobles hélices. Estos patrones de plegamiento obtenidos para las regiones ITS1 e ITS2 coinciden con las estructuras secundarias descritas en diferentes eucariotas para estas regiones. A pesar de que los patrones de plegamiento obtenidos en estas dos regiones han sido altamente variables, el grado de conservación de estas estructuras aumenta a nivel de familia y especie. Además, cambios de base compensados fueron detectados en 13 especies diferentes tanto para la región ITS1 como para la ITS2.

La variación genética presentada por las regiones ITS ha sido mayor que la observada en el ADNr 5.8S por mostrar ambas regiones ITS tasas de evolución mayores que las del ADNr 5.8S. Estas variaciones estructurales debidas a la presencia de inserciones y deleciones producen errores de plegamiento que pueden ser la causa de posibles pseudogenes. En el caso de la región del ADNr 5.8S, han sido detectados seis patrones de plegamiento que podrían indicar la presencia de pseudogenes.

Los análisis llevados a cabo mediante el empleo de marcadores microsatélite desarrollados a partir de las secuencias EST presentaron cuatro marcadores monomórficos y doce polimórficos, los cuales mostraron entre dos y 19 alelos. Los datos de heterocigosidad observada y esperada pusieron de manifiesto las desviaciones del equilibrio Hardy-Weinberg presentadas por nueve marcadores en alguna de las poblaciones estudiadas debido al exceso o defecto de heterocigotos. Las mayores tasas de migración fueron detectadas entre las poblaciones de Brest y Guincho, las cuales presentaron los valores de los índices Fst más bajos.

Los números de alelos y los valores de heterocigosidad mostrados por los marcadores microsatélite empleados en los estudios poblacionales de *P. pollicipes* han sido similares a los detectados tanto en otras especies congenéricas (*P. elegans*) como en otras especies de crustáceos. Las

desviaciones del equilibrio Hardy-Weinberg detectadas en nueve *loci* microsatélites han podido ser debidas tanto a las altas tasas de alelos nulos detectadas como a las presiones pesqueras a las que se encuentra sometido el percebe *P. pollicipes* en ciertas poblaciones. Esta causa ya ha sido reportada en la especie de percebe *P. elegans* como causa de efecto negativo sobre la diversidad genética y el tamaño efectivo.

Los valores de los índices Fst fueron superiores a los detectados en *P. elegans* por Plough y Marko (2014), siendo la población de Roncudo la que presentó mayor grado de diferenciación genética respecto a las demás poblaciones analizadas. Las diferencias presentadas en el grado de diferenciación genética entre poblaciones puede ser debido a que las prácticas de los pescadores incluyen la recolección de los individuos de fenotipo sol y dejan a los de tipo sombra como reproductores, lo que reduce la presión pesquera sobre los percebes de fenotipo sombra y puede cambiar la diversidad genética y frecuencia alélica en las poblaciones salvajes de *P. pollicipes*.

Los estudios de cuellos de botella a lo largo de la historia evolutiva de estas poblaciones no detectaron eventos que pudieran causarlos, aunque algunos marcadores mostraran apoyo estadístico en diferentes poblaciones. Los estudios de identificación de poblaciones mediante ACP y ncMCE permitieron corroborar la existencia de panmixis entre las poblaciones de *P. pollicipes*. Estos estudios, junto con el análisis de agrupamiento bayesiano y la inferencia filogenética, han detectado dos grupos de poblaciones. Por un lado las poblaciones de Safi, Guincho, Islas Cíes y Brest parecen conformar una única unidad. Por otro lado, las poblaciones de Roncudo, Golfo Ártabro, Ortigueira, Andrín y Cabo de Ajo conforman la segunda agrupación de poblaciones.

Estas dos agrupaciones pueden ser debidas a que las poblaciones españolas se encuentran sometidas a presiones pesqueras con la excepción de Islas Cíes que se ubica en una reserva natural. La citada explotación pesquera podría ser causa de selección de ciertos genotipos, provocando una incipiente diferenciación poblacional. Otra posible explicación serían las corrientes oceánicas que se desplazan a lo largo de las costas africana y

europea, así como la variación de temperatura mostrada por la región del golfo Ártabro y el mar Cantábrico que es un par de grados menor que la encontrada en aguas del Atlántico.

El modelo logístico desarrollado en base a los marcadores microsatélite ha permitido determinar el origen geográfico de las poblaciones estudiadas (español o no español) con un 83.7% de precisión.

Las variaciones fenotípicas presentadas por el percebe *P. pollicipes* han sido diferenciadas en base a distintas aproximaciones. Desde el punto de vista morfológico, todas las variables analizadas, con excepción del peso fresco del pedúnculo, han mostrado diferencias significativas entre ambos fenotipos.

La selección de genes de referencia que permitan estandarizar los niveles de expresión génica fue llevada a cabo mediante análisis con cuatro algoritmos diferentes. El primero de ellos, geNorm, determina la estabilidad de los genes en base a la variación media de todos los emparejamientos de cada gen de referencia con los demás. Los genes de referencia que mostraron mayor estabilidad en base a este parámetro fueron albúmina y HSP70. El segundo algoritmo empleado, NormFinder, determinó los mismos genes que geNorm como los más estables mediante la evaluación de la variación intra e intergrupal y el error sistemático asociado al uso de cada gen de referencia. Los análisis realizados con el tercer algoritmo, BestKeeper, determinaron a los genes β-actina y albúmina como los más estables en base a correlaciones de los genes de referencia tomados de forma pareada. Finalmente, el método ΔCt, basado en la medida de las fluctuaciones del valor ΔCt estableció nuevamente la albúmina y el HSP70 como los genes más estables. El gen de referencia escogido en base a los resultados obtenidos fue HSP70 por presentar el menor error sistemático de entre todos los genes de referencia analizados.

Tras su estandarización, los genes de interés analizados en los diferentes tejidos de los percebes de sol y de sombra mostraron diferencias en sus niveles de expresión. Los niveles más bajos fueron presentados por el gen codificante de la proteína cuticular RR-1 en el tejido cuticular del fenotipo de sol, mientras que los mayores valores fueron detectados en el gen codificante de la proteína cuticular 47Ee en el músculo del pedúnculo del fenotipo de sol.

Por otra parte, los genes codificantes de las proteínas cuticulares 11B y RR-1 mostraron niveles de expresión similares a los presentados por el gen control en todos los tejidos. Es remarcable el hecho de que el tejido cuticular y el músculo peduncular del fenotipo de sol mostraron niveles mayores de todos los genes que el fenotipo de sombra, excepto para el gen codificante de la proteína cuticular RR-1.

Los genes analizados para detectar diferencias de expresión entre ambos fenotipos han mostrado que los percebes del fenotipo de sol expresan mayores niveles de los genes relacionados con la musculatura e integridad del pedúnculo que los percebes de sombra. Esto concuerda con el hecho de que los percebes del fenotipo sol muestran un menor contenido de agua en sus pedúnculos y los tejidos del mismo se encuentran conectados entre ellos manteniendo la integridad del pedúnculo. Por otra parte, los percebes del fenotipo sombra, han mostrado menores niveles de expresión de estos genes dado que sus pedúnculos contienen mayores cantidades de agua para mantener su integridad y los diferentes tejidos que presentan no se encuentran tan íntimamente ligados como en el caso del fenotipo de sol.

Las conclusiones de esta tesis doctoral son:

Los análisis filogenéticos realizados empleando los marcadores ADNr 16S, COI, EF1α y ADNr 18S-28S, así como el concatenado de estas secuencias, han corroborado la diferenciación de las cuatro especies del género *Pollicipes*, aunque estas inferencias no han permitido aseverar las relaciones filogenéticas entre estas especies.

Bajo criterios morfológicos, la topología inferida usando el marcador mitocondrial COI ha mostrado las relaciones filogenéticas más adecuadas entre las especies del género *Pollicipes*.

La estructura secundaria del ADNr 5.8S detectada en todas las especies del género *Pollicipes* constó de dos regiones de apareamiento con el ADNr 28S en ambos extremos de la secuencia y tres dobles hélices, la primera de ellas en dirección 5'→3' dividida en otras dos subhélices. Esta estructura fue el

patrón de plegamiento general compartido por las especies del subfilo Crustacea.

Los patrones de plegamiento obtenidos para las regiones ITS1 e ITS2 en especies del género *Pollicipes* fueron similares. La estructura secundaria de la región ITS1 mostró un anillo central con tres a ocho hélices prominentes, mientras que la región ITS2 presentó un anillo central con tres a cinco hélices prominentes dependiendo de cada especie de este género.

La filogenia inferida en base a la estructura secundaria del ADNr 5.8S diferenció a ciertos taxones en el subfilo Crustacea. Estos taxones fueron los órdenes Anostraca y Diplostraca (clase Branchiopoda); familia Darwinulidae (clase Ostracoda); infraórdenes Brachyura, Anomura y Astacidea (orden Decapoda) y familia Mysidae (orden Mysida) en la clase Malacostraca; y finalmente, los órdenes Sessilia y Pedunculata (clase Maxillopoda).

Un conjunto de 16 marcadores microsatélite fue optimizado en la especie *P. pollicipes*, siendo 12 de ellos polimórficos. Los microsatélites Pol003, Pol005, Pol008, Pol011, Pol019, Pol043, Pol044, Pol114 y Pol118 no han presentado equilibrio Hardy-Weinberg en ninguna de las poblaciones analizadas. Los *loci* Pol003, Pol005, Pol011, Pol019, Pol114 y Pol118 exhibieron déficit de heterocigotos en todas las poblaciones de *P. pollicipes*, y Pol005, Pol043 y Pol044 mostraron exceso de heterocigotos en todas las poblaciones. Estos marcadores microsatélite fueron testados en otras especies congenéricas y presentaron amplificación positive en la PCR.

Los análisis poblacionales llevados a cabo en nueve poblaciones de *P. pollicipes* usando estos marcadores microsatélite detectaron dos grupos panmícticos de esta especie, uno constituido por poblaciones del norte de España, incluyendo Roncudo, golfo Ártabro, Ortigueira, Andrín y Cabo de Ajo, y otro grupo formado por percebes de Safi, Guincho, Islas Cíes y Brest. Estas diferencias pueden ser explicadas por las corrientes oceánicas que conectan las poblaciones de cada grupo, y/o la temperatura del agua, la cual es más cálida desde el golfo Ártabro hasta el mar Cantábrico.

Los ensayos de expresión han permitido testar seis genes de referencia: albúmina, HSP70, HSP90, actina, β-actina e histona H3. De estos genes, dos de ellos, HSP70 y albúmina mostraron niveles estables de expresión en todos los tejidos analizados y fueron validados para utilizarlos como genes de referencia en diferentes estudios de expresión.

Los análisis de expresión de cinco genes relacionados con la musculatura y la integridad del pedúnculo de ambos fenotipos fueron realizados en diferentes tejidos pertenecientes al pedúnculo de estos percebes usando el gen HSP70 como gen de referencia. Estos ensayos demostraron que el tejido cuticular y el músculo peduncular mostraron niveles de expresión más elevados de cuatro genes (proteína de unión al nucleótido guanina, unión de cutícula al epitelio basada en quitina, proteína cuticular 47Ee, y proteína cuticular 11B) en el fenotipo de sol que en el fenotipo de sombra, excepto para la proteína cuticular RR-1 que presentó el perfil inverso.

INTRODUCTION

2. Introduction.

The history of pedunculated barnacles goes back to Early Cambrian 520 M.a. ago (Chen 2001), moment when emerged as a taxonomical group. From that time until the present day, their history was surrounded by myths and legends about its origin. Thus, in ancient times it was believed that barnacle's shells hung on trees by the peduncle and when they shed into water transformed into birds such as geese (Heron-Allen, 1928; Gurney, 1947). This is the origin of the common name of this organisms, goose(neck) barnacles (Barnes, 1996). The myth was refuted by different naturalists and scientifics who studied this group of crustaceans, but it was not until Darwin was interested in them in 1851, what not established a classification of cirripedes (Darwin, 1851, 1854). Darwin's classification of Cirripedia has remained until the present day; it was extended by contributions from different authors and being the basis of the current classification of this group.

2.1. Taxonomy and distribution of barnacles of genus *Pollicipes*.

Barnacles are marine crustaceans belonged to infraclass Cirripedia Burmeister, 1834; superorder Thoracica Darwin, 1854; order Scalpelliformes Buckeridge and Newman, 2006. Within order Scalpelliformes, it is worthy distinguishing gooseneck barnacles from family Pollicipedidae Leach, 1817, which is constituted by three genera: *Calantica* Gray, 1825, *Capitulum* Gray, 1825 and *Pollicipes* Leach, 1817. Members of this last genus correspond to four living species, *P. pollicipes* (Gmelin, 1790), *P. elegans* Lesson, 1831, *P. polymerus* Sowerby, 1833 and *P. caboverdensis* Fernandes, Cruz and Van Syoc, 2010. Despite of these are the currently accepted scientific names, across the literature, different authors have used synonym names of these barnacles. Thus, certain studies about *P. pollicipes* describe it as *P. cornucopia* Leach, 1824 or *P. smythii* Leah, 1818. Synonym names of *P. elegans* used by different authors include *P. ruber* Sowerby, 1833 or *P. rigidus* Sowerby, 1839.*P. polymerus* was described in several studies as *P. mortoni* Conrad, 1837. The most recent species discovered, *P. caboverdensis*, has been described also as *P. darwinii* in molecular studies performed by Quinteiro *et al.* (2011).

Geographical distributions of these four *Pollicipes* species ranged from north European coasts of France (Brittany) to African coasts of Dakar (15ºN) in the case of *Pollicipes pollicipes*. *P. elegans* inhabits American pacific coasts from Mexico to Chile while *P. polymerus* is common in intertidal regions of west coasts of North America from 64ºN to 27ºN (Barnes, 1996). Finally, *P. caboverdensis*, inhabit the circumscribed coasts of Cape Verde Islands (Fernandes *et al*., 2010). Global distributions of the different species of *Pollicipes* barnacles are showed in figure 1.

Figure 1. Distribution ranges of the four species of family Pollicipedidae: *P. pollicipes* (blue), *P. caboverdensis* (red), *P. elegans* (yellow), *P. polymerus* (green).

2.2. Habitat and ecology of barnacles of genus *Pollicipes*.

Pollicipes barnacles form dense aggregations of individuals strongly attached to rocky shores of intertidal coasts where waves break with extreme violence (Barnes, 1996) and water was constantly agitated and oxygenated (Joubin 1906, 1907). These crustaceans inhabit open sea parts of the shoreline and fissures and crevices present in cliffs which provide some extra protection for the stalks of these organisms (Barnes, 1996). Communities of *P. pollicipes* and banks of mussels appear in European and African coasts delimitating a zone between the upper chthamalids and below *Balanus* species (*B. perforatus*

in European coasts and *B. tintinnabulum* in African coasts) (Prenant, 1932; Barnes, 1996). The barnacle *P. polymerus* forms dense communities with *Mytilus californianus* in North American coasts. This community is limited by *C. dalli* and *B. glandula* above it and *Semibalanus cariosus* below it (Dayton, 1971), although this zonation is modified by depending on local conditions of exposition or shelter to waves and tides (Barnes, 1960). Few studies have been carried out about ecological communities of *P. elegans* and *P. caboverdensis*. However, several studies reported different species belonged to *Balanus* and *Chthamalus* genera where *P. elegans* inhabits (Laguna, 1990). On the contrary, biological communities where *P. caboverdensis*is allocated have not been reported.

2.3. Morphology and internal anatomy of barnacles of genus *Pollicipes*.

The most remarkable character presented by barnacles of order Scalpelliformes is a muscular and flexible stalk or peduncle. This character differentiate pedunculated barnacles belonged to orders Ibliformes, Lepadiformes and Scalpelliformes from sessile barnacles of order Sessilia. This peduncle shows a cuticle constituted by calcified scales disposed alternately and symmetrically (Barnes, 1996) which protects the internal tissues and maintains peduncle's integrity. Over the peduncle is the capitulum, composed by a variable number of plates which vary from 18 to more than 100 depending on age and species. The morphology of this capitulum and arrangement of their plates which conform it are used to distinguish the different orders above cited and species into the same order. In the particular case of *Pollicipes* species, the size and arrangement of the main plates of the capitulum (*tergum*, *carina*, *scutum*, *rostrum* and *latera*) are used as taxonomical characters to identify *P. pollicipes*, *P. polymerus* and *P. elegans* species (Barnes, 1996), as well as, the number of basal plates of the capitulum is used to distinguish *P. pollicipes* and *P. caboverdensis* (Fernandes *et al*., 2010) (Fig. 2).

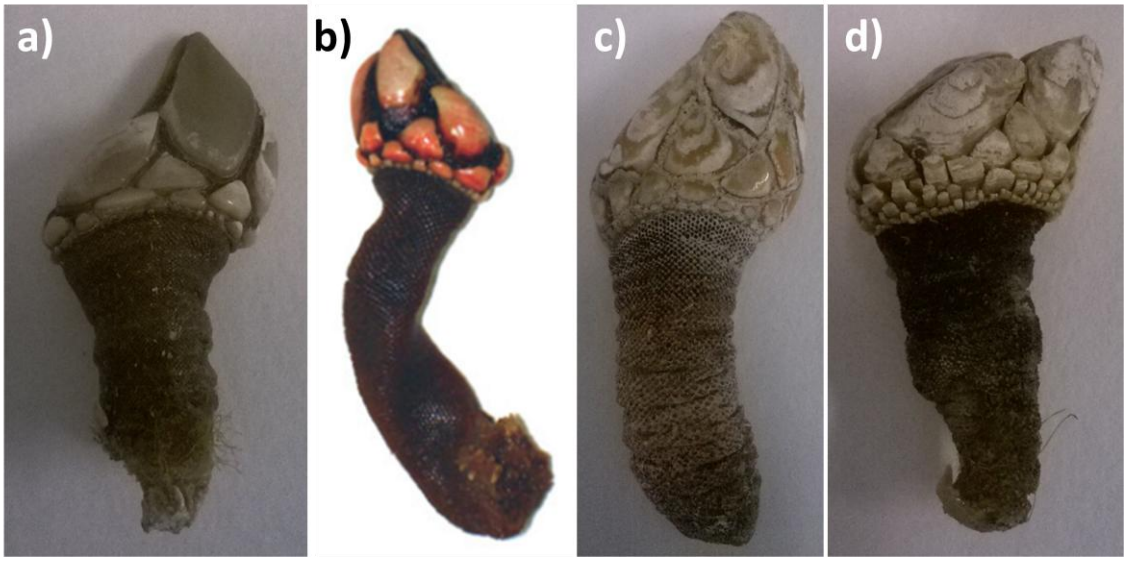


Figure 2. External appearance of each one of the four species of genus *Pollicipes*. a) *P. pollicipes*, b) *P. caboverdensis* taken from Quinteiro *et al*. (2011), c) *P. elegans*, and d) *P. polymerus*.

Gooseneck barnacles of genus *Pollicipes* are characterised by a muscular and flexible peduncle formed by three layers of muscularity, specifically, diagonal, circular and longitudinal from the inside to the outside of the stalk (Molares Vila, 1994). This composition provides barnacle's peduncle enough flexibility and strength to dealing with strong marine waves and tides without suffering damage. The peduncle also contains feminine gonad and cement glands, which produce a proteic adhesive secretion or cement (Barnes, 1996). Above the peduncle, a capitulum, protects digestive and excretory systems, gills, *cirri*, masculine gonad and the ovisac, oviduct and ovisac gland of feminine reproductive system. Nervous and lymphatic systems traverse both capitulum and peduncle, although the main organs of these systems are protected into the capitulum (Molares Vila, 1994).

2.4. Phenotypes of barnacle *P. pollicipes*.

In European barnacle *P. pollicipes* two different phenotypes have been detected (Parada *et al*., 2012) according to ecological, morphological and anatomical characteristics. Thus, barnacles called "sun barnacles'" or solely barnacles inhabit in more exposed to waves parts of the rocky shoreline. Barnacles belonged to this phenotype show a characteristic morphology with short and rough peduncles and they are small in size respect from the other

phenotype. According to their internal anatomy, peduncle of “sun barnacles’” present high density of muscular tissue adhered to cuticle which encircles the peduncle achieving a compact and turgid peduncle. On the contrary, Parada *et al*. (2012) described barnacles of shadow phenotype or “mexóns” as inhabitants of crevices and fissures in coastal riffs which provide extra protection to their peduncles. These barnacles present a long and thin peduncle and are big in size respect from “sun barnacles’”. Anatomy of their peduncles reveals a less compressed muscle tissue inside it and this muscle tissue is disengaged from the cuticle which protects it. As a result, “shadow barnacles’” incorporate water into their peduncles in order to maintain its integrity and turgidity (Fig. 3.).

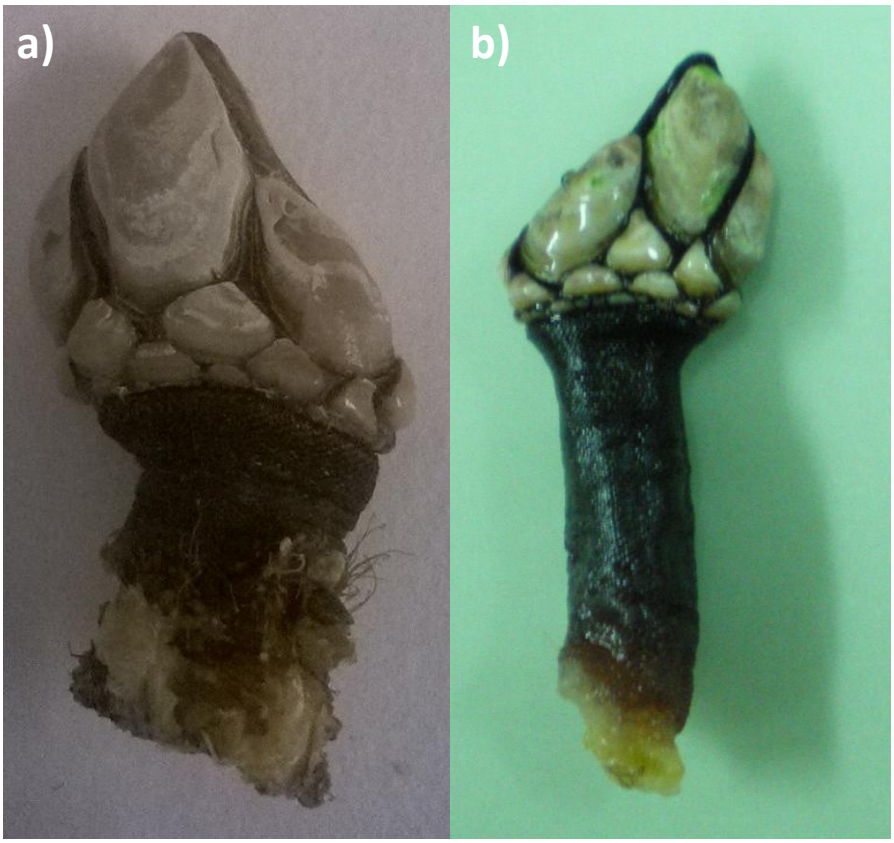


Figure 3. Morphological differences between a) “barnacles of sun”, and b) “barnacles of shadow”.

2.5. Life cycle of barnacles of genus *Pollicipes*.

Gooseneck barnacles of family Pollicipedidae are hermaphroditic organisms with internal fecundation. The life cycle comprises seven larval stages, six *nauplius* and one *cypris*, occurring metamorphosis and moulting

between each stage. *Cypris* larvae attaches to an adult individual in the region of the peduncle nearest to capitulum and metamorphosis into a juvenile specimen. The duration of larval life has been estimated in one month (Molares Vila, 1994). After metamorphosis, the juvenile moves along adult peduncle until the rocky substratum and becomes completely sessile. The cementation process is mediated by the adhesive secretion composed of different proteins produced in cement glands of barnacle peduncle's and excreted outside across its base. Reproductive period of *P. pollicipes* ranges 210 days, from March to September, and includes two larval release periods, one at the end of the winter (March-April) and another one at the beginning of the summer (July-October), the most important in terms of adult population implicated in it (Molares Vila, 1994).

2.6. Economic importance of *P. pollicipes* in European markets.

Barnacle *P. pollicipes* is an economically important marine resource in markets of some countries, especially in Spain and Portugal. Prices that this crustacean can reach in Spanish markets has rangedin past year 2014 between 1.20 and 254.50 €/kg in different parts of the year (Xunta de Galicia). Galicia is the most important supplier of this shellfish providing more than 75% of captures occurring in Spain. As a consequence of this economic demand, captures of *P. pollicipes* in Galicia ranged from 301,734 to 508,963 kg per year in the last 15 years, producing profits due to this marine resource which reach 144 millions of Euros (Xunta de Galicia). The great commercial importance and market demand of this shellfish resource have caused the importation of barnacles of less quality from countries such as France, Portugal, Morocco, and even Canada or Peru (Molares Vila and Freire, 2003).

2.7. Genetics studies performed in barnacle *P. pollicipes*.

2.7.1. Evolutionary relationships between family Pollicipedidae members.

The study of evolutionary relationships among groups of organisms, populations or speciesof crustaceans has developed an important role in ancestry and heritage analyses of this subphylum. Different approaches about the evolutionary history of this group of organisms have been obtained and

used to reconstruct its evolutionary history based on different molecular and morphological characters. Several taxa that throughout history have conformed part of the crustaceans have presented difficult to clarified evolutionary relationships such as Remipedia or Malacostraca (von Reumont, 2009). Controversial results of phylogenetic studies have been obtained generating multiple interpretations of the evolution of Crustacea. Certain studies indicated the paraphyletic origin of Crustacea, which would form a monophyletic group with Hexapoda, named Pancrustacea or Tetraconata. Phylogenetic topologies congruent with this monophyletic origin of Crustacea and Hexapoda have been obtained based on mitochondrial genome (Lavrov *et al*., 2004; Cook *et al*., 2005), protein codifying genes (Garcia-Machado *et al*., 1999; Reiger *et al*., 2001, 2005, 2008), nuclear ribosomal RNA genes (Zrzavy *et al*., 1998; Mallat *et al*., 2004; Mallat and Giribet, 2006), nervous system characters (Harzsch 2004, 2006), a combination of morphologic and molecular data (Zrazy *et al*., 1998; Giribet *et al*., 2005), and based on expression sequence tags (ESTs) (Meusemann, 2010).

Regardless these studies which agree with a monophyletic position of Pancrustacea into the Athropoda phylogeny, different crustacean clades have occupied different phylogenetic positions along the evolutionary history of these organisms. Between all these lineages, subclass Thecostraca, the lineage where barnacles are included, has showed a monophyletic origin based on morphological and molecular data (Pérez-Losada *et al*., 2004, 2008).Included into Thecostraca (subclass), Cirripedia (infraclass), Thoracica (superorder), gooseneck barnacles of family Pollicipedidae conform a monophyletic clade whose three species may all emerged at about the same time during a species radiation event in the proto-Atlantic as the Tethys was closing (Van Syoc *et al*., 2010).

Phylogenies of family Pollicipedidae were reconstructed several times by different authors based on different molecular markers. Thereby, phylogenies inferred using a 37 sequences dataset from COI, 16S and H3 (Van Syoc *et al*., 2010), from a 40 sequences of COI and 27 sequences of ITS1, 5.8S rDNA and ITS2 (Quinteiro *et al*., 2011), or form a dataset of 99 sequences of 5S ribosomal DNA (Perina *et al*., 2011) showed incongruent tree topologies between the four

species of *Pollicipes*. Tree topologies presented in the results obtained by Van Syoc *et al*. (2010) showed three lineages, one derived in current *P. polymerus* species, another in *P. elegans* and the third one is constituted by *P. pollicipes* and *P. caboverdensis* based on COI region. However, the other mitochondrial gene analysed by these authors, 16S rDNA, showed two lineages, one conformed by *P. polymerus* and another constituted by the other three species of genus *Pollicipes* in which *P. pollicipes* diverged earlier than the clade constituted by *P. elegans* and *P. caboverdensis*. Phylogeny based on nuclear histone H3 resulted in a tree topology with a basal polytomy divided in three lineages, *P. polymerus*, *P. caboverdensis*, and the tandem *P. pollicipes* and *P. elegans*. The topology obtained by these authors using the three genes concatenated showed incongruence with previous topologies. This concatenated region generated a phylogeny with a mixed lineage which originated *P. polymerus* and *Capitulum* species, and other lineage constituted by the other three *Pollicipes* species, in which *P. elegans* diverged earlier than the clade formed by *P. pollicipes* and *P. caboverdensis*. Phylogenetic reconstructions performed by Quiteiro *et al*. (2011) based on COI presented two *Pollicipes* lineages, one constituted by *P. polymerus* and another conformed by *P. elegans* and the clade *P. pollicipes-P.caboverdensis*. However, reconstructions inferred based on ITS1-5.8S-ITS2 region showed three lineages: *P. polymerus*, *P. elegans* and the clade *P. pollicipes* - *P. caboverdensis*. Finally, reconstructions carried out by Perina *et al*. (2011) based on 5S rDNA achieve isolate *Pollicipes* depending on their origin (Atlantic or Pacific) instead of specific taxa, being sequences of this genetic region were not suitable for phylogeny inferences.

2.7.2. Secondary structure of 5.8S major ribosomal gene and internal transcribed spacers.

Nucleic acids show a specific secondary structure which is needed for the correct development of their biological functions such as regulation of gene expression, processes of splicing or interactions between macromolecules. Secondary structure depends directly on the primary structure, i.e., the lineal sequence of nucleotides which constitute its primary structure. As a consequence, mutations of primary structure provoke highly alterations in the

secondary structure, although certain changes occurred in both chains of the DNA, which maintain the pair bonding between the two bases of the same position (compensatory base changes or CBCs), do not alter the structure. Structural data are useful to refine and increase the accuracy of phylogenetic trees topologies (Wang *et al*., 2007; Dohrmann, 2014) and assist in the identification or reinforcement of new species (Wolf *et al*., 2013).

One of the most used gene region in secondary structure studies includes ribosomal RNA genes and their internal transcribed spacers 1 and 2. Major ribosomal genes appear repeated in tandem from hundred to thousand times in units formed by three rRNA genes (18S, 5.8S, and 28S) and internal (ITS1 and ITS2) and external (ETS) transcribed spacers. Each unit is separated from the contiguous one by an intergenic spacer (IGS) (Eickbush and Eickbush, 2007). The number of times that the rDNA unit appears repeated varies between species and individuals (Fig. 4). Secondary structures of each major rRNA gene (18S and 28S) are well known due to crystallographic data (Ben-Shem *et al*., 2010) and folding patterns for these regions are described for many species. In the case of 5.8S rRNA and both internal transcribed spacers, their reduced length (in base pairs) allows determine the secondary structure by inferences of folding patterns. The secondary structure of 5.8S rDNA showed by different eukaryotic taxa is constituted by two regions of pairing with 28S rRNA and three helices, being the first one formed by another two subhelices (Wei *et al*. 2012; Petrov *et al*., 2014). In the case of internal transcribed spacers, secondary structures of these spacers were examined among different taxa looking for a general folding pattern in eukaryotes (Ursi *et al*., 1982; Coleman 2007; Koetschan *et al*., 2014), which have showed similar structures formed by an open ring with a variable number of protruding helices. Differences between both ITS regions and even species concern the number and subdivision degree of protruding helices. In crustaceans, secondary structures of these regions were slightly studied and only 5.8S rDNA (Ursi *et al*., 1982) and ITS2 (Kornobis and Pálsson, 2013) secondary structure of *Artemia salina* have been reported.

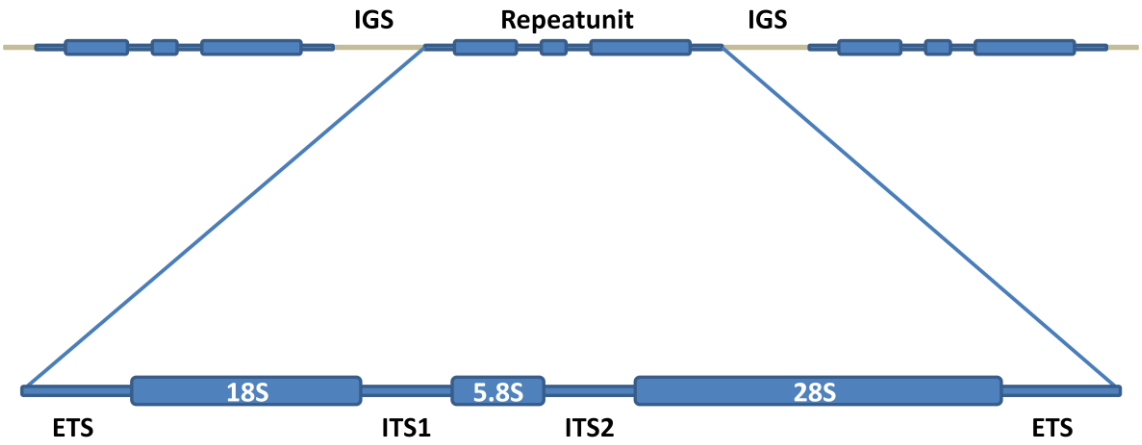


Figure 4. Major ribosomal genes array organization. Each repeat unit is constituted (in 5'-3' direction) by external transcribed spacer (ETS), 18S rDNA (18S), internal transcribed spacer 1 (ITS1), 5.8S rDNA (5.8S), internal transcribed spacer 2 (ITS2), 28S rDNA (28S), and another external transcribed spacer (ETS). Each repeat unit is separated from the contiguous one by an intergenic spacer (IGS).

2.7.3. Population genetics of Atlantic barnacle P. pollicipes.

Genetic analysis of different populations using certain molecular markers let study genetic diversity, population structure, effective size and putative bottlenecks or migration rates. One of the most used molecular markers in population genetic studies are microsatellites. These makers, tandem repeats of one to six nucleotides found at high frequency in the nuclear genomes of most taxa (Selkoe and Toonen, 2006), show some characteristics which make them ideal markers in population studies such as, they are selectively neutral, co-dominant, highly polymorphic, follow Mendelian inheritance, abundant and distributed throughout the genome, and might be species-specific (Selkoe and Toonen, 2006; Miah *et al*., 2013).

In the case of *Pollicipes*, such as other marine organisms, population structure is driven by oceanic currents which, at the same time, are corridors for larvae dispersion and invisible barriers for gene flow (Palumbi, 1994). Using mitochondrial markers, Quinteiro *et al*. (2007) demonstrated that *P. pollicipes* individuals along its distribution range show a high degree of genetic homogeneity and these populations might behave as a panmictic population. Other factor that may affect the present distribution of *P. pollicipes* populations are glacial and interglacial events during the Pleistocene, which were analysed

by Campo *et al*. (2010). Using COI mitochondrial marker these authors detected three potential glacial refugia for this species, North African coasts, northwestern Iberian Peninsula and English Channel/Brittany, as well as, a pre-last glacial maximum pattern of demographic expansion, in concordance with many other North Atlantic marine species.

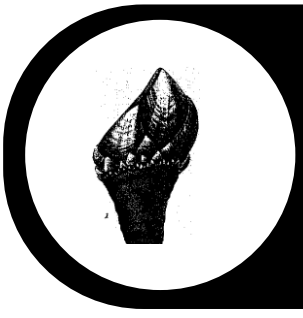
2.7.4. Reference genes and gene expression assays developed in crustacean organisms.

Phenotypic variation observed between individuals of the same species is caused by different factors. One of the most important are differences in expression levels of codifying genes. These variable levels of gene expression cause that individuals with the same genetic endowment generate individuals phenotipically different. The need to quantify the expression levels of certain genes of interest in different tissues, organisms or biological conditions require the use of a reference gene (or housekeeping gene) which present a stable expression in the biological conditions used in the experiment (Silver *et al*., 2006). Reference genes are any gene or group of genes which show a stable expression patterns in all studied conditions and will use to standardise the expression levels of genes of interest. Characteristics of ideal reference gene include constitutive expression and constant expression levels between different samples (Chandna *et al*., 2012). Commonly, reference genes used in expression quantifying experiments carried out in crustacean organisms are listed in table 1.

Table 1. List of reference genes used by different authors in real-time PCR experiments.

Reference gene	Gene symbols	Authors
18S ribosomal RNA	18S rRNA	Dhar *et al*. (2009), Cottin *et al*. (2010), Leelatanawit *et al*. (2012)
Actin	ACT	Lind *et al*. (2013)
Anti-lipopolysaccharide factor isoform 3 from *Penaeus monodon*	ALF*Pm* 3	Somboonwiwat *et al*. (2006)
DNA Meiotic Recombinase 1	Dmc1	Leelatanawit *et al*. (2012)
Glucose transporter 1	GLUT1	Somboonwiwat *et al*. (2006)
Glyceraldehyde-3-phosphate-dehydrogenase	GAPD, GAPDH	Vandesompele *et al*. (2002), Frost and Nielsen (2003), Dhar *et al*. (2009), Cottin *et al*. (2010), Barman *et al*. (2012), Leelatanawit *et al*. (2012)
Hydroxymethyl-bilane synthase	HMBS	Vandesompele *et al*. (2002)
Hypoxanthine phosphoribosyl-transferase I	HPRTI	Vandesompele *et al*. (2002)
Interferon-related developmental regulator	IFRD1	Somboonwiwat *et al*. (2006)
Lysozyme	Lysozyme	Somboonwiwat *et al*. (2006)
Nicotinamide adenine dinucleotide dehydrogenase subunit 1	NADHd1	Lind *et al*. (2013)
Profilin	Profilin	Somboonwiwat *et al*. (2006)
Ribosomal LI3a	RPLI3a	Vandesompele *et al*. (2002)
Ribosomal protein L18	RPL18, RpL18	Sharp *et al*. (2010), Barman *et al*. (2012)
Ribosomal protein L8	RPL8, RpL8	Cottin *et al*. (2010), Barman *et al*. (2012), Lind *et al*. (2013)
Ribosomal protein P0	RPLP0, 36B4	Lind *et al*. (2013)
Serpin Peptidase Inhibitor, Clade B (Ovalbumin), Member 3	SERPINB3	Somboonwiwat *et al*. (2006)
Structural ribosomal protein S20	RPS20	Frost and Nielsen (2003)
Succinate dehydrogenase complex (subunit A)	SDHA	Vandesompele *et al*. (2002)
TATA box binding protein	TBP	Vandesompele *et al*. (2002)
Translation elongation factor 1α	EF1α, eEF1α, Elfα, EF1, efa-1α	Frost and Nielsen (2003), Somboonwiwat *et al*. (2006), Dhar *et al*. (2009), Barman *et al*. (2012), Leelatanawit *et al*. (2012), Lind *et al*. (2013), Petkeviciute *et al*. (2015)
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (zeta polypeptide)	YWHAZ	Vandesompele *et al*. (2002)
Ubiquitin C	UBC	Vandesompele *et al*. (2002)
Vitellogenin	Vitellogenin	Leelatanawit *et al*. (2012)
β-2-microglobulin	B2M	Vandesompele *et al*. (2002)
β-actin	ACTB	Vandesompele *et al*. (2002), Dhar *et al*. (2009), Cottin *et al*. (2010), Barman *et al*. (2012), Leelatanawit *et al*. (2012), Petkeviciute *et al*. (2015)

Frequently, studies of gene expression quantification are carried out using real-time reverse transcription PCR or quantitative reverse transcription PCR technology (qRT-PCR). This technology is based on PCR technique but including a fluorescent reagent whose intensity of fluorescence is proportional to accumulation of specific PCR products during the amplification process (Saunders, 2009). Reverse transcription PCR characterises by its accuracy, speed, throughput and degree of automation respect from conventional techniques such as northern-blot, ribonuclease protection assay or competitive RT-PCR (Vandesompele *et al*., 2002) what make it favourable for assays of gene expression.



OBJECTIVES

3. Objectives.

The main objectives in the current Ph.D. research developed in barnacle species of family Pollicipedidae and, particularly, in *P. pollicipes* species, are:

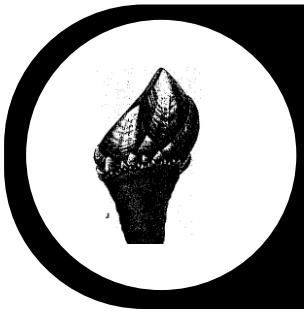
1. Infer phylogenetic relationships between members of crustacean family Pollicipedidae. Elaboration of phylogenetic reconstructions based on two nuclear markers, 18S-28S rDNA region and EF1α, and another two mitochondrial markers, COI and 16S rDNA, which allow resolving the incongruent results of phylogenetic inferences carried out by different authors between the four living species of this family.

2. Determination of folding patterns of 5.8S rDNA and ITS regions belonged to major ribosomal genes array in subphylum Crustacea, with special focus on family Pollicipedidae. Detection of putative pseudogenes will be carried out based on secondary structures of these rRNA regions and the structural information obtained will be used to corroborate crustacean phylogeny.

3. Detection, characterization and optimization of microsatellite markers in *P. pollicipes*, based on EST libraries, and tested them in other congeneric species in order to use these markers in further population studies.

4. Analysis of *P. pollicipes* populations using optimised microsatellite markers based on its geographical origin, and determination of population structure of this barnacle along its distribution range.

5. Differentiation of two phenotypic variants, “sun barnacles” and “shadow barnacles”, reported in *P. pollicipes* using gene expression analysis. Development and optimization of reference genes will be carried out in order to determine the differences in gene expression levels of genes related to peduncle integrity and muscularity in certain tissues from both phenotypes.



METHODOLOGY

4. Methodology.

4.1. Reconstructing phylogenetic relationships of family Pollicipedidae.

4.1.1. Sampling of Pollicipes specimens.

Individuals of four *Pollicipes* species were analysed. Specimens of *P. pollicipes* were sampled in four populations, Balcobo, Bens and Ortigueira (NW of Spain) and Safi (Morocco). Specimens of *P. elegans* were collected from two populations of Peru, Lobos de Afuera and Lobos de Tierra Islands. Specimens of *P. polymerus* were sampled in Olympic National Park (NW of USA) and specimens of *P. caboverdensis* came from Cape Verde Island (Fig. 5). Additional samples of crustacean species *Chthamalus montagui* Southward, 1976 (Mera, Spain) and *Lepas anatifera* Linnaeus, 1758 (Brazil) were also collected to act as outgroup species. Three specimens of each population were included in the analysis. Genomic DNA extraction were performed with the kit Genomic DNA from Tissue, NucleoSpin Tissue (Macherey Nagel) following the instructions of the manufacturer. DNA was extracted using soft tissue from the peduncle in the case of *Pollicipes* and *Lepas* species, and using all soft tissue from the capitulum in the case of *C. montagui*.

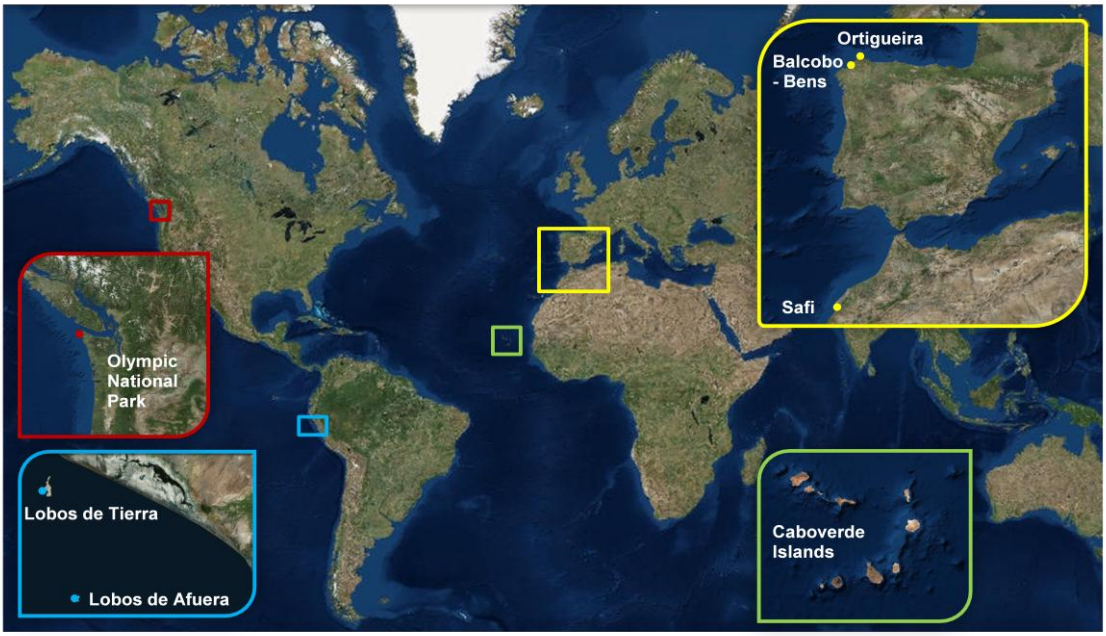


Figure 5. Map of sampled populations included in phylogenetic analyses. Coloured dots indicate points of recollections of each species: *P. pollicipes* (yellow), *P. caboverdensis* (light green), *P. elegans* (light blue), and *P. polymerus* (red).

4.1.2. Design of primers used to reconstruct the Pollicipedidae phylogeny.

Amplifications of four gene regions (Table 2) were carried out by polymerase chain reaction (PCR). Primers 1971 and 1972 (Table 2) from Crandall and Fitzpatrick (1996) were used to amplify the mitochondrial 16S rDNA in all analysed species.

Table 2. Gene regions amplified for phylogenetic reconstructions. For each gene region (Gene), primers used in PCR amplification experiments (Primers name), forward and reverse primer sequences in 5'→3' (Primers sequence), melting temperature in Celsius degrees (Tm (ºC)), and authors who have developed the primers (Authors) are shown.

Gene	Primers name	Primer sequence	Tm (ºC)	Authors
16S rDNA	1971	F: CCTGTTTANCAAAAACAT	50	Crandall and Fitzpatrick (1996)
	1972	R: AGATAGAAACCAACCTGG		
18S-28S rDNA	18S-Pol	F: TGTTTTCCGTAGGTGA	43	This study
	28S-Pol	R: TCTGATCTGAGGTCGAATCGA		
	d18S-Crustac	F: TGAACCCTTTTCGTGATGG	53	This study
	d28S-Crustac	R: CGAACTCCTTTCCAGGA		
EF1a	EF1A-F	F: GTCTGGGCTTGTGTAGTGAC	48	This study
	EF1A-R	R: GCAGCTCCTTCACAGATACG		

Nuclear 18S-28S rDNA region was amplified using different primer pairs. Firstly, degenerated primers d18S-Crustac and d28S-Crustac (Table 2) were designed using consensus sequences from *Calanus finmarchicus* (AY446904, AY446906, AF367714, AF367719 for 18S and EU375491, AY455948 for 28S) and *Asellus aquaticus* (AJ287055, AF255701 for 18S and AY739195 for 28S) with GeneFisher 2.0. (Giegerich *et al*., 1996). Once several sequences of 18S-28S rDNA region from *P. pollicipes* were obtained using d18S-Crustac and d28S-Crustac primer pair, a new inner primer pair, 18S-Pol and 28S-Pol, was designed using Primer3 0.4.0. (Untergrasser *et al*., 2012).

Once both primer pairs were obtained, nuclear 18S-28S rDNA amplifications of each species were carried out using the pair which presented better amplification rates: d18S-Crustac and d28S-Crustac in specimens of *P. polymerus* and *P. elegans*, and 18S-Pol and 28S-Polin specimens of *P. pollicipes* and *P. caboverdensis* (Table 2). Specimens of *L. anatifera* and *C. montagui* were amplified with both primer pairs.

Nuclear EF1α gene region was amplified with primers pair EF1a-F and EF1a-R (Table 2) designed from unigene UN0120 from an EST sequence obtained by Perina *et al*. (2014) using Primer3 0.4.0. (Untergrasser *et al*., 2012).

4.1.3. PCR amplifications and Sanger sequencing.

PCR reactions performed in a 25 µl of final volume using a reaction mixture with 1X PCR reaction buffer (Roche), 1.5 mM $MgCl_2$, 200 µM of every dNTP (Roche), 0.5 µM of each primer and 0.625 U of Taq polymerase (Roche). PCR reactions were carried out in a MyCycler^{TM} (BIO-RAD) in different conditions depending on the genetic region amplified.

Thus, PCR amplifications of 16S rDNA for all crustacean species were performed using 1471 and 1472 primer pair (Crandall and Fitzpatrick, 1996) at 95ºC during 5 min, followed by 35 cycles of 95ºC, 30 s; 50ºC, 30 s and 72ºC, 30 s, and finally, a extension to 72ºC during 7 min.

Nuclear 18S-28S rDNA region was amplified using two primer pairs. On one hand, *P. pollicipes* and *P. caboverdensis*, *L. anatifera* and *C. montagui* species were amplified using 18S-Pol and 28S-Pol primer pair in the following conditions: 95ºC during 4 min, followed by 35 cycles of 95ºC, 30 s; 43ºC, 30 s and 72ºC, 45 s, and finally, an extension to 72ºC during 5 min. On the other hand, amplifications for *P. polymerus* and *P. elegans*, *L. anatifera* and *C. montagui* were performed using d18S-Crustac and d28S-Crustac primer pair at thermal conditions of 94ºC during 3 min, followed by 35 cycles of 94ºC, 20 s; 53ºC, 20 s and 72ºC, 45 s, and a final extension step of 72ºC during 5 min.

Finally, EF1α for all analysed species was amplified using EF1A-F and EF1A-R primer pair in the following conditions: 95ºC during 7 min, followed by 35 cycles of 95ºC, 45 s; 48ºC, 45 s and 72ºC, 90 s, and finally, an extension to 72ºC during 7 min.

Amplified PCR fragments for all gene regions were cloned using Strataclone PCR Cloning Kit (Stratagene) following the manufacturer instructions and sequenced in a3130xl genetic analizer (Applied Biosystems) in Servicios Apoyo a la Investigación (Universidade da Coruña). Cytochrome oxidase I sequences were obtained from NCBI for the four *Pollicipes* species and the two outgroup species *C. montagui* and *L. anatifera* included into the analysis.

4.1.4. Bioinformatic analyses of phylogenetic sequences dataset.

Different sequence datasets of each region were aligned using Clustal W algorithm implemented in MEGA 6 (Tamura *et al*., 2013) using a gap opening penalty of 15 and a gap extension penalty of 6.66 for both pairwise and multiple alignments, the DNA weight matrix was IUB and the transition weight of 0.5. After that, best substitution model were determined using MEGA 6 (Tamura *et al*., 2013) under Akaike Information Criterion corrected (AICc) and using default parameters. Phylogenetic reconstructions were carried out using default parameters for maximum likelihood algorithm, the substitution model previously selected and with 1000 replicates of bootstrap in MEGA 6 (Tamura *et al*., 2013). Finally, a concatenated phylogenetic tree was inferred using sequences of three genetic regions (COI+16S+rDNA) after aligning each region separately.

4.2. Inferring secondary structures of 5.8S rRNA and both ITS regions in subphylum Crustacea.

A dataset of 2,806 sequences of ITS1, 5.8S rRNA and ITS2 regions, belonged to 329 species of crustaceans, were obtained from NCBI (Genbank) (Annex1) and extended with sequences of major ribosomal genes obtained in the previous section of this thesis. Each of these three regions was established following the guidelines of original authors, in the cases which these regions were previously described. In the cases where boundaries were not described by original authors, the ITS2 database III (Koetschan *et al*., 2010) and alignments of these regions belonged to related species were performed with ClustalX2 (Larkin *et al*., 2007) and BioEdit (Hall, 1999) in order to determine the boundaries of each region. Once each region of each species was determined, folding patterns in a range of temperatures from 5ºC to 37ºC were obtained using default parameters of Vienna RNAfold server (Gruber *et al*., 2008) and 4SALE (Seibel *et al*., 2006). CBCs searching was carried out using 4SALE software (Seibel *et al*., 2006) and probability of CBCs were associated with different species were calculated using algorithms described by Müller *et al*. (2007). TF's searching was performed using tfsearch tool implemented in Geneious 5.6.7 (Kearse *et al*., 2012) using a allow mismatches parameter limited to 5 and limit minimum length of matches to 7.

Neighbor-Joining phylogenetic reconstructions of different classes of subphylum Crustacea were performed based on both nucleotidic sequence and secondary structure using ProfDistS software (Wolf *et al*., 2008) with 1000 replicates of bootstrap and a Jukes-Cantor model of molecular evolution.

4.3. Developing and optimizing microsatellite markers in barnacle *P. pollicipes*.

Specimens obtained from natural banks of the Cíes Islands (north-western Spain), Safi (Morocco), Brest (France), and Guincho (Portugal) for *P. pollicipes* species, from the Cabo Verde Islands for *P. caboverdensis*, from Lobos de Afuera Island (Peru) in the case of *P. elegans*, and from Olympic National Park (USA) for *P. polymerus* (Fig. 6) were include into the analysis.

Figure 6. Map of *P. pollicipes* populations sampled for their use in microsatellite amplification.

Genomic DNA from 64 individuals belonging to these species and populations was extracted using the kit Genomic DNA from tissue, NucleoSpin Tissue (Macherey-Magel GmbH and Co.) following the manufacturer's instructions. Microsatellite markers (Table 3) were obtained from an expressed sequence tags (ESTs) library (Meusemann *et al*., 2010) using Tandem Repeats Finder (Benson, 1999) with values of alignment parameters for match, mismatch and indels of 2, 3 and 5, respectively, minimum alignment score to report repeat of 30 and maximum period size of 500.

Primers were designed using Primer3 (Untergrasser *et al*., 2012) and each reverse primer was labelled with one fluorescent dye, FAM or HEX. Microsatellite markers were amplified by polymerase chain reaction (PCR) in a MyCycler thermal cycler (BIO-RAD) using Type-it Microsatellite PCR Kit (Qiagen) following manufacturer's conditions with slight modifications. Specifically, PCR reactions were performed in a 12.5 µl final volume with 15 ng of genomic DNA per reaction.

Thermal cycler protocol included an initial DNA polymerase activation step during 5 min at 95ºC, following by 30 cycles of 30 s at 95ºC, 90 s at different annealing temperature (Table 3) and 30 s at 72ºC; and a final extension of 30 min at 60ºC. Microsatellite fragments were sequenced using a 3130xl genetic analyser (Applied Biosystems) in Servicios de Apoyo a la Investigación (Universidade da Coruña) and analysed with Genemapper v3.7 software (Applied Biosystems).

Table 3. Characterization of 16 microsatellite *loci* introducing *locus* name, EST accesion number which the microsatellite motif was obtained (EST), repeat motif, primer sequences in 5'→3', annealing temperature (T_a) and allele size range (bp).

Locus	EST	Repeat motif	Primer sequence	T_a (ºC)	Allele size range (bp)
Pol001	FN246803	GTG	F: GACATGGCGGATATCAAG	57.0º	417-450
			R: GAACTGAATGACGCTGTC		
Pol003	FN246779	AGC	F: AAGAACAAGACTCCCAAGC	57.0º	210-304
			R: GCACGAGTTTCTTCACCT		
Pol004	FN246748	GAC	F: ACGAGTTGCTGGTTGACGAC	57.0º	151-184
			R: GAGAGGGCGCAGCAAAAG		
Pol005	FN246719	CGG	F: ACTGTACGACGCAGGAA	57.0º	182-239
			R: GCTAGTGGTCGCAGGA		
Pol008	FN246665	GGA	F: CGCAAAAGCACGTCTGCCCA	48.0º	516-561
			R: AGGGAGACAGCTCACACACGCA		
Pol011	FN244203	AAG	F: GCGACATCATGGCTGAC	57.0º	484-520
			R: ACCTCCTTGGGCGTGA		
Pol013	FN246659	TCG	F: ACCTTACACAACACTGACTGAG	57.0º	400-403
			R: TGCACGTAATCCAGCTGCA		
Pol014	FN246655	CTG	F: GATGGGTCACACGGTCA	57.0º	237-240
			R: CTTCCCTTCACGCACCT		
Pol019	FN244825	GCC	F: CCCGACCAAATCATCACT	57.0º	505-511
			R: TCTGGAAGACAGTGCTGA		
Pol025	FN247045	ATG	F: GGTGTCTGCCATTGAACAGG	57.0º	226-232
			R: TGCCTCATCATCACTGCCAA		
Pol043	FN244226	GGC	F: GATGATCCGCACGGCTTT	57.0º	276-279
			R: CCTTCTTCTGGTTCGTCTTGA		
Pol044	FN244192	CGA	F: CCGAGAAGTTCAAGACGCCGGA	57.0º	91-136
			R: CTTCAACGCCCGTCTCGTCGAT		
Pol114	FN247251	GCG	F: AGCGGCCCGAGTTTGTCGAGTA	57.0º	154-208
			R: AACTATCTTCTGGCCAGCTCCCCC		
Pol118	FN245700	CGG	F: TCCGCTCCAGCACATTTCCACG	57.0º	263-287
			R: CGGGCCCGCGTAACACTTTCAA		

4.4. Performing population genetic analyses in wild stocks of barnacle *P. pollicipes*.

Adult individuals of *P. pollicipes* were collected in nine wild populations along its distribution range. Analysed populations included some of the previously used to microsatellite markers optimization such as Safi (Morocco), Guincho (Portugal), Cíes Islands (Spain), and Brest (France), and six more wild populations from different locations of Spanish coast were added: Roncudo, Artabrian Gulf, Ortigueira, Andrín, and Cape of Ajo (Fig. 6). Genomic DNA was extracted using the kit Genomic DNA from tissue, NucleoSpin Tissue (Macherey-Magel GmbH and Co.) following the manufacturer's instructions. Microsatellite markers used in this population analysis were optimised in the previous section and amplifications carried out by polymerase chain reaction

(PCR) in a MyCycler thermal cycler (BIO-RAD) using Type-it Microsatellite PCR Kit (Qiagen) under conditions specified before. Thermal cycler protocol was exactly the same than it was used to microsatellite optimization except for Andrín and Cape of Ajo populations which amplified temperature was 48ºC instead 57ºC. Microsatellite fragments were sequenced using a 3130xl genetic analyser (Applied Biosystems) in Servicios de Apoyo a la Investigación (Universidade da Coruña).

Microsatellite allele determination was performed using software Geneious (Kearse *et al*., 2012).Estimations of allele number, observed and expected heterozygosities, deviations of H-W equilibrium, private alleles, Fst indices and number of migrants (Nm) were performed using GenAlEx 6.5 (Peakall and Smouse, 2012) under default conditions. Software FreeNA (Chapuis and Estoup, 2007) was used to determine frequencies of null alleles. Possible bottleneck events were looked for using bayesian algorithms implemented in Bottleneck 1.2.02 (Cornuet and Luikart, 1997), under three available mutational models (IAM, TPM, SMM) provided by the program and using default 1000 replicates. Principal component analysis (PCA) were carried out using software Genetix 4.05 (Belkhir *et al*., 1996-2004) and non-centred minimum curvilinear embedding (ncMCE) algorithm described by Alanis-Lobato *et al*. (2015) was applied using MatLab (The MathWorks Inc., 2015) after transform microsatellite data into qualitative variables and plotted using IBM-PASW package version 19 (IBM Corp., 2010). Bayesian clustering of *P. pollicipes* populations was performed with software Structure 2.3.4 (Hubisz *et al*., 2009) and Neighbor-Joining phylogenetic reconstruction was inferred using Mega6 (Tamura *et al*., 2013). Logistic model to assign individuals to populations was calculated with software IBM-PASW package version 19 (IBM Corp., 2010).

4.5. Developing gene expression analyses in two phenotypes of barnacle *P. pollicipes*.

Morphological data measured by Eugenia Rodriguez and Ricardo Arnáiz (personal communication) from individuals of two phenotypes (“sun” and “shadow”) collected in wild populations of rocky coast and crevices from O

Roncudo (NW Spain) were statistically analysed in order to perform comparisons between phenotypes for variables measured by them using T-test implemented in IBM-PASW package version 19 (IBM Corp., 2010).

Different individuals of both phenotypes from O Roncudo (NW Spain) were collected and preserved into RNA*later*™ (QIAGEN) and liquid nitrogen. RNA extractions were carried out from different tissues of both phenotypes: capitulum tissue, peduncular muscle, cuticular tissue from sun barnacles and capitulum tissue, peduncular muscle, cuticular tissue, capitulum-peduncle limit and cuticle-penduncular tissue from shadow barnacles. RNA extractions were performed following the instructions of the manufacturer using Aurum™ Total RNA Mini Kit (BIO-RAD Laboratories) freezing the tissues in liquid nitrogen and pulverized with a pestle. RNA quantifications were measured in a 2100 Bioanalyser Instrument (Agilent Technologies). Reverse transcription were carried out using iScript™ cDNA Synthesis Kit (BIO-RAD Laboratories) following the instructions of the manufacturer in a thermocycler MyCycler™ (BIO-RAD Laboratories) with a thermal program of 5 min at 25ºC, 30 min at 42ºC and 5 min at 85ºC.

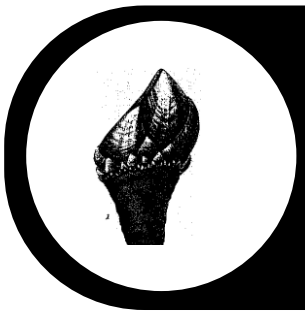
Reference genes were designed from ESTs sequences developed by Meusemann *et al*. (2010) and from nucleotide sequences of Pérez-Losada *et al*. (2004). Primers for genes albumin, actin, β-actin, histone H3, HSP70, HSP90 (Table 4) were designed using Primer3 (Untergrasser *et al*., 2012) and amplified from 150 to 200 nucleotides of each housekeeping gene annealing in conserved regions. A calibration line was estimated for housekeeping genes using four different cDNA concentrations (100, 20, 5 and 1 ng/µl). Real-time PCR amplifications were carried out using FastStart SYBR Green Master (Roche Diagnostic) following the instructions of the manufacturer and using an annealing temperature of 53ºC.

Target genes selected to compare between both phenotypes were obtained from unigenes assembled by Perina *et al*. (2014). Target genes include guanine nucleotide-binding protein, chitin-based cuticle attachment to epithelium, cuticular protein 47Ee, cuticular protein 11B and cuticular protein RR-1. Primers to amplify selected target genes were designed in conserved

regions of these unigenes and its amplification products ranging between 150 and 200 nucleotides (Table 4). Three replicates were carried out for all amplifications (both housekeeping and target genes). Amplifications were performed in all tissues from both phenotypes as it was described above for housekeeping genes.

Table 4. Reference and target genes used in the study including gene name, gene name abbreviation (Code), primer sequences in 5'→3', size in base pairs of the PCR product, and accession numbers of the sequences used to primer design.

Reference genes				
Gene	Code	Primers sequence	Size	Accession numbers
Albumin	ALB	F: CCTTGGTGAACATTTGAGTGC R: CTGTTGTTGACCGGACTGT	200	FN246676
Heat shock protein 70	HSP70	F: GCTGATCAAGCGCAACACG R: GTCAGCTCGAACTTGCCGA	150	FN246260
Heat shock protein 90	HSP90	F: AGAAGGAAGAGGACAAGGACA R: CTCCATACTCCTCCTGGCTAA	183	FN244265
Histone H3	H3	F: CGTCGCTACCAGAAGAGCAC R: ATGGCACACAGGTTCGTGTC	182	AY520718
β-Actin	β-ACT	F: CCAGATCATGTTCGAGACCTT R: GGCAGAGCATAACCCTCATAG	156	FN244041, FN244645, FN245194, FN245876, FN246602, FN247145
Actin	ACT	F: GACCGTGTACAACAGCATCAT R: TCTTGATCTTGATGGTGCTCG	158	FN245194, FN245493
Target genes				
Gene		Primers sequence	Size	Accession numbers
Guanine nucleotide-binding protein subunit beta-like protein-like		F: TTTCGCTGTGATAGTGTCGT R: ACATGATCAGCGATTTGTCG	161	UN0024
Chitin-based cuticle attachment to epithelium		F: CAAGGTCAGAAGTGCGTTCA R: CGTTCACGTTGTTGCCAGTT	170	UN0076
Cuticular protein 47Ee		F: ATCACGAGCCAGAACTTCG R: GTCGGGATCCTGATACGAGT	159	UN0113
Cuticular protein 11B		F: AAAATGTTTGTGCTCGCCTG R: TATCCGTACTCGTAGTTGCC	164	UN0196
Cuticular protein RR-1 motif 52		F: ATTTTTCGTCCCTGTAAGGC R: CGAGAAGTAGAAACGGAGCA	156	UN0179



RESULTS

5. Results.

5.1. Phylogenetic relationships between current species of family Pollicipedidae based on nuclear and mitochondrial markers.

Phylogenetic inferences based on sets of sequences belonged to mitochondrial and nuclear markers have showed both different evolutionary models for each set and different tree topologies. Thus, estimations of molecular evolutionary models were carried out under AICc detecting general time reversible model in 16S, 18S-28S and EF1α, and Tamura model in COI gene.

Sets of sequences from mitochondrial genes generated similar tree topologies. Reconstructions carried out with 16S rDNA dataset have showed two lineages, one represented by *P. caboverdensis* and another constituted by the other three species of this genus which showed an earlier divergence of *P. pollicipes* respect from the clade *P. elegans-P. polymerus*. However, two important branches of this topology have not showed enough statistical support (Fig. 7). On the contrary, statistical support obtained by branches which define each *Pollicipes* species was higher than 96%.

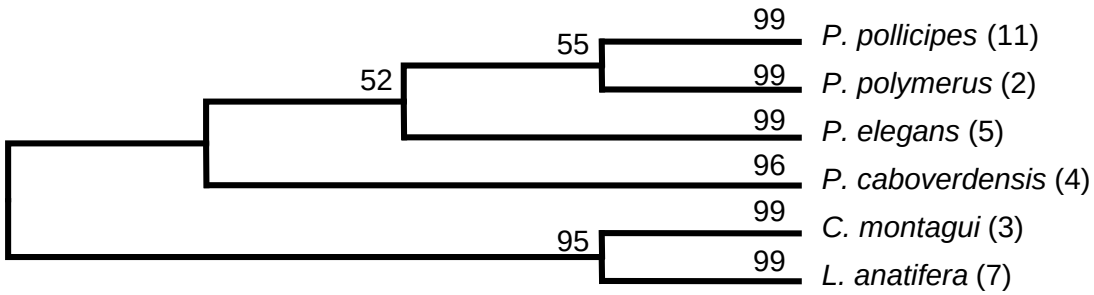


Figure 7. Phylogenetic reconstruction of family Pollicipedidae based on 16S rDNA region using 1000 bootstrap replicates. Only values of bootstrap higher than 50 are showed. Numbers between parentheses indicate the number of sequences in each collapsed branch.

Phylogenetic reconstruction obtained using COI gene showed a high number of branches with low bootstrap support. However, this tree topology showed the lineage constituted by *P. polymerus* isolated from the other three lineages and supported by an 89% of bootstrap value. Other lineages arise from a 90% supported branch where *P. caboverdensis* and *P. elegans* were

statistically isolated (99% and 97% of bootstrap support, respectively) but *P. pollicipes* showed a polytomy without enough statistical support to form a specific clade (Fig. 8).

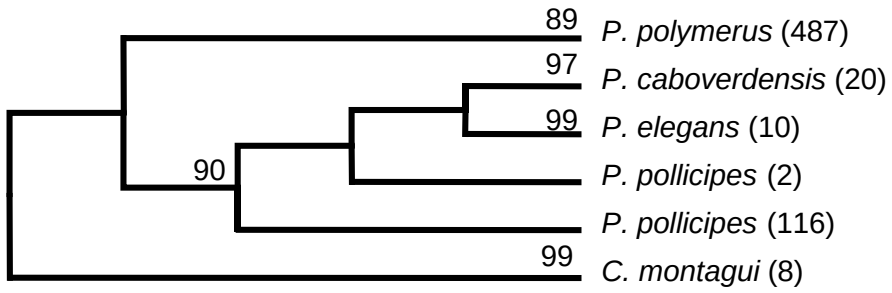


Figure 8. Phylogenetic reconstruction of family Pollicipedidae based on COI region using 1000 bootstrap replicates. Only values of bootstrap higher than 50 are showed. Numbers between parentheses indicate the number of sequences in each collapsed branch.

Phylogenetic trees reconstructed based on nuclear markers EF1α and 18S-28S rDNA regions have showed similar topologies and let differentiate all the four species of family Pollicipedidae. On the one hand, phylogenetic reconstruction based on EF1α presented two differentiated lineages, one corresponding to *P. elegans*, and the other lineage included *P. pollicipes* and *P. polymerus* (Fig. 9).Sequences belonged to *P. caboverdensis* could not be obtained. Bootstrap values were higher than 93% in all branches which defined clades in this tree, although the cluster conformed by *P. polymerus* and *P. pollicipes* showed a bootstrap value not enough robust to support this branch(73%).

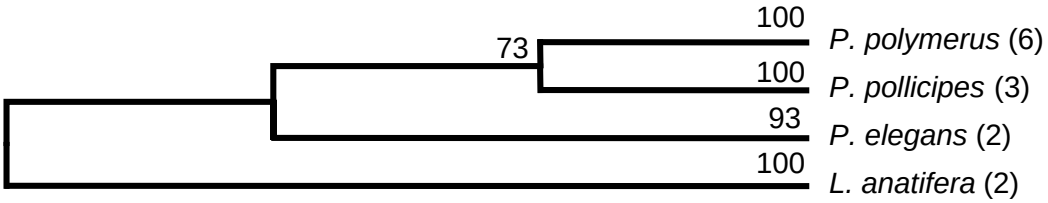


Figure 9. Phylogenetic reconstruction of family Pollicipedidae based on EF1α region using 1000 bootstrap replicates. Only values of bootstrap higher than 50 are showed. Numbers between parentheses indicate the number of sequences in each collapsed branch.

Topology showed by reconstructed tree based on ribosomal genes distinguished four lineages, each one corresponding to one species of genus

Pollicipes. Tree topology showed two lineages, one conformed by *P. caboverdensis* and another constituted by the other three *Pollicipes* species, which, subsequently, is divided into two clades one formed by *P. polymerus* and another by *P. pollicipes* and *P. elegans* (Fig. 10). However, bootstrap support is not enough strong in the two branches which determine these lineages, so the tree topology corresponds to a polytomy constituted by four lineages, each one corresponding to one species of genus *Pollicipes*. Statistical support showed by each branch which defines a lineage is higher than 94%.

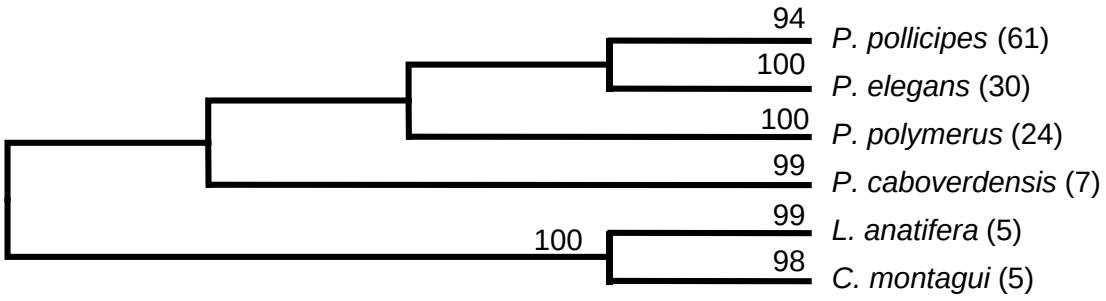


Figure 10. Phylogenetic reconstruction of family Pollicipedidae based on 18S-28S rDNA region using 1000 bootstrap replicates. Only values of bootstrap higher than 50 are showed. Numbers between parentheses indicate the number of sequences in each collapsed branch.

A consensus tree was inferred using a combination of three genes amplified in all four species of genus *Pollicipes*: 16S, COI and 18S-28S region. Topology obtained with this sequences dataset showed two lineages, one constituted by *P. pollicipes* and *P. elegans* and another one conformed by *P. polymerus* and *P. caboverdensis* (Fig. 11). Although branches which support these lineages presented bootstrap values lower than 50%, being a polytomy the topology which better fits with this phylogenetic inference. In this polytomy, each of the four clades is constituted by sequences belonging to one species of genus *Pollicipes* and is supported by bootstrap values higher than 95%.

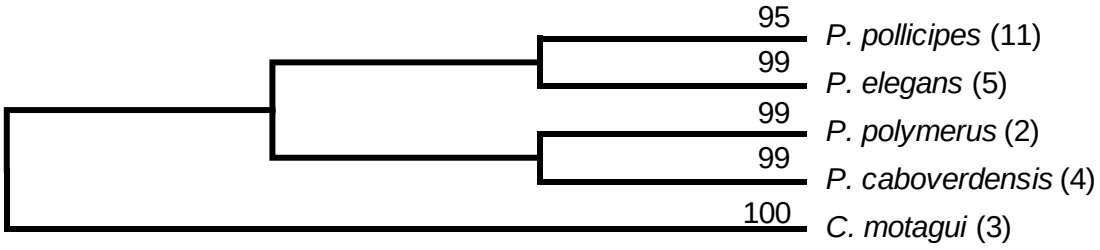


Figure 11. Phylogenetic reconstruction of family Pollicipedidae based on concatenated sequences of 16S, COI and 18S-28S regions using 1000 bootstrap replicates. Only values of

bootstrap higher than 50 are showed. Numbers between parentheses indicate the number of sequences in each collapsed branch.

5.2. Secondary structures predicted for internal transcribed spacer 1, 5.8S rDNA and internal transcribed spacer 2 among Crustacea: general folding patterns.

5.2.1. 5.8S rRNA: secondary structure general model, CBCs and TFs.

Secondary structures of 5.8S rRNA were predicted for 1,543 sequences belonged to 128 crustacean species, and 319 different haplotypes were detected on the basis of the nucleotidic sequence. A general folding pattern was detected and consisted in three double-stranded helices with helix I composed by two subhelices, and two 28S rRNA pairing regions in both ends (Fig. 12).

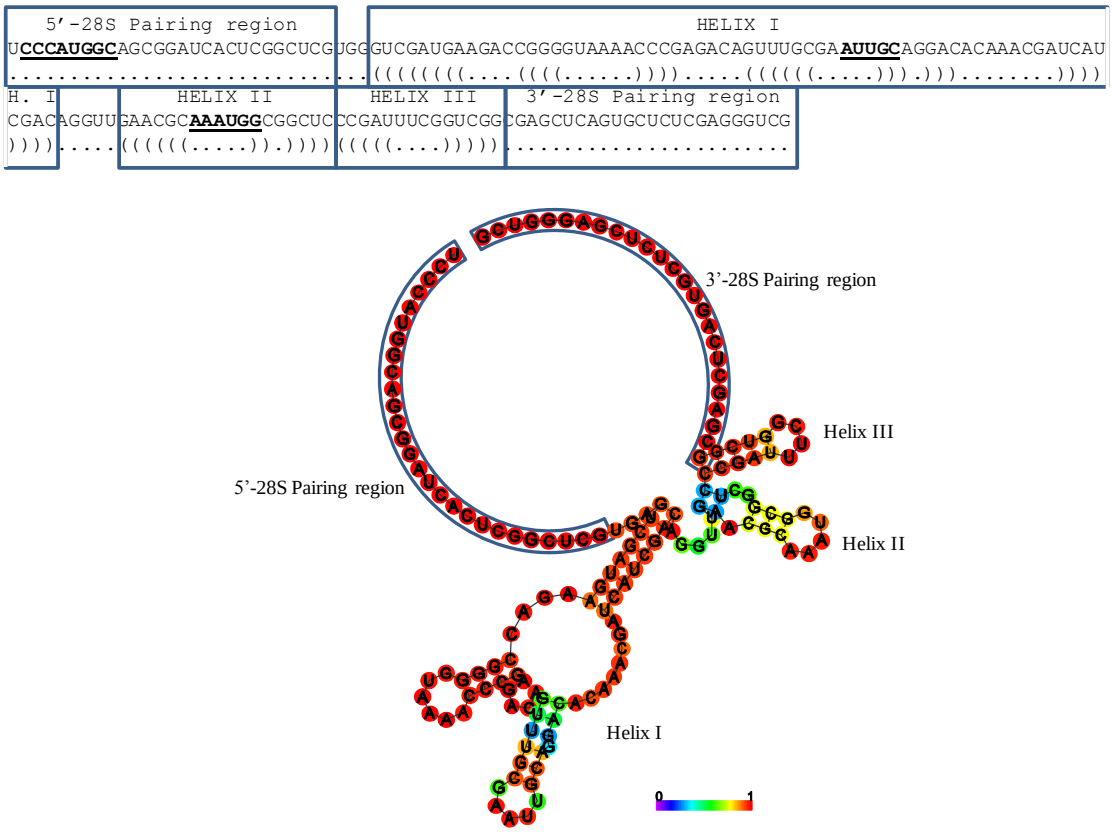


Figure 12. Secondary structure predicted for 5.8S rDNA from *P. pollicipes*. Boxes show 5.8S-28S pairing regions and the different helices domains and bolded underlying nucleotides correspond with transcription factors binding regions. Scale represents the base pair probabilities.

At morphologic level, secondary structures detected in crustacean species have showed structural similarities which evince their conservation degree. Putative secondary structures were also detected including:

a) Helix I composed by three subhelices instead of two, and helices II and III (might either be absent or constituting the same helix) (Fig. 13a).

b) Helix I folded in two helices, one of them longer, and might display two subhelices; and helices II and III folded as in the general model, absent or constituting the same helix (Fig. 13b).

c) A secondary folding pattern with helix I shorter and without showing subhelices, helix II displaying two subhelices and helix III might be absent (Fig. 13c).

d) Three helices without subhelices but with helix II the longest one (Fig. 13d).

e) Helix I with two subhelices, one of them significantly longer, and helices II and III constituting the same helix (Fig. 13e).

f) Helix I composed by four subhelices instead of two, and helices II and III absent (Fig. 13f).

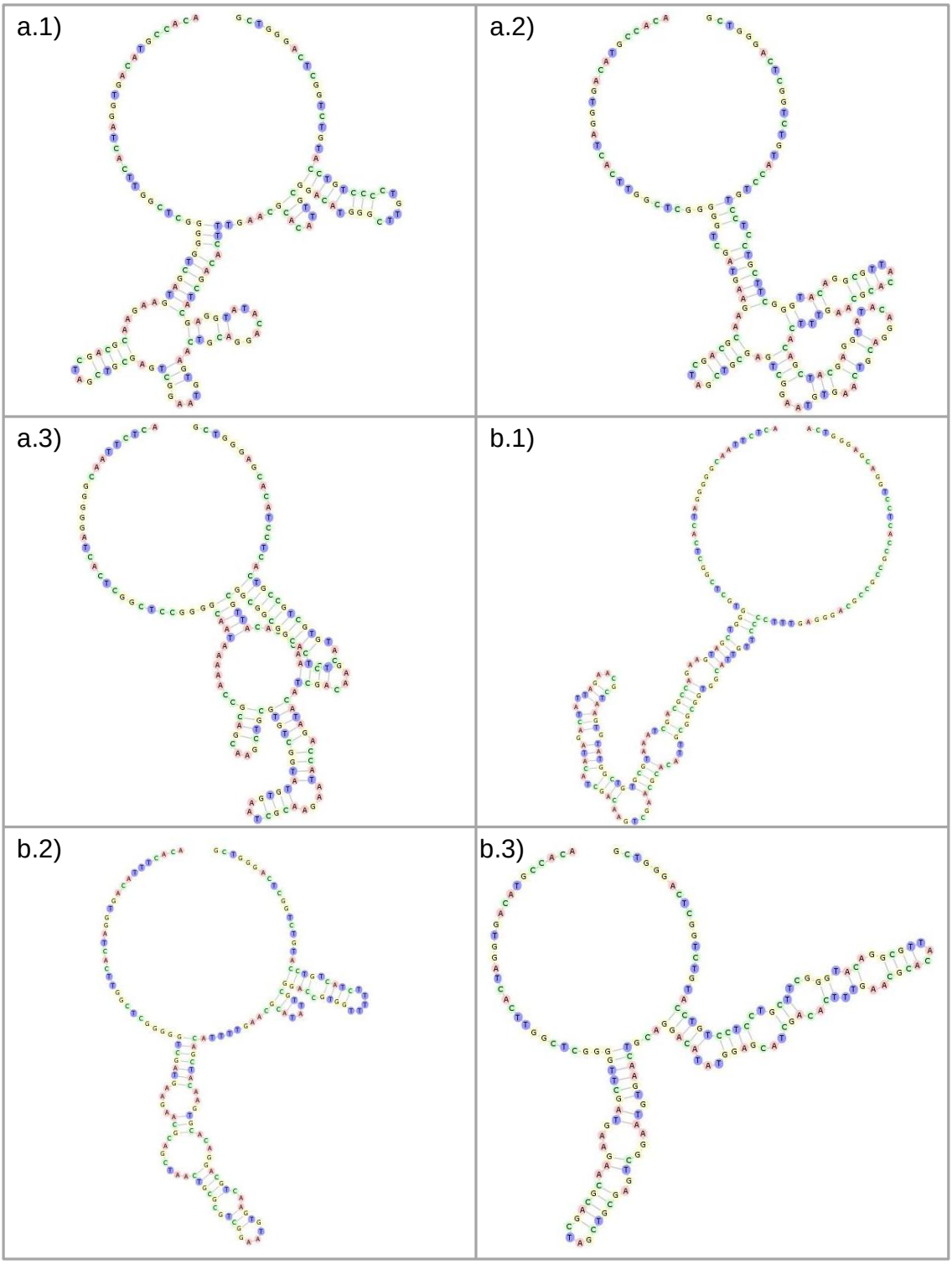


Figure 13. Putative secondary structures detected for 5.8S rDNA in analysed crustacean species.

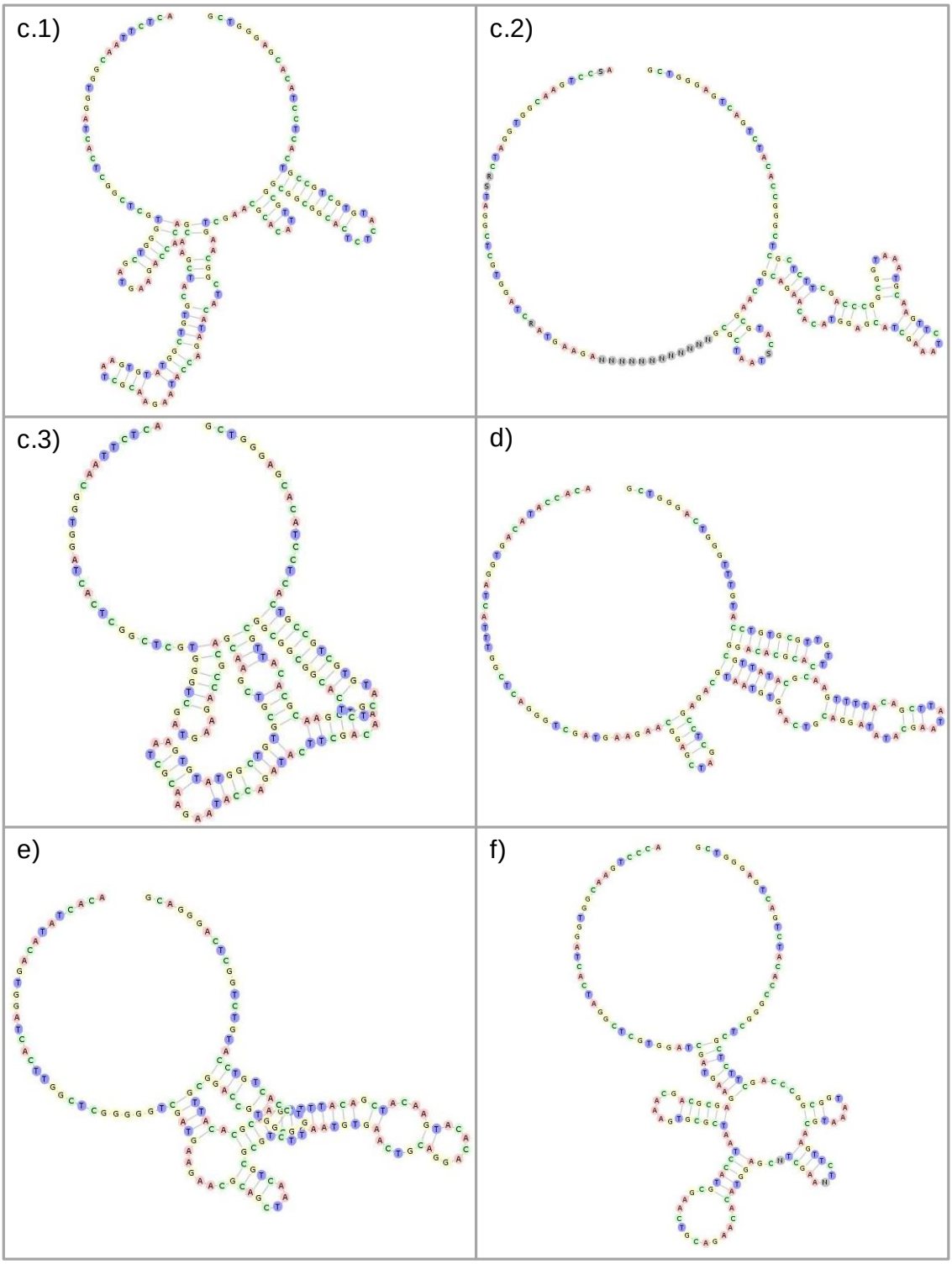


Figure 13. Cont.

The minimum free energy (MFE) obtained for each secondary structure ranged between -50.50 and -12.40 kcal/mol. Folding pattern of 5.8S rDNA maintained its secondary structure with temperature diminution. However, folding variants were significantly affected by temperature. Specifically, variant with helix I composed by four subhelices which showed at low temperatures

only two helices. CBCs were detected within the same species solely in *Acanthodiaptomus pacificus* of family Diaptomidae, which showed the compensatory changes G-C/A-U in positions 113/131 and A-U/G-C in positions 115/129, and *Epilobocera sinuatifrons* of family Pseudothelphusidae, which showed the compensatory change C-G/U-A in positions 34/44 and G-C/U-A in positions 71/111. The mean probability that the CBCs found in this region were associated to different species was 0.9359. Different species presented between 2 to 16 regions which showed homology with TFs target sites along 5.8S rRNA sequence.

Phylogenetic inferences were carried out using only 5.8S rDNA due to ITS regions sequences were too genetically divergent to be aligned. Secondary structures and nucleotidic sequences of 5.8S rDNA were used to infer four Neighbor-Joining phylogenies corresponding to four classes of subphylum Crustacea: Ostracoda, Branchipoda, Malacostraca and Maxillopoda. A general reconstruction including all sequences and structural data could not be inferred due to genetic distances between different taxa which conform subphylum Crustacea were so high. Topologies of phylogenetic reconstructions of each class inferred independently let identified certain taxa. Thus, in class Branchiopoda (Fig. 14) two orders were monophyletic: Anostraca (represented by family Branchipodidae) and Diplostraca (constituted by family Bosminidae); in class Ostracoda reconstruction (Fig. 15) only family Darwinulidae showed a monophyletic origin; in class Malacostraca (Fig. 16) sequences of infraorders Brachyura, Anomura and Astacidea belonged to order Decapoda, and family Mysidae from order Mysida appeared correctly defined; finally, in class Maxillopoda (Fig. 17),orders Sessilia (with family Chthamalidae) and Pedunculata (including family Pollicipedidae) showed monophyletic origins.

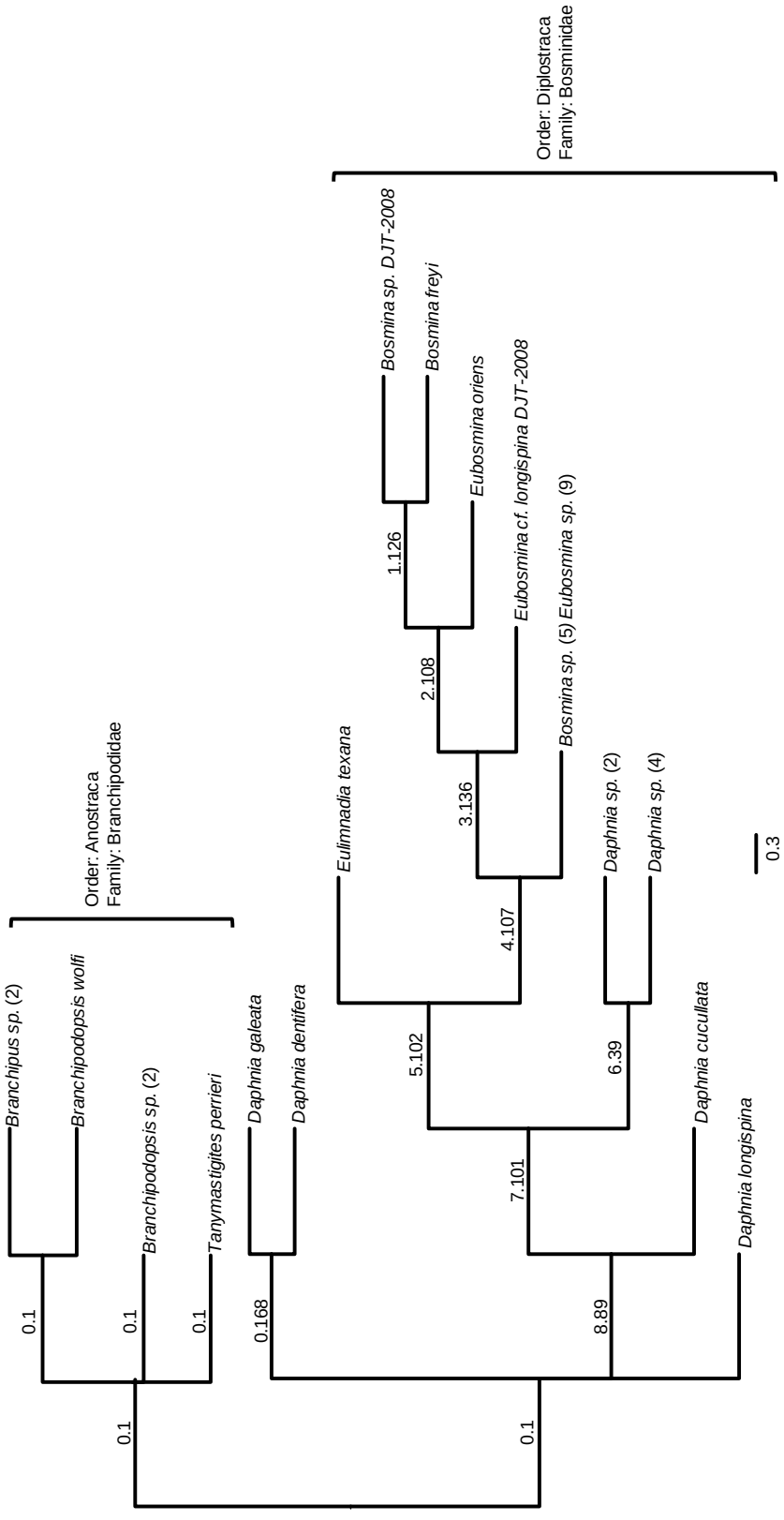


Figure 14. Phylogenetic reconstruction of class Branchiopoda based on sequence and secondary structure of 5.8S rDNA gene. Numbers between parentheses indicate the number of different species with the same sequence and secondary structure for this region. The number of bootstrap replicates is 1000.

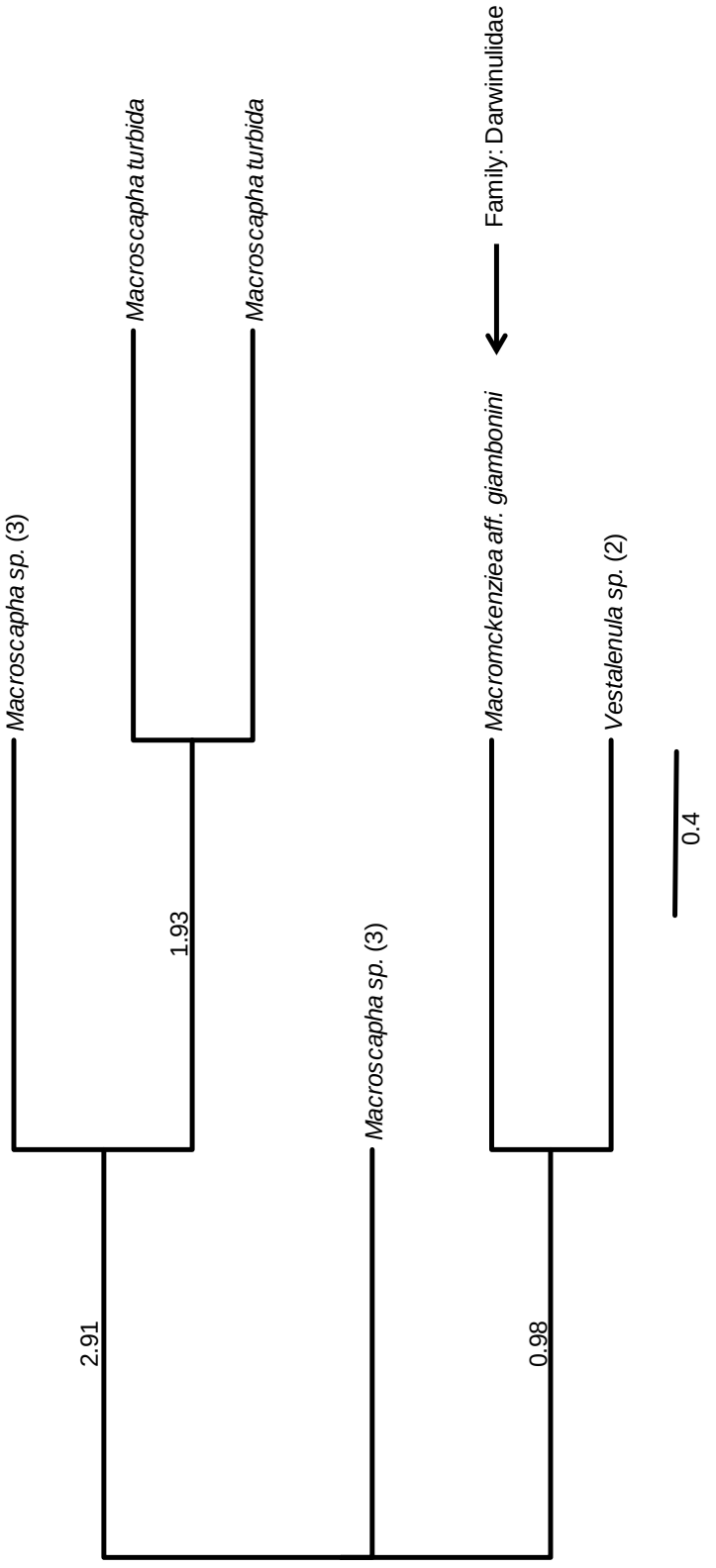


Figure 15. Phylogenetic reconstruction of class Ostracoda based on sequence and secondary structure of 5.8S rDNA gene. Numbers between parentheses indicate the number of different species with the same sequence and secondary structure for this region. The number of bootstrap replicates is 1000.

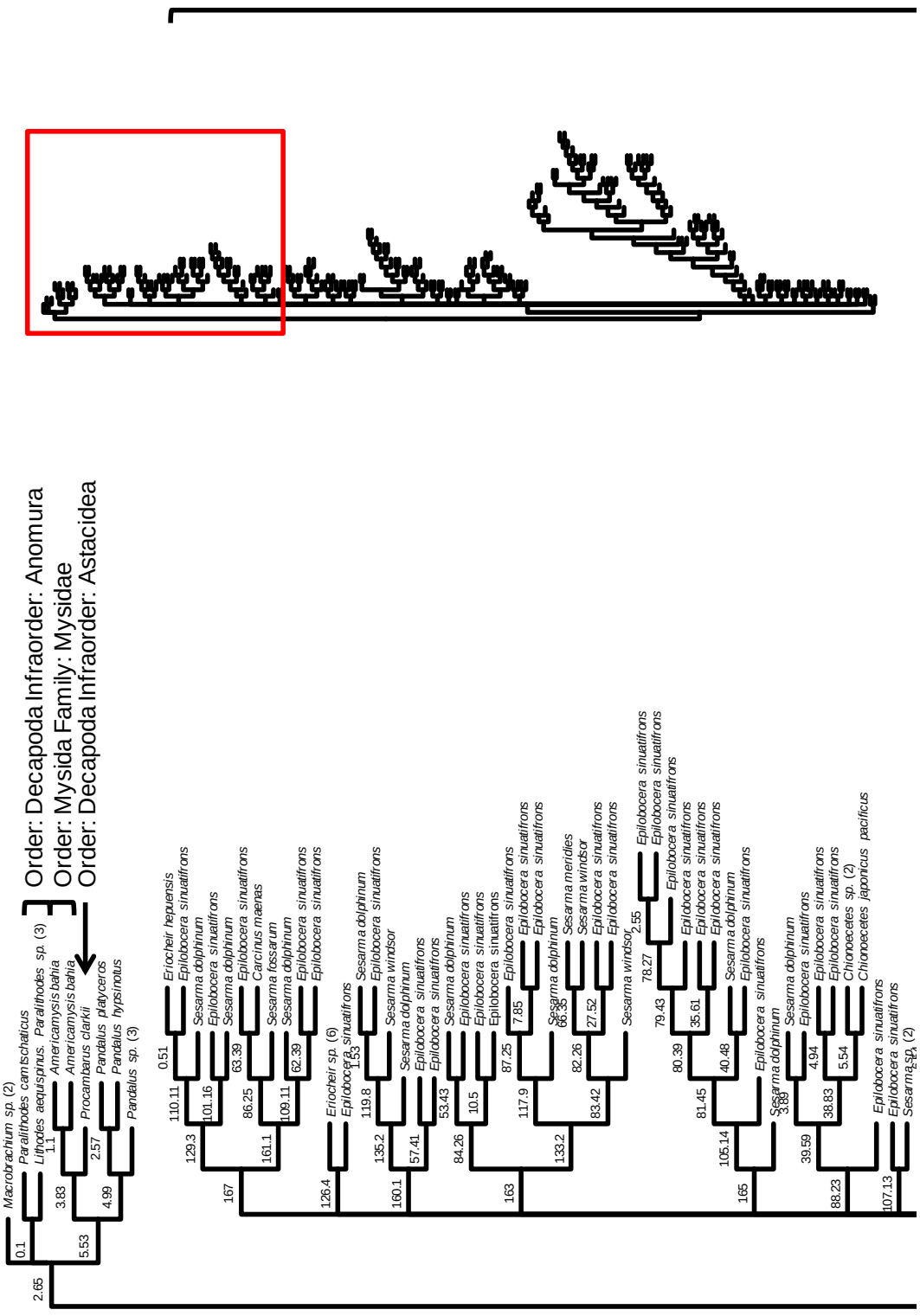


Figure 16. Phylogenetic reconstruction of class Malacostraca based on sequence and secondary structure of 5.8S rDNA gene. Numbers between parentheses indicate the number of different species with the same sequence and secondary structure for this region. The number of bootstrap replicates is 1000.

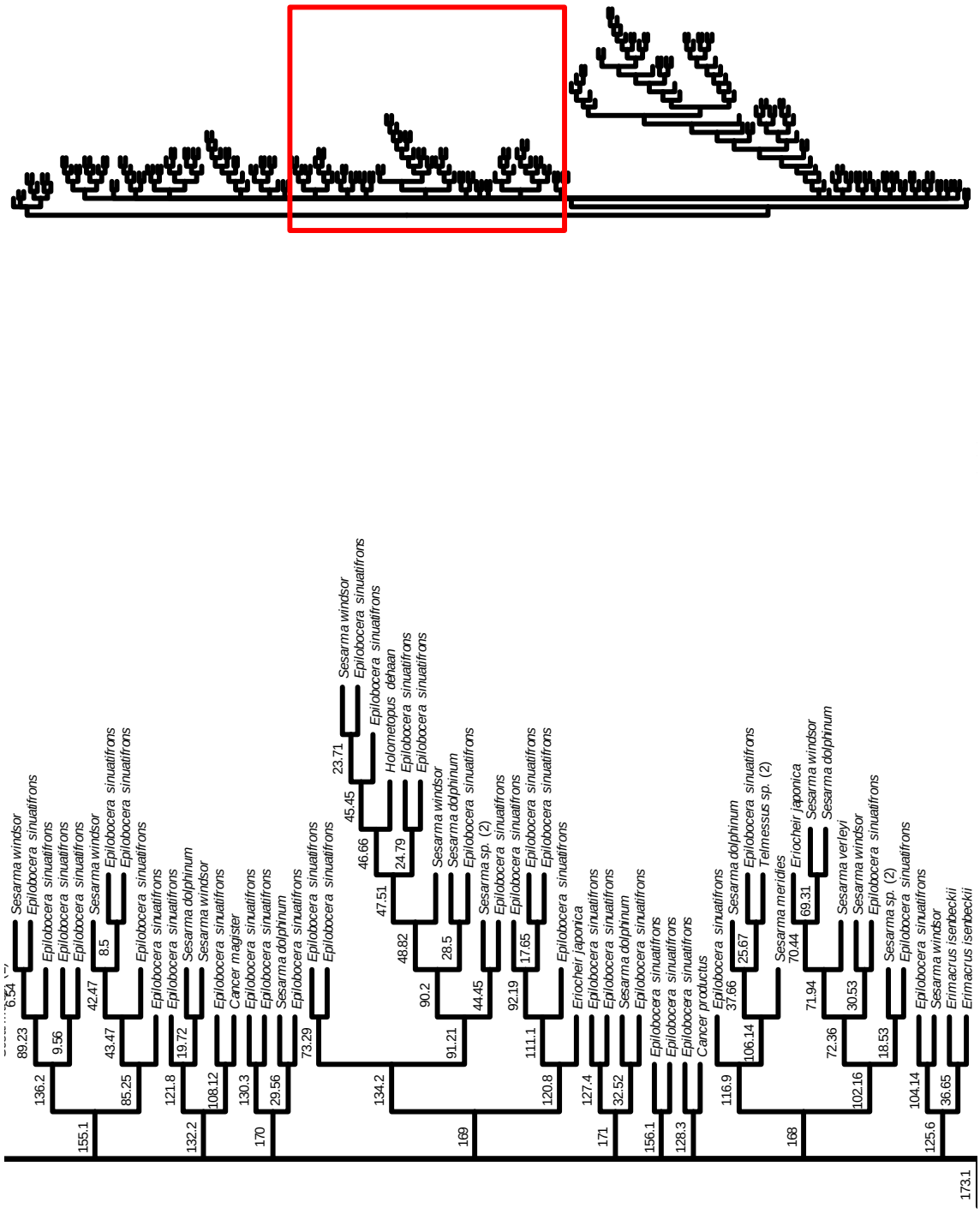


Figure 16. Cont.

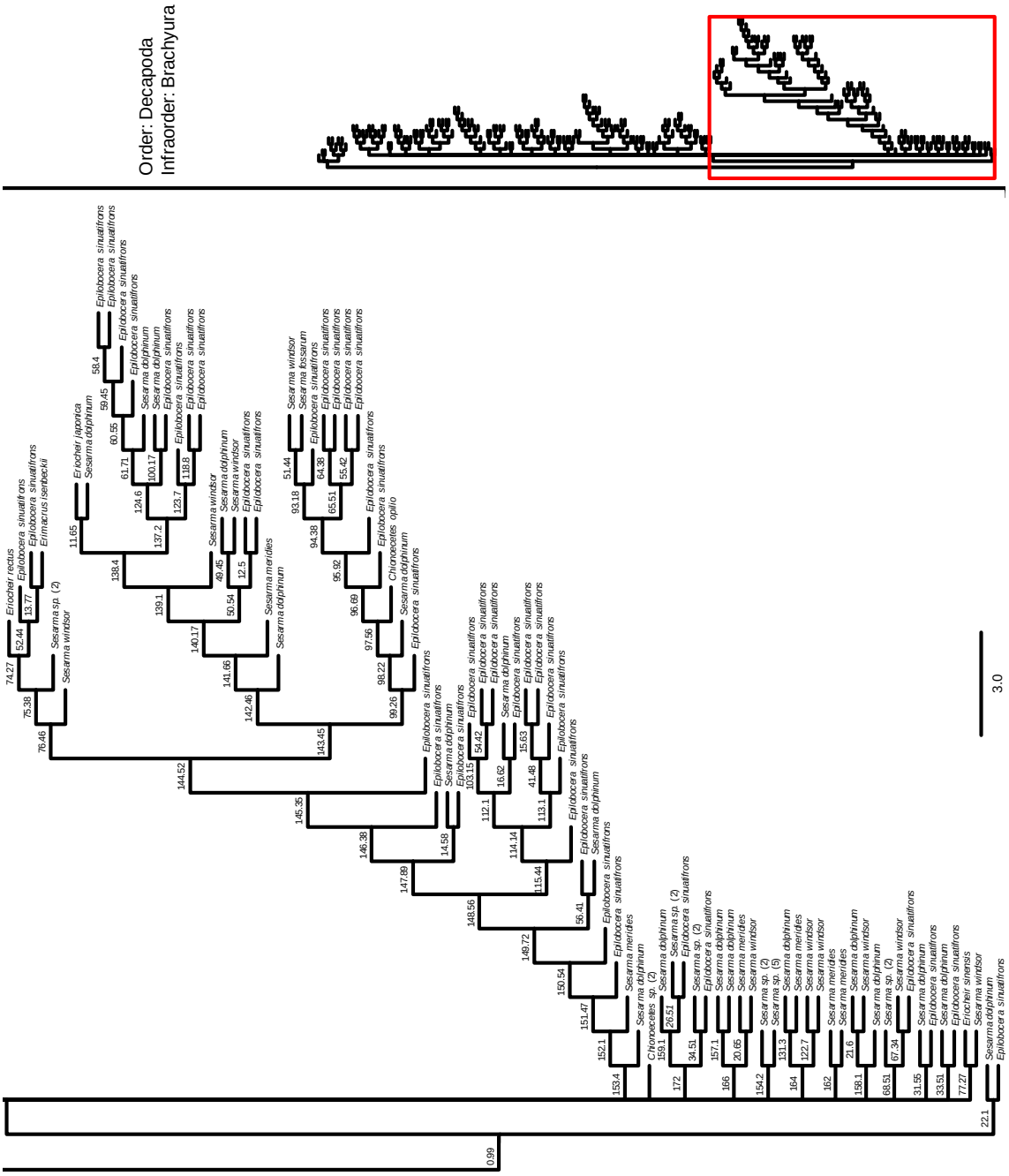


Figure 16. Cont.

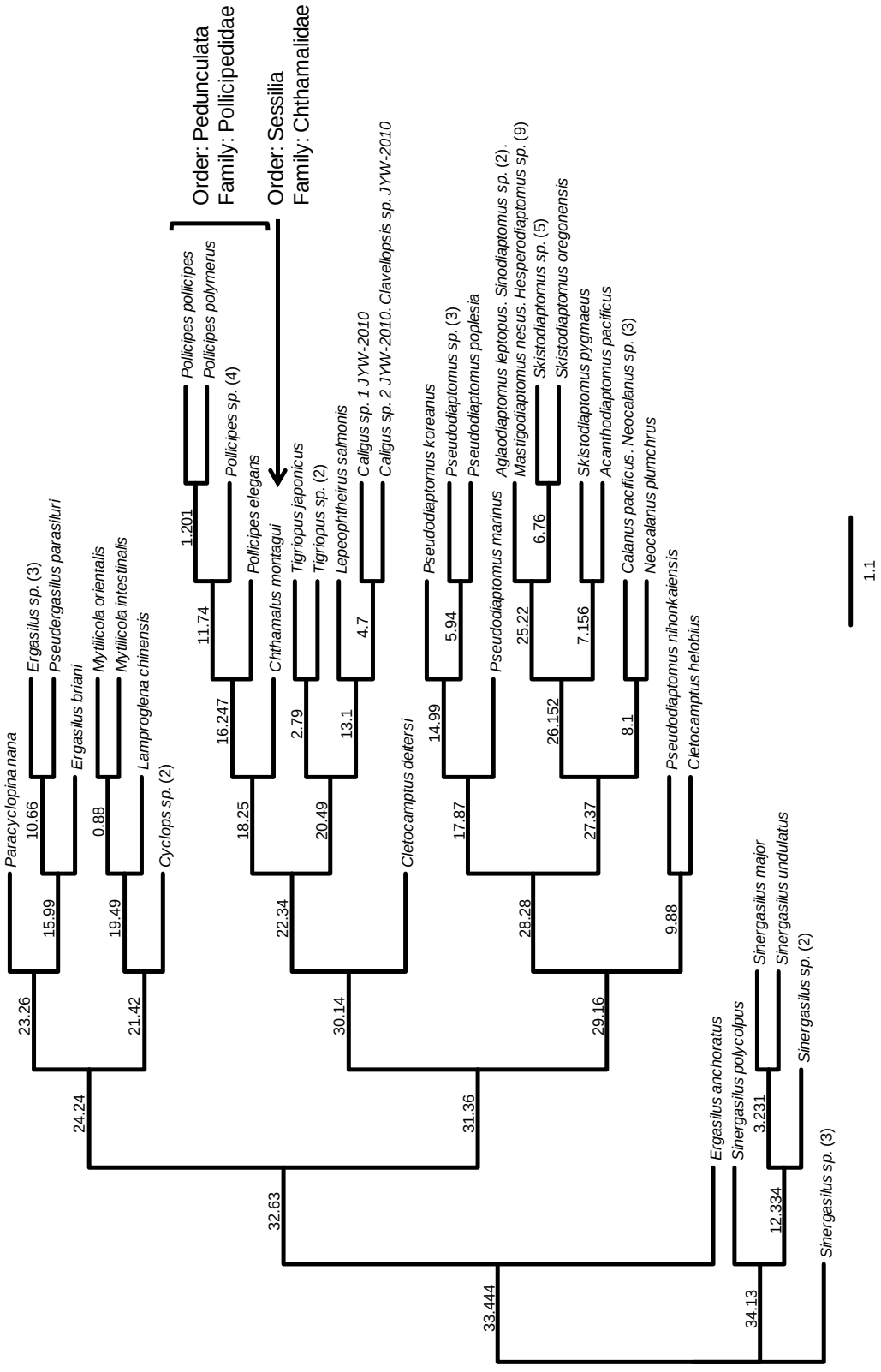


Figure 17. Phylogenetic reconstruction of class Maxillopoda based on sequence and secondary structure of 5.8S rDNA gene. Numbers between parentheses indicate the number of different species with the same sequence and secondary structure for this region. The number of bootstrap replicates is 1000.

5.2.2. ITS1: secondary structure general model and CBCs and TFs.

Secondary structures of ITS1 were predicted for 1,283 sequences and 614 different haplotypes belonged to 190 crustacean species were detected. This folding pattern consisted in a variable number of double-stranded helices protruding from an open ring structure, how is showed in figure 18, which represent the ITS1 folding pattern presented by the main species of this study, *P. pollicipes*. Putative secondary structures detected showed between two and 11 double-stranded helices along the ring core (data not shown), which indicate that ITS1 is not highly conserved on this taxa. Nevertheless, the conservation degree increase at family level. The MFE obtained for each secondary structure ranged between -726.90 and -41.20 kcal/mol. Decline of folding temperature increased the number of doubled-stranded helices protruding from the open ring structure, and these helices were shorter and showed a reduced number of sub-helices. CBC detected in the *Ampithoe longimana*, *Cletocamptus deitersi*, *Skistodiaptomus pallidus*, *Acanthodiaptomus pacificus*, *Euphausia recurva*, *Tigriopus japonicus*, *Macrobrachium nipponense*, *M. rosenbergii*, *Jasus edwardsii*, *Paracalanus parvus*, *Marsupenaeus japonicus*, *Pseudocyclops cf. juanibali*, *Eriocheir sinenesis* species varying from one to ten (Annex 2). The mean probability that the CBCs found in this region correspond with different species was 0.9545. Target sites of TFs found along ITS1 sequence, ranging between seven and 75 TF motifs per sequence.

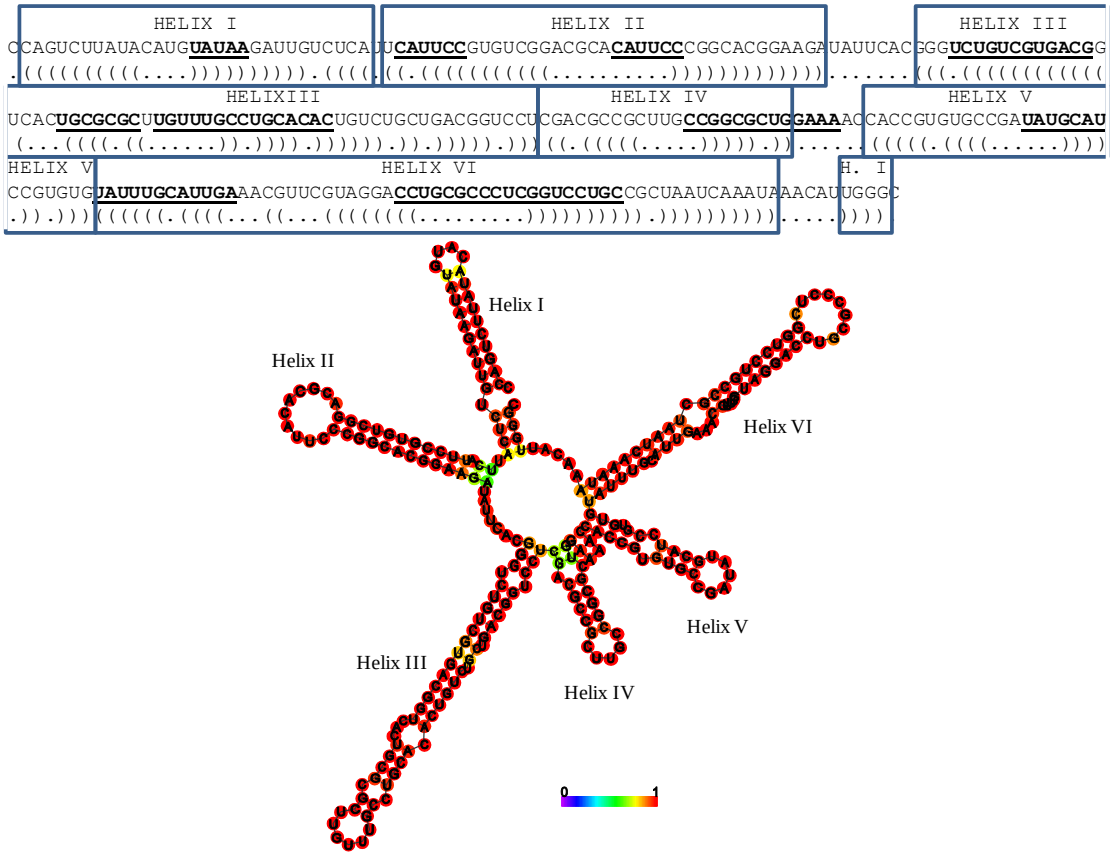


Figure 18. Secondary structure predicted for ITS1 spacer from *P. pollicipes*. Boxes show the different helices domains and bolded underlying nucleotides correspond with transcription factors binding regions. Scale represents the base pair probabilities.

5.2.3. ITS2: secondary structure general model and CBCs and TFs.

Secondary structures of ITS2 region were predicted for 2,063 sequences and 1107 different haplotypes belonged to 208 crustacean species were found based on the nucleotidic sequence. A maximum of 27 different folding patterns were obtained. A general secondary structure for ITS2 region consists in two to 12 doubled-stranded helices separated by a single-stranded structure which constitute an open ring. Figure 19 shows the secondary structure of ITS2 region of *P. pollicipes*, main species object of this study. Albeit different putative variants were detected, the conservation degree showed at family level was higher than for ITS1.

Putative secondary structures detected were consistent within different taxa (data not shown), mainly at species level and at family level. MFE obtained for each secondary structure ranged between -880.20 and -57.60 kcal/mol.

Similar to ITS1, reduction in folding temperature produced secondary structures of ITS2 region with an increased the number of doubled-stranded helices protruding from the open ring structure. CBC were detected in *Calanus sinicus*, *C. deitersi*, *Mesocyclops longisetus*, *Daphnia longispina*, *A. pacificus*, *T. japonicus*, *P. pollicipes*, *P. elegans*, *Labidocera acuta*, *Pseudodiaptomus koreanus*, *Epilobocera sinuatifrons*, *E. sinensis*, *E. japonica* species ranging between one and ten (Annex 2). The mean probability that the CBCs found in this region belong to different species was 0.9165. Different regions along ITS2 sequence presented homology with TFs target sites, ranging between five and 36 TF motifs.

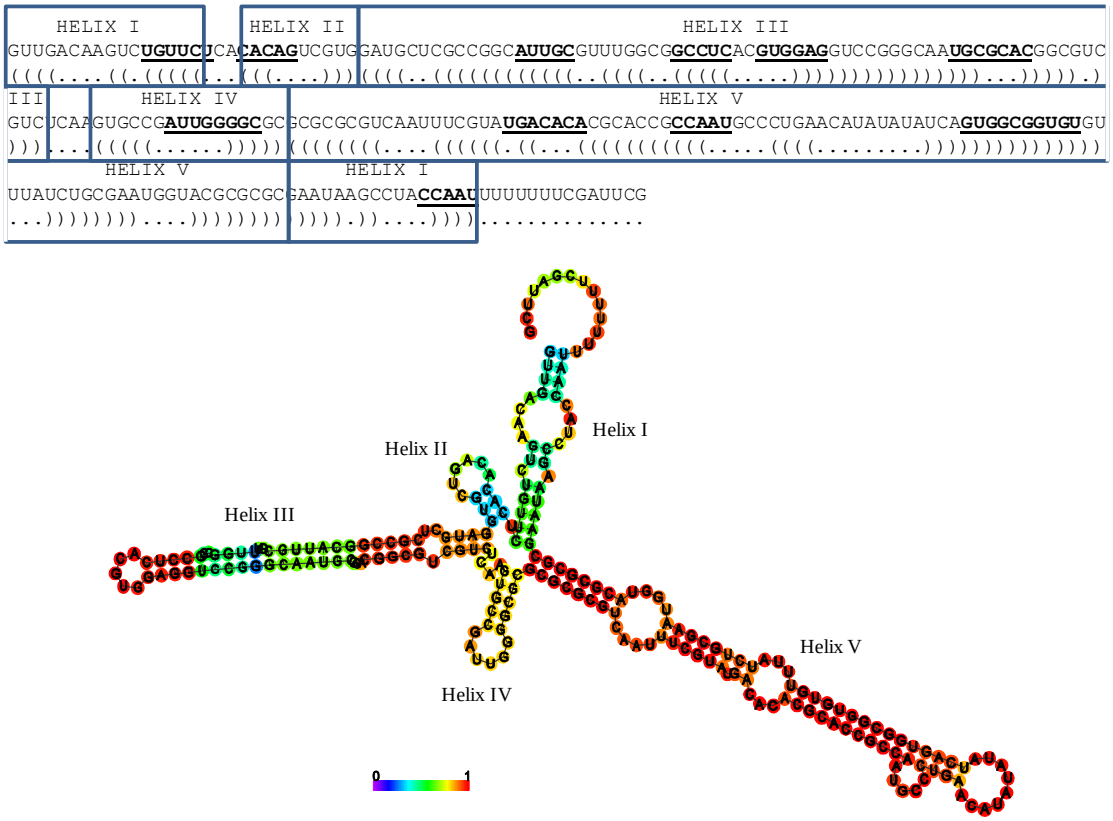


Figure 19. Secondary structure predicted for ITS2 spacer from *P. pollicipes*. Boxes show the different helices domains and bolded underlying nucleotides correspond with transcription factors binding regions. Scale represents the base pair probabilities.

5.3. Characterization of 12 polymorphic microsatellite *loci* in gooseneck barnacle *P. pollicipes* (Gmelin, 1789), and cross-amplification in other *Pollicipes* species.

Microsatellite markers were amplified in 16 individuals of four populations of *P. pollicipes* with different amplification success. The number of alleles detected for each *locus* ranged between two and 20. Only some of these markers showed deviation of H-W equilibrium after Bonferroni's correction due to deficit of heterozygotes. Frequencies of null alleles are higher than 10% in *loci* Pol003, Pol004, Pol014, Pol019, Pol043 and Pol118 (Table 5).

The half analysed microsatellite markers presented private alleles in some of four populations. Cross-specific amplifications of these microsatellite *loci* performed in other three species of family Pollicipedidae showed positive amplification for four to six markers in each other barnacle species (Table 6).

Table 5. Statistical assessment of 16 microsatellite *loci* in each of the four population including number of alleles presented in each *locus* (NA) and frequency of null alleles in each *locus* (between parentheses), observed heterozigosity (Ho) and expected heterozigosity (He).

Locus	All populations			Cíes Islands		Safi		Brest		Guincho	
	NA	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He
Pol001	5 (0.04862)	0.125	0.148	0.063	0.061	0.000	0.000	0.125	0.119	0.313	0.363
Pol003	20 (0.16378)	0.484	0.737	0.688	0.826	0.438	0.623	0.250	0.619	0.571	0.699
Pol004	4 (0.20993)	0.063	0.300	0.063	0.170	0.125	0.430	0.063	0.463	0.000	0.000
Pol005	13 (0.03630)	0.667	0.785	0.438	0.369	0.929	0.645	0.667	0.727	0.667	0.583
Pol008	6 (0.00000)	0.754	0.603	0.875	0.643	0.429	0.452	0.909	0.657	0.813	0.617
Pol011	7 (0.00001)	0.237	0.220	0.125	0.119	0.267	0.238	0.333	0.300	0.231	0.210
Pol013	2 (0.00001)	0.484	0.446	0.625	0.469	0.250	0.219	0.250	0.492	0.813	0.482
Pol014	2 (0.32145)	0.000*	0.464	0.000*	0.492	0.000*	0.219	0.000*	0.391	0.000*	0.469
Pol018	1 (0,00100)	0.000*	0.000	0.000*	0.000	0.000*	0.000	0.000*	0.000	0.000*	0.000
Pol019	3 (0.28272)	0.000*	0.348	0.000*	0.430	0.000*	0.227	0.000*	0.219	0.000*	0.430
Pol025	3 (0.00000)	0.403	0.342	0.250	0.219	0.500	0.398	0.500	0.406	0.375	0.320
Pol043	2 (0.30484)	0.016	0.446	0.000	0.000	0.000*	0.375	0.063	0.482	0.000	0.000
Pol044	5 (0.00000)	1.000	0.739	1.000	0.746	1.000	0.576	1.000	0.629	1.000	0.557
Pol114	10 (0.00000)	0.969	0.824	1.000	0.652	0.875	0.760	1.000	0.729	1.000	0.699
Pol115	2 (0.00006)	0.031	0.031	0.063	0.061	0.000	0.000	0.000	0.000	0.063	0.061
Pol118	4 (0.23945)	0.066	0.350	0.063	0.174	0.188	0.451	0.000*	0.556	0.000	0.000

* significant frequencies after Bonferroni correction.

Table 6. Microsatellite markers cross-specific amplification.

Locus	*P. elegans*	*P. polymerus*	*P. caboverdensis*
Pol001	-	-	-
Pol003	-	-	-
Pol004	+	+	-
Pol005	+	+	+
Pol008	+	+	-
Pol011	-	-	-
Pol013	-	+	+
Pol014	+	+	+
Pol018	+	+	+
Pol019	+	-	-
Pol025	+	+	+
Pol043	-	+	+
Pol044	+	+	+
Pol114	-	+	+
Pol115	-	+	+
Pol118	-	+	+

-: no amplification, +: positive amplification.

Once the microsatellite markers were characterised and optimised, the number of individuals per population and populations were increased to 222 individuals distributed in nine populations along *P. pollicipes* distribution range in order to approach population studies in this species. The next chapter expose the results obtained concerning this population-based study.

5.4. Population genetic structure of barnacle *P. pollicipes* (Gmelin 1789) along its distribution range based on microsatellite markers.

5.4.1. Determination of genetic variability in populations of P. pollicipes.

Analysis of microsatellite markers in nine different populations of *P. pollicipes* was useful to assess genetic variability of this crustacean across its distribution range. Microsatellite markers Pol013, Pol014, Pol018 and Pol115 were monomorphic, and number of alleles of polymorphic *loci* ranged between 2 (Pol019 and Pol025) and 19 (Pol003) (Table 7). Values of observed heterozygosity were highly variables across *loci* and populations. In fact, comparison between observed and expected heterozygosities revealed H-W deviations in different *loci* and populations of *P. pollicipes*. *Locus* Pol019 presented H-W deviations in all nine populations. These deviations were also detected in different *loci* in populations from Safi (Pol005, Pol011, Pol044), Guincho (Pol003), Cíes Islands (Pol003, Pol008, Pol118), Roncudo (Pol005, Pol043, Pol044), Brest (Pol003, Pol044, Pol114, Pol118) (Table 7).

Table 7. Determination of genetic variables at population level of observed and expected heterozygosities (Ho, He), deviations of H-W equilibrium (HWe), frequency of null alleles (NA) and number of private alleles (PA).

Locus	Cape of Ajo					Andrín				
	Ho	He	HWe	NA	PA	Ho	He	HWe	NA	PA
Pol001	0.000	0.000	mono	N/A	0	0.000	0.000	mono	0.001	0
Pol003	0.000	0.000	mono	N/A	0	1.000	0.806	ns	0	1
Pol004	0.048	0.046	ns	0.00004	0	0.000	0.000	mono	0.001	0
Pol005	0.000	0.000	mono	N/A	0	1.000	0.625	ns	0	0
Pol008	0.000	0.000	mono	N/A	0	0.000	0.000	mono	N/A	0
Pol011	0.000	0.000	mono	N/A	0	0.000	0.000	mono	0.001	0
Pol013	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol014	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol018	0.000	0.000	mono	N/A	0	0.000	0.000	mono	N/A	0
Pol019	0.000	0.408	***	0.30237	0	0.000	0.320	***	0.26895	0
Pol025	0.000	0.000	mono	N/A	0	0.000	0.000	mono	N/A	0
Pol043	0.000	0.000	mono	N/A	0	0.000	0.000	mono	N/A	0
Pol044	0.000	0.000	mono	0.001	0	0.000	0.444	ns	0.315	0
Pol114	0.000	0.000	mono	N/A	0	0.000	0.000	mono	N/A	0
Pol115	0.000	0.000	mono	N/A	0	0.000	0.000	mono	N/A	0
Pol118	0.000	0.000	mono	N/A	0	0.000	0.000	mono	N/A	0

mono: monomorphic *locus* in certain population; ns: no significant; *: significant at 0.05; **: significant at 0.01; ***: significant at 0.001.

Table 7. Cont.

Locus	Brest					Cies Islands				
	Ho	He	HWe	NA	PA	Ho	He	HWe	NA	PA
Pol001	0.067	0.064	ns	0.00003	0	0.000	0.000	mono	0.001	0
Pol003	0.667	0.853	**	0.11734	1	0.214	0.260	**	0.00001	0
Pol004	0.053	0.051	ns	0.00004	0	0.000	0.000	mono	0.001	0
Pol005	0.313	0.279	ns	0	0	0.750	0.611	ns	0	0
Pol008	0.333	0.403	ns	0.00001	0	0.750	0.656	*	0.0005	0
Pol011	0.000	0.000	mono	0.001	0	0.083	0.080	ns	0.00002	0
Pol013	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol014	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol018	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol019	0.000	0.500	***	0.33333	0	0.000	0.198	**	0.21307	0
Pol025	0.333	0.278	ns	0	0	0.111	0.105	ns	0.00002	0
Pol043	0.533	0.391	ns	0	0	0.500	0.375	ns	0	0
Pol044	1.000	0.611	*	0	0	1.000	0.645	ns	0	0
Pol114	0.333	0.751	*	0.231	0	0.400	0.587	ns	0.10016	1
Pol115	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol118	0.000	0.219	**	0.22384	0	0.000	0.370	***	0.29764	0

Locus	Artabrian Gulf					Safi				
	Ho	He	HWe	NA	PA	Ho	He	HWe	NA	PA
Pol001	0.000	0.000	mono	0.001	0	0.043	0.043	ns	0.00004	0
Pol003	0.682	0.752	ns	0.06875	2	0.500	0.687	ns	0.10806	0
Pol004	0.083	0.081	ns	0.00002	1	0.050	0.049	ns	0.00004	0
Pol005	0.429	0.737	ns	0.18377	2	0.429	0.763	**	0.20474	0
Pol008	0.000	0.000	mono	N/A	0	0.000	0.000	mono	0.001	0
Pol011	0.000	0.000	mono	0.001	0	0.091	0.170	**	0.11734	1
Pol013	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol014	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol018	0.000	0.000	mono	N/A	0	0.000	0.000	mono	0.001	0
Pol019	0.000	0.083	***	0.13997	0	0.000	0.480	***	0.32686	0
Pol025	0.000	0.000	mono	N/A	0	0.250	0.219	ns	0.00001	0
Pol043	0.000	0.000	mono	N/A	0	0.545	0.397	ns	0	0
Pol044	0.000	0.000	mono	0.001	0	1.000	0.594	*	0	0
Pol114	0.000	0.000	mono	N/A	0	0.455	0.678	ns	0.16545	0
Pol115	0.000	0.000	mono	N/A	0	0.000	0.000	mono	0.001	0
Pol118	0.000	0.000	mono	N/A	0	0.143	0.500	ns	0.2381	0

Locus	Ortigueira					Guincho				
	Ho	He	HWe	NA	PA	Ho	He	HWe	NA	PA
Pol001	0.048	0.046	ns	0.00004	0	0.083	0.080	ns	0.00002	1
Pol003	0.850	0.676	ns	0	1	0.333	0.556	*	0.09858	0
Pol004	0.043	0.043	ns	0.00004	0	0.000	0.000	mono	0.001	0
Pol005	0.647	0.682	ns	0.03425	0	0.545	0.562	ns	0	2
Pol008	0.000	0.000	mono	N/A	0	0.800	0.580	ns	0	0
Pol011	0.048	0.046	ns	0.00004	1	0.250	0.226	ns	0.00001	0
Pol013	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol014	0.000	0.000	mono	0.001	0	0.000	0.000	mono	0.001	0
Pol018	0.000	0.000	mono	N/A	0	0.000	0.000	mono	0.001	0
Pol019	0.000	0.495	***	0.33173	0	0.000	0.463	***	0.32119	0
Pol025	0.000	0.000	mono	N/A	0	0.182	0.165	ns	0.00001	0
Pol043	0.000	0.000	mono	N/A	0	0.625	0.430	ns	0	0
Pol044	0.000	0.000	mono	0.001	0	0.909	0.562	ns	0	0
Pol114	0.000	0.000	mono	N/A	0	0.563	0.689	ns	0.06344	0
Pol115	0.000	0.000	mono	N/A	0	0.000	0.000	mono	0.001	0
Pol118	0.000	0.000	mono	N/A	0	0.000	0.000	mono	0.001	0

Table 7. Cont.

Locus	Roncudo				
	Ho	He	HWe	NA	PA
Pol001	0.000	0.000	mono	0.001	0
Pol003	0.704	0.588	ns	0	1
Pol004	0.000	0.000	mono	0.001	0
Pol005	0.786	0.601	**	0.01186	1
Pol008	0.882	0.548	ns	0	2
Pol011	0.000	0.000	mono	0.001	0
Pol013	0.000	0.000	mono	0.001	0
Pol014	0.000	0.000	mono	N/A	0
Pol018	0.000	0.000	mono	0.001	0
Pol019	0.000	0.452	***	0.31751	0
Pol025	0.185	0.168	ns	0.00001	0
Pol043	0.724	0.504	***	0.01554	1
Pol044	0.963	0.630	***	0	0
Pol114	0.069	0.067	ns	0.00003	0
Pol115	0.000	0.000	mono	0.001	0
Pol118	0.560	0.480	ns	0	0

Loci Pol003, Pol011, Pol114 and Pol118 showed deficit of heterozygotes, and Pol043 and Pol044 presented excess of heterozygotes in this populations which showed H-W deviations. *Locus* Pol005 showed deficit of heterozygotes in Safi and excess of heterozygotes in Roncudo. *Locus* Pol019 showed deficit of heterozygotes in all populations, introducing its two alleles in homozygosis condition. Frequencies of null alleles agreed with H-W deviations detected in different populations. Different populations showed rates of null alleles higher than 10% like Safi (Pol003, Pol005, Pol011, Pol114, Pol118), Cíes Islands (Pol114, Pol118), Artabrian Gulf (Pol005), Andrín (Pol044), and Brest (Pol003, Pol114, Pol118). *Locus* Pol019 presented frequencies of null alleles higher than 10% in all populations analysed (Table 7).

Pairwise population differentiation was assessed using Fst indices which ranged from 0 (between Brest and Guincho populations) to 0.707 (between Cape of Ajo and Roncudo) (Table 8). As a consequence, Brest and Guincho were the populations which share a higher Nm (12.461) and Cape of Ajo and Roncudo were the populations with fewer Nm 0.104.

Table 8. Pairwise values of Fst indices between populations.

Population	Cape of Ajo	Andrín	Brest	Cies Islands	Artabrian Gulf	Safi	Ortigueira	Guincho	Roncudo
Cape of Ajo	0.000	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Andrín	0.070	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Brest	0.327	0.213	0.000	0.001	0.001	0.050	0.001	0.378	0.001
Cies Islands	0.598	0.472	0.114	0.000	0.001	0.001	0.001	0.001	0.001
Artabrian Gulf	0.413	0.193	0.195	0.439	0.000	0.001	0.001	0.001	0.001
Safi	0.402	0.254	0.020	0.132	0.133	0.000	0.001	0.010	0.001
Ortigueira	0.549	0.324	0.216	0.459	0.067	0.149	0.000	0.001	0.001
Guincho	0.313	0.226	0.000	0.142	0.237	0.049	0.270	0.000	0.001
Roncudo	0.707	0.625	0.314	0.208	0.583	0.333	0.590	0.328	0.000

Fst values below diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

5.4.2. Determination of genetic structure in populations of P. pollicipes.

Genetic structure of barnacle populations was analysed using microsatellite markers. Possible bottleneck events occurred in studied populations were analysed under three mutational models, IAM, SMM and TPM. Only several microsatellite markers showed statistical significance in different populations, as it appears indicated in table 9.

Table 9. Microsatellite which have showed statistical significance in putative bottleneck events in different analysed populations under the three mutational models.

Population	IAM	TPM	SMM
Artabrian Gulf		Pol003, Pol004	Pol003, Pol005, Pol114
Brest	Pol003, Pol019	Pol005, Pol019	Pol005, Pol019
Cíes Islands	Pol003, Pol044	Pol003	Pol003
Guincho		Pol005	Pol003, Pol005, Pol114
Ortigueira		Pol003	Pol003
Roncudo		Pol114	Pol003, Pol004
Safi	Pol005, Pol011, Pol118	Pol011, Pol118	Pol003, Pol011

IAM=Infinite Allele Model, TPM= Two Phase Model, SMM= Stepwise Mutation Model.

PCA was carried out in order to distinguish populations along its range of distribution (Fig. 20). The four principal components explained 87.37% of observed variability in *P. pollicipes* populations. PCA detected two clearly isolated groups of populations. Individuals from Roncudo clustered in one separated group, and another four populations from Spain grouped into another well defined cluster. Individuals from populations of Safi, Guincho, Cíes Islands and Brest showed a dispersed distribution respect from the other populations.

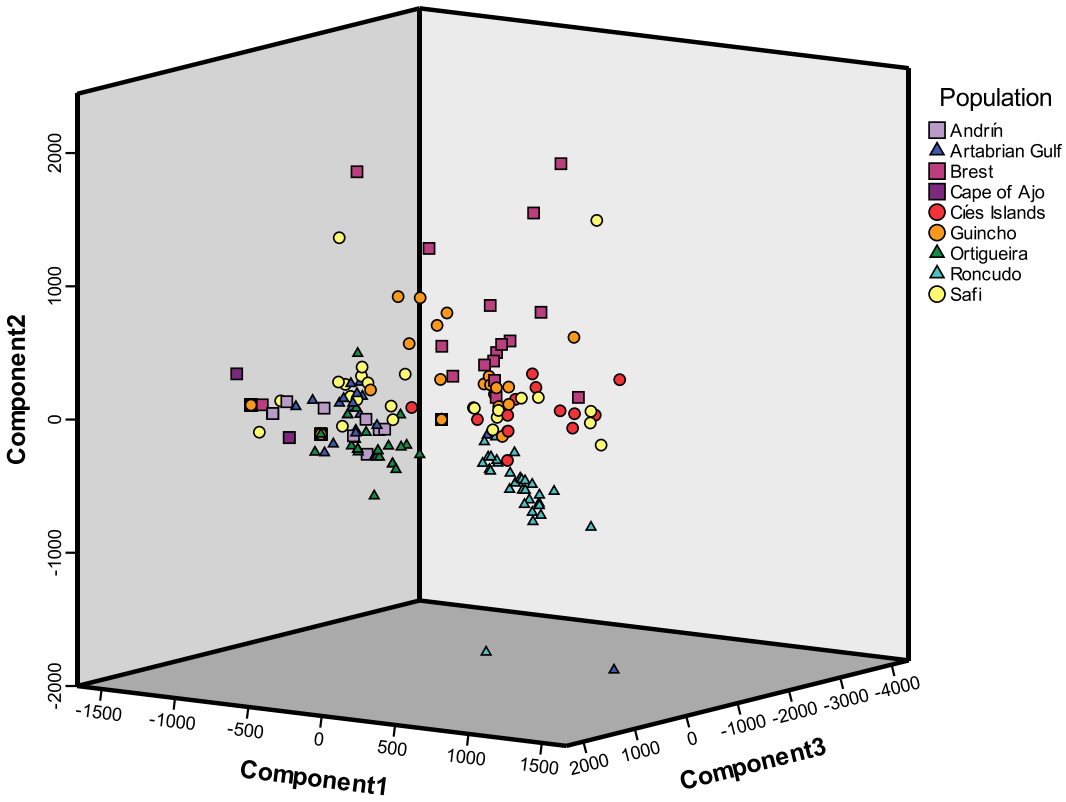


Figure 20. Representation of different *P. pollicipes* populations in the dimension reduced space carried out under PCA algorithm.

A non-lineal method was also used (ncMCE) to reduce dimensions of obtained dataset and results showed seven well delimited groups. However, a mixture of individuals from different populations constituted the detected groups (Fig. 21).

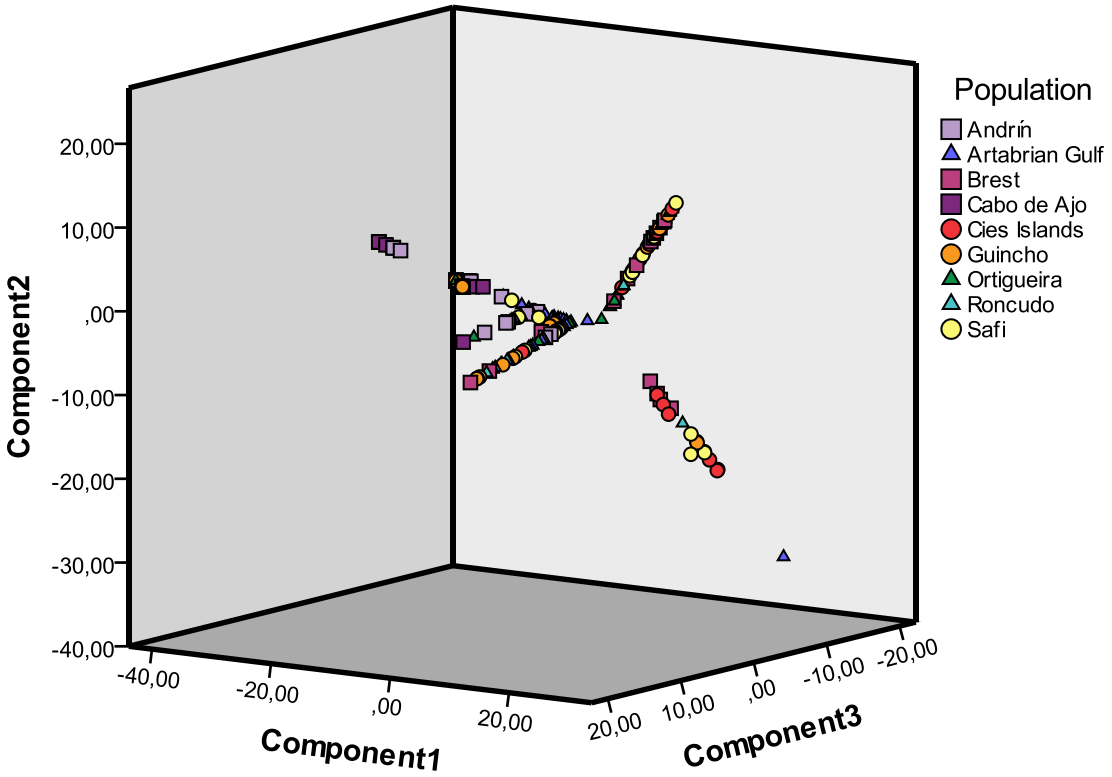


Figure 21. Representation of different *P. pollicipes* populations in the dimension reduced space carried out under ncMCE algorithm.

Bayesian inference of genetic structure across all populations was performed using Structure. Bayesian analysis of populations showed the high probability with K=7, which indicate that more probable number of real populations is seven. However, individuals from the same populations appeared grouped into different clusters. Phylogenetic reconstructions based on genetic distances between populations support this dispersal pattern. Tree topology showed two clades, one constituted by five populations from Spain (Cape of Ajo, Ortigueira, Roncudo, Andrín, Artabrian Gulf), and another clade including populations from Safi, Guincho, Spain (Cíes Islands) and Brest.

5.4.3. Logistic model of assignation of P. pollicipes individuals to populations.

Microsatellite markers were also used to perform traceability approaches and a logistic model was developed and validated in order to assign *P. pollicipes* individuals to Spanish populations. The logistic regression is used due to the discrete nature of the outcome variable (origin Spanish or non-Spanish of the barnacle populations) and predictor variables are categorical (genotype of

each microsatellite *loci*), reason why the assumption of linearity in normal regression is violated. Different component included in the resultant model appear defined in table 10.

Table 10. Component included in the logistic regression model where B is the coefficient for the constant in the null model; S.E. is the standard error associated to coefficient for the constant; Wald is the value of the Wald chi-square test; df is the degrees of freedom for the Wald chi-square test; Sig. is the p-value associated to Wald chi-square test; Exp(B) is the exponentiation of the B coefficient.

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Pol001	0.00	0.00	0.06	1.00	0.81	1.00
	Pol003	0.00	0.00	0.80	1.00	0.37	1.00
	Pol004	0.06	0.03	2.83	1.00	0.09	1.06
	Pol005	0.00	0.00	1.00	1.00	0.32	1.00
	Pol008	0.00	0.00	0.12	1.00	0.73	1.00
	Pol011	0.00	0.00	0.10	1.00	0.76	1.00
	Pol013	0.40	0.32	1.56	1.00	0.21	1.50
	Pol014	-0.04	0.02	3.48	1.00	0.06	0.96
	Pol018	0.01	0.02	0.45	1.00	0.50	1.01
	Pol019	-0.32	0.26	1.61	1.00	0.21	0.72
	Pol025	-0.02	0.02	2.02	1.00	0.15	0.98
	Pol043	0.00	0.01	0.02	1.00	0.90	1.00
	Pol044	0.04	0.02	5.43	1.00	0.02	1.04
	Pol114	-0.02	0.02	1.61	1.00	0.20	0.98
	Pol115	-0.01	0.01	0.55	1.00	0.46	0.99
	Pol118	0.00	0.01	0.01	1.00	0.90	1.00
	Constant	1.97	0.68	8.29	1.00	0.00	7.17

a: Variables entered on step 1: Pol001, Pol003, Pol004, Pol005, Pol008, Pol011, Pol013, Pol014, Pol018, Pol019, Pol025, Pol043, Pol044, Pol114, Pol115, Pol118.

This model was able to distinguish individuals from different analysed populations based on microsatellite genotypes and assign these individuals to Spanish or non-Spanish populations with 83.7% of average accuracy. In the case of Spanish populations, 139 of a total of 178 were correctly predicted, which suppose an 89.6% of accuracy. On the other hand, 22 of a total of 38 individuals from non-Spanish populations were correctly predicted (72.6%) (Fig. 22).

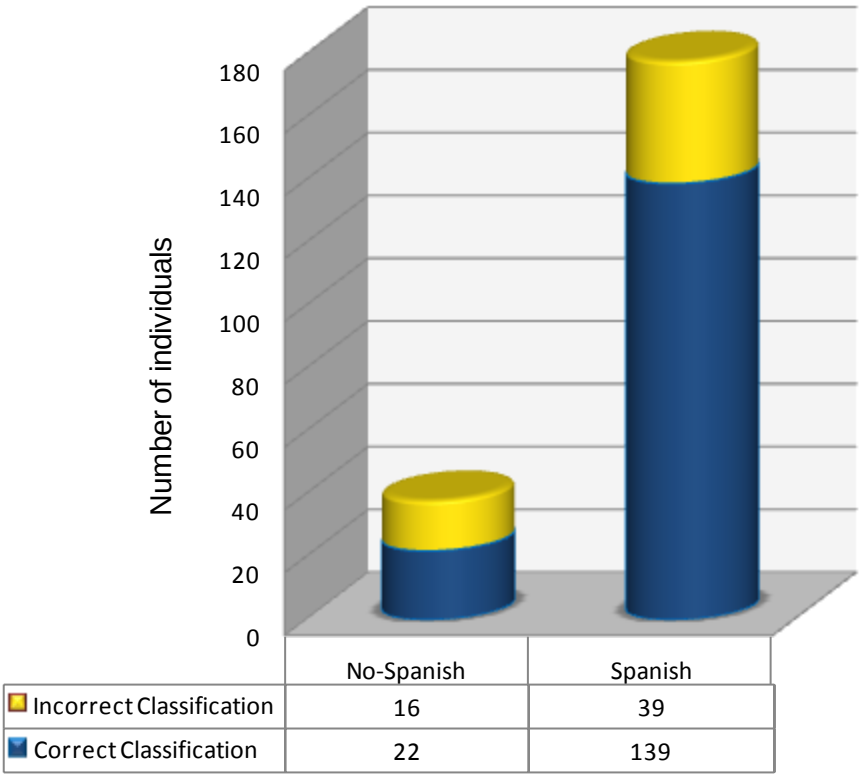


Figure 22. Representation of assignation accuracy of the logistic model developed based on microsatellite markers in *P. pollicipes* populations.

5.5. Differential gene expression patterns between two phenotypes of gooseneck barnacle *P. pollicipes*.

5.5.1. Morphological differences between phenotypes.

Morphological comparisons performed between barnacles of sun and shadow phenotypes, carried out in collaboration with Eugenia and Ricardo (Xunta de Galicia), contribute to differentiation of both phenotypes based on variables describe above (Figure 23).

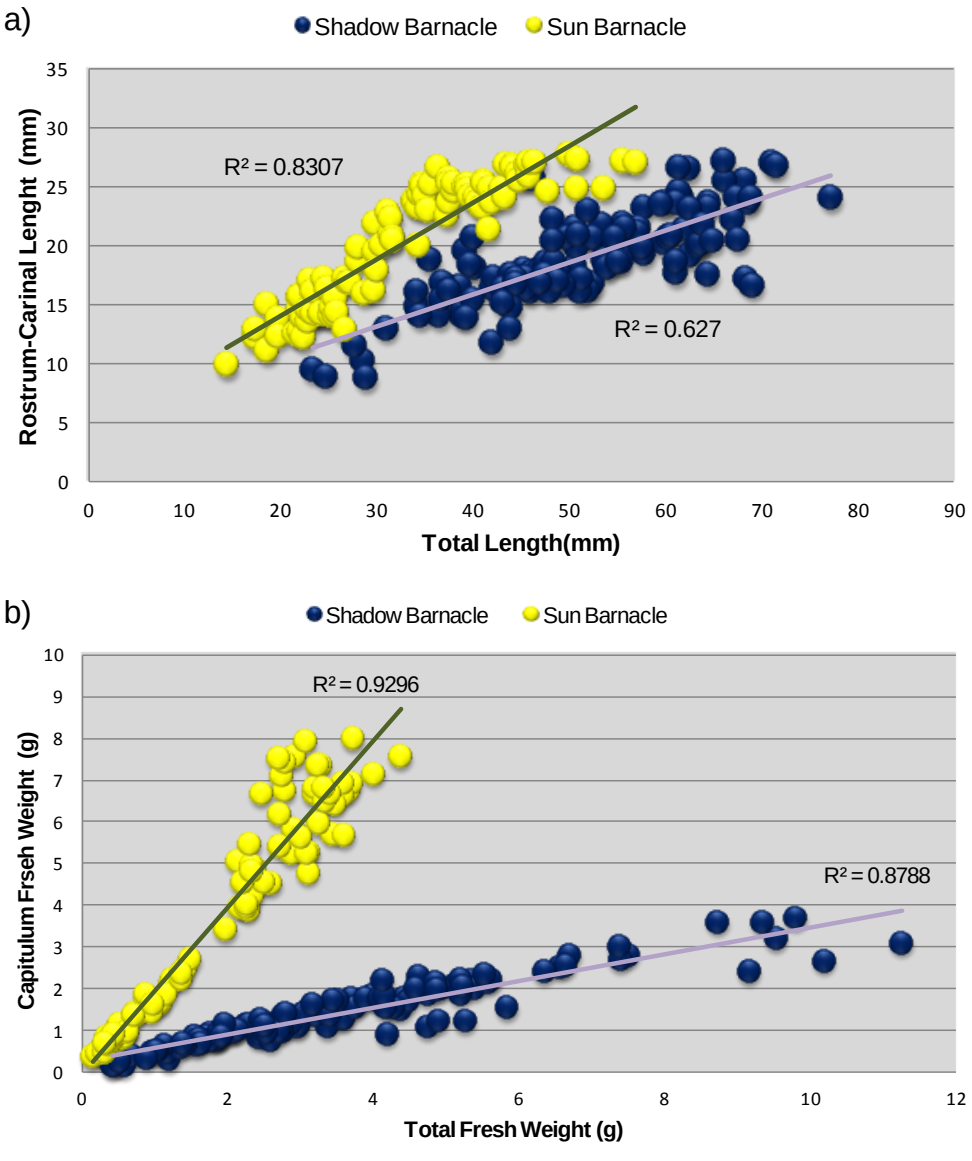


Figure 23. Morphological differences between sun and shadow barnacles. a) Scatter plot shows relation between Rostrum-Carinal Length and Total Length, and b) Scatter plot shows relation between Capitulum Fresh Weight and Total Fresh Weight. R^2 coefficient is given for each calibration curve.

Statistical support was presented by all analysed variables between phenotypes, except for PFW, which not showed significant differences between them (Table 11).

Table 11. T-Test for equality of means of different morphological variables from both barnacles' phenotypes. Different parameters are showed: t is the value of T-Student estimator; gl are the degrees of freedom; Sig. (bilateral) is the p-value; Average difference is the difference of the averages between both phenotypes for each variable; Standard Error is the standard error associated to average difference; and 95% Confidence shows the inferior and superior values of the confidence limit for average difference.

Morphological variables	t	gl	Sig. (bilateral)	Means difference	Standard error	95% confidence	
						Inferior	Superior
Rostrum-Carinal Length	12.480	2280.000	0.000	3.413	0.273	2.877	3.949
Capitulum Length	11.813	662.186	0.000	4.809	0.407	4.010	5.608
Total Length	33.580	1202.932	0.000	23.292	0.694	21.931	24.653
Capitulum Width	2.308	1837.356	0.021	0.475	0.206	0.071	0.878
Total Fresh Weight	17.219	1533.892	0.000	1.723	0.100	1.526	1.919
Capitulum Fresh Weight	-3.305	143.729	0.001	-0.486	0.147	-0.777	-0.195
Peduncle Fresh Weight	1.239	210.000	0.217	0.237	0.191	-0.140	0.614

5.5.2. RNA quality and selection of housekeeping genes.

Barnacle *P. pollicipes* is a protostome organism which showed a hidden break in 28S rRNA (Ishikawa, 1977) what impede the correct determination of RNA integrity in these organisms. However, our results displayed enough RNA concentration to perform the cDNA synthesis nevertheless RNA Integrity Number (RIN) (Schroeder *et al*., 2006) ranged between 3 and 7.

Reference genes selected in order to normalise expression levels of genes of interest in different tissues were albumin, actin, β-actin, histone H3, HSP70, and HSP90. Primer efficiency for these reference genes was determined by a 3 point calibration curve which showed qPCR efficiencies values of 0.705, 0.814, 0.869, 0.863, 0.822, and 0.780 for albumin, actin, β-actin, histone H3, HSP70, and HSP90, respectively. Selection of the best housekeeping gene was carried out by algorithms implemented in geNorm (Vandesompele *et al.*, 2002), Normfinder (Andersen *et al.*, 2004), BestKeeper (Pfaffl *et al.*, 2004) and the comparative ΔCt method (Silver *et al.*, 2006) using the Ct values showed by each reference gene (Fig. 24).

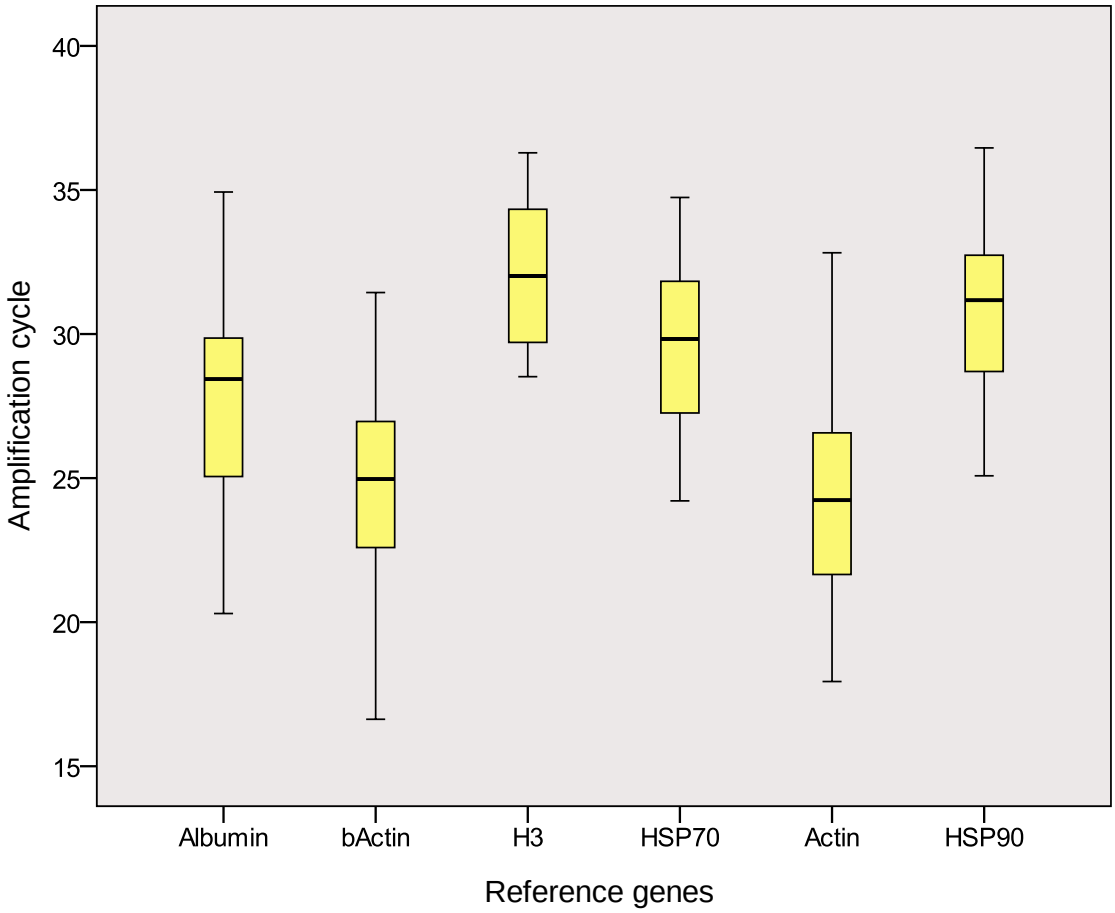


Figure 24. Expression levels of candidate reference genes across all datasets. Lines across the boxes depict the medians, boxes indicate the interquartile range, and whiskers represent 95% confidence intervals.

5.5.3. Expression profiling and stability of the reference genes.

Analysis performed by geNorm determines the most stable expressed genes bases on the parameter M, which is determined for each reference gene as the average pairwise variation between this reference gene respect from all the other reference genes included in the study (Vandesompele *et al.*, 2002). The most stable genes revealed by geNorm algorithm in our experiments are showed in table 12 and figure 25. Albumin was the most stable gene (M=1.394) follow by HSP70 (M=1.483) and least stable gene was histone H3 (M=2.013) with M parameter higher than 1.5, value consider the default limit of stability. Analysis including data belonged to only one of the two phenotypes presented the similar results. Dataset of sun phenotype revealed albumin as the most stable gene, follow by HSP90. In the case of shadow phenotype, albumin and HSP70 were the most stables genes, and β-actin and histone H3 showed M

values of 1.737 and 2.007 in the case of the analysis of this shadow phenotype dataset. All reference genes showed M values less than 1.5 in both conditions except for histone H3 whose M values exceeded 1.5. Analysis of the whole set of data revealed that three reference genes are necessary to normalise genic expression. In the dataset from sun phenotype three genes are required to normalise genic expression (albumin, HSP90 and HSP70), and other three genes are necessary also in the case of the shadow phenotype data set (albumin, HSP70 and HSP90).

Table 12. Ranking of all reference genes expressed in three tissue datasets (both phenotypes, only sun phenotype and only shadow phenotype) under all four evaluation algorithms.

Rank	geNorm[a]			NormFinder[b]		
	Both	Sun	Shadow	Both	Sun	Shadow
1	ALB (1.394)	ALB (1.208)	ALB (1.037)	HSP70 (0.044)	HSP90 (0.881)	HSP70 (0.581)
2	HSP70 (1.483)	HSP90 (1.216)	HSP70 (1.073)	ALB (0.050)	HSP70 (0.886)	HSP90 (0.618)
3	HSP90 (1.583)	HSP70 (1.221)	HSP90 (1.080)	HSP90 (0.055)	β-ACT (1.017)	ALB (0.981)
4	ACT (1.588)	β-ACT (1.285)	ACT (1.271)	β-ACT (0.056)	ALB (1.242)	ACT (1.311)
5	β-ACT (1.706)	ACT (1.466)	β-ACT (1.737)	ACT (0.065)	ACT (1.452)	β-ACT (1.395)
6	H3 (2.013)	H3 (1.910)	H3 (2.007)	H3 (0.080)	H3 (1.907)	H3 (2.505)

Rank	BestKeeper[c]			ΔCt[d]		
	Both	Sun	Shadow	Both	Sun	Shadow
1	ACT (0.930)	ALB (0.945)	ACT (0.934)	ALB (1.810)	HSP70 (1.813)	ALB (2.167)
2	ALB (0.924)	ACT (0.935)	ALB (0.926)	HSP70 (1.848)	ALB (1.847)	HSP70 (2.222)
3	HSP70 (0.773)	β-ACT (0.907)	β-ACT (0.852)	HSP90 (1.990)	HSP90 (1.917)	H3 (2.595)
4	HSP90 (0.712)	HSP90 (0.874)	HSP70 (0.619)	β-ACT (2.022)	β-ACT(2.189)	ACT (2.641)
5	β-ACT (0.666)	HSP70 (0.833)	HSP90 (0.467)	ACT (2.030)	ACT (2.513)	HSP90 (2.749)
6	H3 (0.619)	H3 (0.685)	H3 (0.459)	H3 (2.485)	H3 (2.945)	β-ACT(2.854)

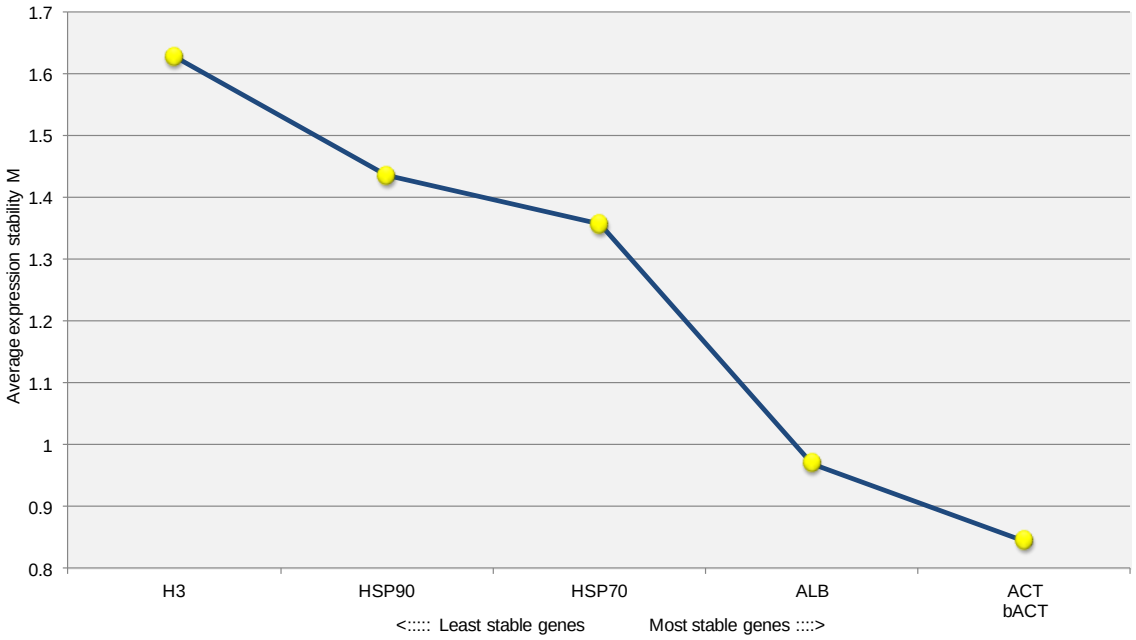


Figure 25. Average expression stability values of control genes revealed by geNorm algorithm.

Analysis performed by NormFinder is rooted in a mathematical model that assess intragroup and intergroup variation of the sample set in order to measure the systematic error introduced when using each gene (Andersen *et al.*, 2004). Results of gene stability under NormFinder model revealed HSP70 as the most stable gene (0.044), followed by albumin (0.050). Stability values and gene ranking are showed in table 12. Slight differences of NormFinder respect from geNorm were detected. Ranks of the two most stable references genes, albumin and HSP70, are exchange, and the ranks of genes actin and β-actin are also exchanged. Using NormFinder to detect the most stable genes in each phenotype showed HSP90 (first rank in sun phenotype and second rank in shadow phenotype) and HSP70 (second rank in sun phenotype and first rank in shadow phenotype) as the most stable genes in both cases.

Analysis performed by BestKeeper bases on repeated pair-wise correlations between analysed genes (Pfaffl *et al*., 2004). Stability showed by reference genes and assessed by BestKeeper introduced β-actin and albumin as the two reference genes that showed less standard deviation. Taking in account Pearson coefficients of correlation, the best genes assessed by BestKeeper were actin and albumin (Table 12). Classification obtained using BestKeeper showed differences respect from the classifications obtained using

geNorm and NormFinder. Genes actin and β-actin occupied higher ranks in BestKeeper classification although genes HSP70 and HSP90 occupied higher ranks in geNorm and NormFinder classification. The three classifications showed H3 as the worst reference gene. Focus on each phenotype and based on standard deviations, classifications obtained using BestKeeper were different in each phenotype dataset. In the case of sun phenotype, reference genes ranking was exactly equal than the ranking presented above, with β-actin as the best reference gene (Table 12). However, for shadow phenotype, the best reference genes were HSP90 and HSP70 (Table 12).

Analysis performed by ΔCt method bases on measures of ΔCt value fluctuations between pairs of genes (Silver *et al.*, 2006). Ranking of reference genes based on mean of standard deviation presented albumin and HSP70 as the genes with most stable expression. Although general ranking showed similar results to geNorm and NormFinder, different genes showed more stable expression levels in the case of each phenotype. Sun phenotype dataset presented HSP90 as the most stable gene but shadow phenotype dataset showed HSP70 as the most stable one.

5.5.4. Reference genes validation.

Once assessed the candidate reference genes in all datasets, albumin and HSP70 were the two more stable genes selected by different algorithms used in this study. NormFinder algorithm provided a ranking of gene stability which includes the systematic error introduced in the results of the expression study when each gene is used (Table 12). Thus, the subsequent expression studies in *P. pollicipes* were carried out using HSP70 as reference gene in basis on NormFinder results for this gene.

In order to perform an appropriate interpretation of expression assays results carried out in different tissues of *P. pollicipes*, two facts must be taken into account. On the one hand, main differences detected between both phenotypes are related to the proportion of muscular tissue and water included into the peduncle, being both capitula similar in composition. On the other hand, the integrity of the peduncle from sun barnacles lie in an interconnection between cells from all the tissues of the peduncle what prevents obtain samples

of tissue which separates the muscle and the cuticle. For this reason, main comparisons between phenotypes will focus on cuticle tissue and peduncular muscle.

After standardisation, genes of interest showed variation in expression level in the analysed tissues. Minimum expression levels were presented by cuticular protein RR-1 gene (0.482) in cuticular tissue of sun barnacle phenotype and maximum expression levels were determined for cuticular protein 47Ee gene (3.125) in peduncular muscle of sun phenotype barnacles. In contrast, cuticular protein 11B gene and cuticular protein RR-1 gene showed expression similar than expression levels of control gene in all tissues. It is remarkable that cuticle tissue and peduncular muscle from sun phenotype showed higher levels of all genes than in shadow phenotype, except for cuticular protein RR-1 which showed the inverse profile (Fig. 26).

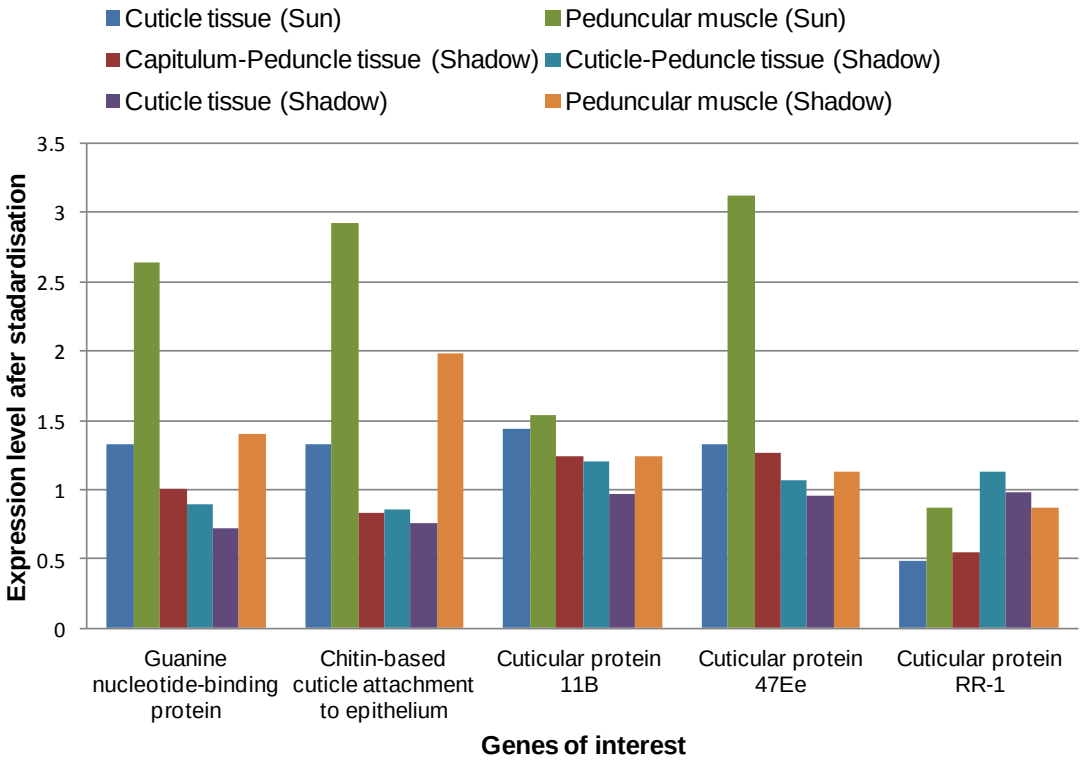


Figure 26. Expression level showed by all candidate to reference genes in all analysed tissues from both phenotypes after normalisation and respect for the control expression level of each gene in capitulum tissue (control).

DISCUSSION

6. Discussion.

6.1. Phylogenetic relationships in family Pollicipedidae.

Phylogenetic reconstructions carried out using different mitochondrial and nuclear markers have showed tree topologies with certain differences. Evolutionary pressures drive the molecular evolution of each genetic region and affect the evolutionary model selected to reconstruct each phylogeny. In fact, two different models were used in phylogenetic inferences: general reversible time model was used in four datasets (16S rDNA, 18S-28S rDNA, EF1α and concatenated tree) while Tamura was used with COI dataset.

Phylogenies inferred with these molecular markers have let distinguish the four species belonged to family Pollicipedidae by grouping of all sequences of each species in the same tree branch and these branches showed bootstrap support higher than 89% in all phylogenetic trees. However, statistical support showed by branches which include different species have been insufficient, ranging from 52 to 73, and it has impeded establish adequate phylogenetic relationships between species of this family. These values of bootstrap less than 80% have been obtained also by Van Syoc *et al*. (2010) and Quinteiro *et al*. (2011) in their phylogenetic reconstructions of family Pollicipedidae based.

Evolutionary inferences based on mitochondrial and nuclear markers carried out in the current study have reported tree topologies different than topologies obtained by Van Syoc *et al*. (2010), Perina *et al.* (2011), and Quinteiro *et al*. (2011). So, phylogenetic reconstruction based on COI region, which includes all sequences used by Van Syoc *et al*. (2010) and Quinteiro *et al*. (2011) and other sequences from different authors obtained for this gene from GenBank, showed the same tree topology than they were inferred them based on this marker, and which was inferred by Quinteiro *et al*. (2011) based on 18S-28S rDNA region. These similar topologies showed *P. polymerus* lineage diverged earlier during the evolutionary history of family Pollicipedidae, follow by *P. elegans*, and being *P. pollicipes* and *P. caboverdensis* the two most related species.

A priori, concatenated tree inference must show the more accurate phylogeny reconstruction due to include nucleotide sequence from different genomic regions under different evolutionary pressures. However, results obtained from this inference must be treated carefully in order to avoid misinterpretations. On the one hand, certain genetic regions could be overrepresented in terms of nucleotide length.

In the concatenated reconstruction, genetic regions COI (1524 bp) and 18S-28S rDNA (1276 bp) genes have lengths higher than 16S rDNA (881 bp) and EF1α (805 bp), being the two first genetic regions overrepresented in terms of sequence length respect from 16S rDNA and EF1α. On the other hand, results could be affected by variable sites contributed by each region to the concatenated sequence. The inference showed that, a 32% of total sites for 18S-28S rDNA were variable sites, 23% in the case of 16S rDNA, 17% for COI gene, and 12% for EF1α, presenting a higher contribution by 18S-28S rDNA region in the complete concatenated sequence.

Topology obtained in this concatenated tree disagrees with morphological similarities observed between *Pollicipes* barnacles. Based on morphological characters such as shape, size, number and colour of capitulum plates, *P. pollicipes* and *P. caboverdensis* are the two more similar species. This clade would be close to *P. elegans* which shows a little less morphological similarity based on these characters, and being *P. polymerus* the more discrepant species in terms of morphology. Concatenated tree topology disagrees with these interspecific relationships, showing two lineages, one constituted by *P. pollicipes* and *P. elegans* and other containing *P. polymerus* and *P. caboverdensis*.

6.2. Predictions of secondary structures of ribosomal regions ITS1, 5.8S and ITS2 in crustacean organisms.

6.2.1 Secondary structure conservation and evolutionary mechanism.

Secondary structures for regions ITS1, 5.8S rDNA, and ITS2 were described in different crustacean organisms. Folding pattern showed by 5.8S rDNA consisted in three helices (helix I branched) and two 28S pairing regions although deviations of this pattern were identified, which consist in structural modifications related to the number of subhelices showed by each helix and the absence of one of the helices. In eukaryote organisms, Ursi *et al*. (1982) reported the same conserved folding pattern as the one presented by crustaceans for 5.8S rDNA. Taking into account structural folding patterns predicted for 5.8S rDNA in Crustacea, the general folding pattern detected in eukaryotic organisms was confirmed in this subphylum.

Secondary structures described above in crustaceans for both ITS regions displayed a single-stranded ring structure and two to 12 double-stranded helices agreeing with secondary structures described in different eukaryote organisms (Coleman 2007, Koetschan *et al*. 2014). Notwithstanding folding patterns of the ITS regions in the subphylum Crustacea are highly variable in terms of structural morphology. However, at family level secondary structure are highly conserved in these regions, e.g., the secondary structure common for all species belonged to family Pollicipedidae presented in figure 18 for ITS1 and figure 19 for ITS2. Temperature affected secondary structures of these regions altering the secondary structure of certain 5.8S rDNA variants, and increasing the number of helices protruding the open ring in the case of both ITS regions, being these helices shorter and presented a reduced number of sub-helices.

CBCs were also detected in 27 species indicating that secondary structure is necessary for the fulfilment of the biological function of ITS regions. Otherwise, the existence of CBCs between nucleotide sequences is correlated to taxonomical differentiation (Müller *et al*. 2007). Obtained results in crustaceans for the probability that the detected CBCs in crustaceans belonging

to different species is 0.9359, 0.9545 and 0.9165 for 5.8S rDNA, ITS1 and ITS2, respectively, in accordance with results of Müller *et al*. (2007).

6.2.2. Genetic and structural diversity. Pseudogenes.

The ITS1-5.8S-ITS2 region is commonly used in phylogenetic reconstructions (Álvarez and Wendel 2003) due to it combines regions with fast evolving rates (both internal transcribed spacers) and others with slow evolution rates (5.8S rDNA). The genetic variation, measured such as the number of different haplotypes, detected in both ITS regions (176 and 238 haplotypes, respectively) was higher than genetic variation found in 5.8S rDNA (54 haplotypes) and agreed with rapid evolutionary rates in ITS regions and slow evolutionary rates in 5.8S rDNA (Suh *et al*. 1993).

Different evolutionary rates are explained due to the 5.8S rDNA is subject to a stronger purifying selection than ITS regions (Ironside 2013). This same case of purifying selection was reported by Perina *et al*. (2011) in 5S rDNA of *P. pollicipes*. According to Bailey *et al*. (2003), presence of insertions or deletions in regions ITS1, 5.8S rDNA or ITS2 which produce mistaken folding patterns were described as a cause of pseudogenic sequences. These altered folding patterns were detected in 5.8S rDNA sequences of Crustacea which showed six alternative folding patterns incongruent with general structure found for this region transforming these patterns into putative pseudogenes (Fig. 13). Alternative folding patterns showed a lack of conserved domains characteristics of 5.8S rDNA secondary structure, such as helix II, helix III, or branches of helix I, constituting putative pseudogenes. On the one hand, detection of pseudogenes in 5.8S rDNA regions is explained due to its putative recent origin and purifying selection had no time to remove the pseudogenes. On the other hand, the different genetic variants present by 5.8S rDNA region could be explained by means of differential expression of these genetic variants in specific tissues or along different development stages.

Phylogeny reconstructed based on 5.8S rDNA secondary structure showed four well defined clusters corresponding to classes Ostracoda, Branchiopoda, Maxillopoda and Malacostraca and certain inferior taxa such as orders Anostraca, Diplostraca, Mysida, Sessilia and Pedunculata, and three

infraorders belonged to order Decapoda. These groups corresponding to four classes of crustaceans described in Pancrustacea phylogenetic reconstructions carried out by different authors (Zrzavy *et al*., 1998; Garcia-Machado *et al*., 1999; Reiger *et al*., 2001, 2005, 2008; Harzsch 2004, 2006; Lavrov *et al*., 2004; Mallat *et al*., 2004; Cook *et al*., 2005; Giribet *et al*., 2005; Mallat and Giribet, 2006; Meusemann, 2010). However, inferior taxa of the taxonomic scale, such as species or families, presented sequences in different clusters. The presence of sequences belonged to different families in the same cluster is a proof of the high conservation degree of 5.8S rDNA secondary structure respect for primary sequence. This conservation degree is because of the changes in primary sequence do not always alter the folding patterns of secondary structure, making possible that sequences of different species or families grouped together.

6.3. Preliminary evaluation of microsatellite markers developed in *P. pollicipes*.

Certain microsatellite markers developed in *P. pollicipes* showed deviation of H-W equilibrium after Bonferroni's correction due to deficit of heterozygotes. These H-W deviations are possibly due to high frequencies of null alleles detected in those *loci* and being *loci* Pol014, Pol019, Pol043 and Pol118 which presented of H-W deviation in different populations. Multiple alleles showed by these four *loci* appeared in homozygosis (Ho=0) despite heterozygosis is significantly different than 0 and their frequencies of null alleles are the highest of all analysed *loci*. Until six microsatellite *loci* developed in *P. pollicipes* can be used in population studies in other species of family Pollicipedidae due to they have showed successful amplification in different species of this family.

6.4. Population genetics in *P. pollicipes* based on microsatellite markers.

Microsatellite markers were a powerful tool to analyse current genetic condition in different populations of barnacle *P. pollicipes* along its distribution range. These microsatellite markers allowed quantify different population genetic variables and study *P. pollicipes* genetic structure. Four microsatellite markers were monomorphic and 12 presented a variable grade of polymorphism showed from two to 19 alleles per *locus* in a dataset of 217 analysed individuals.

This allele number agreed allele diversity detected in other *Pollicipes* species, *P. elegans* showed between two and 22 alleles per *locus* in a dataset of 95 individuals from two populations of America (Plough and Marko, 2014). Microsatellites used to characterise populations of other crustaceans reported similar number of alleles ranging from nine to 25 in *Nematocarcinus lanceopes* (Dambach *et al.*, 2103) and four to 14 in *Austropotamobius torrentium* (Iorgu *et al.*, 2011), despite of the sample size used by this authors was much smaller than ours. Other similar values of number of alleles reported in crustaceans ranged from six to 23 in *Semibalanus balanoides* (Flight *et al.*, 2012), and two to 24 in *Melicertus kerathurus* (Arculeo *et al.*, 2010), authors who used sample sizes bigger than ours. However, microsatellite marker used in *P. pollicipes* showed a less grade of polymorphism than microsatellite markers reported for other crustaceans like *Litopenaeus schmitti* which showed from 13 to 70 alleles per *locus* (Maggioni *et al.*, 2003), and *Callichirus islagrande* from 14 to 51 alleles per *locus* (Bilodeau *et al.*, 2005), results obtained from datasets smaller than ours; or *Penaeus monodon* from 34 to 84 alleles per *locus* reported by Brooker *et al.* (2000), who analysed 312 individuals.

Observed heterozygosity detected in populations of *P. pollicipes* ranged from 0.043 to 1, similar to values reported in *P. elegans* (from 0.17 to 0.83) by Plough and Marko (2014). Other crustacean species presented also high values of expected heterozygosity, from 0.415 to 0.987 in *S. balanoides* (Flight *et al.*, 2012), from 0.432 to 0.976 in *P. monodon* (You *et al.*, 2008), from 0.091 to 0.952 in *M. kerathurus* (Arculeo *et al.*, 2010), from 0.545 to 0.927 in *N. lanceopes* (Dambach *et al.*, 2103), from 0.125 to 0.771 in *A. torrentium* (Iorgu *et*

al., 2011), from 0.000 to 0.429 in *Triops cancriformis* (Cesari *et al.*, 2004), or from 0.662 to 0.944 in *L. schmitti* (Maggioni *et al.*, 2003). Plausible explanations of H-W deviations detected in nine *loci* might be null alleles, inbreeding, selection or Wahlund effect (Wahlund, 1928). Frequencies of null alleles detected in microsatellite *loci* of *P. pollicipes* ranged from 0.00001 to 0.33333. These values are higher than frequencies of null alleles reported in *P. elegans* which showed a frequency of null alleles from 0.04 to 0.19 (Plough and Marko, 2014), or another crustacean species as *N. lanceopes* with a frequency of null alleles from 0 to 0.052 (Dambach *et al.*, 2103). These slightly high frequency of null alleles detected in *P. pollicipes* might affected H-W equilibrium of analysed microsatellite markers.

Gooseneck barnacle *P. pollicipes* is an important fishing resource and several analysed populations could be under fishing pressure which alters H-W equilibrium of these *loci*. This fishing pressure was reported in other congeneric species, *P. elegans*, as a putative responsible factor for fluctuations in population sizes which might cause negative effects on genetic diversity and effective population size (Plough and Marko, 2014). Significant excess of homozygotes was detected in two *loci*, Pol118 in populations from Cíes Islands and Brest, and Pol119 in all nine analysed locations (Wahlund effect), contributing to H-W deviations detected in analysed microsatellites.

Genetic scars related to historical events of bottleneck, which might affect to current allelic frequencies of analysed populations, were not detected with the microsatellite set using in this study. According to Wright (1978) Fst interpretation, certain genetic structure was detected between analysed populations. Roncudo population showed higher values of Fst indices which indicate great genetic differentiation respect for the other populations. On the other hand, Morocco showed fewer Fst values than most of the analysed populations (except for Roncudo, Andrín and Cape of Ajo). Fst values detected in *P. pollicipes* populations were significantly higher than Fst values detected in the congeneric barnacle *P. elegans* (Plough and Marko, 2014). Fst highest value presented by *P. elegans* using microsatellite markers was 0.097 which indicate moderate genetic differentiation. In the case of *P. pollicipes*, Fst average of all microsatellite *loci* in all analysed populations was 0.296, which

indicates a great genetic differentiation. Traditionally, local fishermen leave shadow barnacles as larvae producers in order to maintain and restock wild populations of this barnacle and they recollect sun barnacles for commercial purposes. This reduced fishing pressure over shadow barnacles might change genetic diversity and allelic frequencies in wild populations of *P. pollicipes*.

Once analysed populations of *P. pollicipes* were genetically characterised using microsatellite markers, clustering analysis were carried out with different algorithms in order to differentiate populations. Linear clustering models based on PCA showed dispersed patterns presented in Safi, Guincho, Cíes Islands and Brest populations contrasting with delimited patterns detected in Roncudo population (constituting a single group isolated from the others), and with the rest of the analysed populations, which clustered in another group constituted by four Spanish populations (Artabrian Gulf, Ortigueira, Andrín, and Cape of Ajo). These two patterns showed by PCA might be due to fishing pressures. All Spanish populations analysed except for Cíes Islands population, which is protected in a natural reserve, are shellfish resources recurrently exploited by local fishermen. This fishing pressure might be responsible of positive selection over certain genotypes, causing incipient population differentiation. Other possible explanation for this population clustering pattern is oceanic currents which flow along the west coasts of Africa and Europe connecting the different locations of *P. pollicipes*, as well as the temperature of the water in Cantabrian Sea and Artabrian Gulf which is a few degrees less than the temperature of the rest of Atlantic Ocean and might determine the development and settlement of *P. pollicipes* larvae.

Based on results displayed by ncMCE algorithm, a disagreement with PCA results were detected and seven groups of individuals were defined based on genetic composition. These groups agree with the number of Bayesian clusters obtained by Structure software, which grouped individuals into seven clusters statistically differentiated, although individuals from different populations grouped in the same clusters. This reflects certain admixture structure in *P. pollicipes* populations. Adults of *P. pollicipes* develop sessile habits of life, constituting dense aggregations and presenting internal fecundation. In contrast, larvae of *P. pollicipes* migrate long distances through

oceanic currents (Quinteiro *et al.*, 2007) which might explain population admixture model. The two clusters detected by Neighbor-Joining tree inferred from distance matrix obtained from microsatellite data, isolate all Spanish populations (except for Cíes Islands) and populations from Safi, Guincho and Brest. Fishing pressure might be an explanation for this grouping pattern due to barnacle *P. pollicipes* is exploited as a valuable shellfish resource in all analysed populations except for the individuals from Cíes Islands, which is a natural reserve where *P. pollicipes* fishing is prohibited. In contrast, logistic model identified individual's origin (Spanish - non Spanish) with high probability, although exclusion of the model the Cíes Islands population produced a reduced effect over probability estimations.

6.5. Gene expression levels in *P. pollicipes* barnacles from both phenotypes.

Different hypotheses were postulated to explain these phenotypical variations based on the composition and integrity of musculature of the peduncle. Parada *et al.* (2012) have reported phenotypic variations between barnacles from Galicia (NW Spain) coasts. They had described small and thick barnacles inhabit in exposed rocky coasts (sun phenotype), and other barnacles elongated and thin circumscribed to rock's crevices (shadow phenotype). Morphological and genetic analyses carried out in the current research in barnacles of both phenotypes led determine what differences are due to, which are unknown until now. On the one hand, samples of shadow barnacles used in this study where collected in Roncudo (NW Spain) from the same habitat than samples of sun phenotype, which indicate that shelter degree is not decisive to condition the larval metamorphosis to sun or shadow phenotype. On the other hand, genetic analyses carried out in the current research do not detect differences based on microsatellite and nuclear markers between phenotypes, residing these differences in morphological characters and expression levels of certain genes. At morphological level, differences between phenotypes were detected in different anatomic variables, and, at expression level, these

differences lied in higher levels of genes related to muscularity in sun phenotype respect from shadow phenotype.

Despite the hidden break of protostome organisms distorts values of RIN, results obtained with our real-time PCR experiments were congruent. Real-time PCR is widely used for gene expression assays and can combine both high sensitivity and reliable specificity (Bustin, 2002). However, statistical analysis might show different results depending on used algorithms. Current study assessed both reference genes previously used in expression assays in crustaceans, like actin and β-actin (Dhar *et al.*, 2009; Cottin *et al.*, 2010; Leelatanawit *et al.*, 2012; Petkeviciute *et al.*, 2015), as well as new reference genes candidates, like albumin, HSP70, HPS90 and histone H3 obtained from Meusemann *et al.* (2010) EST library and Pérez-Losada *et al.* (2004) nucleotide sequences. Reference genes which will be use in the qPCR experiment must be validated in the dataset object of study in order to demonstrate their gene stability, although these genes were used by other authors in similar conditions or species. Different algorithms used to determine reference gene stability across all tissues and phenotypes analysed presented different gene ranking. However, two genes, HSP70 and albumin, filled the highest ranks with three algorithms used (geNorm, NormFinder, and ΔCt method). BestKeeper detected β-actin and actin as the two more stable genes. Reference gene HSP90 filled first rank under NormFinder and delta-Ct method algorithms in the tissues dataset of shadow phenotype and under BestKeeper algorithm in the tissues dataset of sun phenotype. All four algorithms detected histone H3 gene as invalid reference gene in all analysed datasets due to its unstable expression and high deviation levels. Different algorithm used to assess genic expression stability generate their genes ranking basing on different statistics. In the current research it was considered important the fact that NormFinder determined the systematic error associated to use each reference gene in the study. The HSP70 reference gene showed the lowest values of systematic error if it was used in experimental studies (Table 12). For this reason, HSP70 gene was used as reference gene in the subsequent studies.

Differences detected between barnacle phenotypes based on morphological characters were also detected with gene expression. Different

tissues used in expression analyses are also a proof to demonstrate differences in peduncle integrity between both phenotypes. Thus, different tissues from sun barnacles appeared strongly attached to each other preventing obtain certain tissues from this phenotype such as cuticular-peduncular tissue. On the contrary, shadow barnacles showed a reduced integrity of their peduncles appearing certain tissues detached which facilitate their sampling. Four of the five analysed genes, guanine nucleotide-binding protein, chitin based cuticle attachment to epithelium, cuticular protein 11B and cuticular protein 47Ee genes, presented overexpression in peduncular muscle of sun phenotype respect from the same tissue from shadow phenotype. The analysed gene cuticular protein RR-1 showed similar values in both peduncular muscles and achieved the highest value in cuticular-peduncular tissue of shadow barnacle. These differences agreed with the explanation propose to explain morphological differences. The higher expression of these genes related to muscular development and cuticular integrity in the peduncle of sun phenotype provides the barnacles the enough strength to maintain themselves attached to rocky shore when they are beaten by marine waves. The shadow phenotype barnacles showed a reduced expression of these genes related to muscular development and cuticular integrity and in order to compensate this fact, shadow barnacles include higher contents of water in their peduncles to maintain their integrity.

CONCLUSIONS

7. Conclusions.

Different results reached with studies performed in this Ph.D. research have let obtain the following conclusions:

Phylogenetic analyses performed using mitochondrial 16S rDNA, COI genes and nuclear EF1α and 18S-28S rDNA region, and the concatenated sequences have corroborated the differentiation of the four species of genus *Pollicipes*, although, these inferences have not let asseverate phylogenetic relationships between these species unambiguously.

Under morphological criteria analysed in *Pollicipes* barnacles, tree topology inferred using mitochondrial marker COI has showed the most adequate phylogenetic relationships between *Pollicipes* species.

Secondary structure of 5.8S rDNA detected in all species of genus *Pollicipes* consisted in two 28S-pairing regions in both sequence ends and three doubled-helices, the first of them in direction 5'→3' divided into another two subhelices. This structure was the general folding pattern shared by species of subphylum Crustacea.

Folding patterns obtained for ITS1 and ITS2 regions in *Pollicipes* species were similar. Secondary structure of ITS1 showed a ring core with three to eight protruding helices and ITS2 presented a ring core with three to five protruding helices depending on each species of this genus.

Phylogeny inferred based on secondary structure of 5.8S rDNA showed certain taxa differentiated in subphylum Crustacea. These taxa were orders Anostraca and Diplostraca (class Branchiopoda); family Darwinulidae (class Ostracoda); infraorders Brachyura, Anomura and Astacidea (order Decapoda) and family Mysidae (order Mysida) in class Malacostraca; finally, orders Sessilia and Pedunculata (class Maxillopoda).

A set of 16 microsatellite markers were optimised to use them in *P. pollicipes* species, being 12 of them polymorphic. Microsatellite markers Pol003, Pol005, Pol008, Pol011, Pol019, Pol043, Pol044, Pol114, and Pol118 have not showed HW equilibrium in any of the analysed populations. *Loci* Pol003,

Pol005, Pol011, Pol019, Pol114, and Pol118 exhibit deficit of heterozygotes in any population of *P. pollicipes*, and Pol005, Pol043, and Pol044 demonstrate excess of heterozygotes in any analysed population. These microsatellite markers were tested in other congeneric species and showed positive PCR amplification.

Population analyses performed in nine populations of *P. pollicipes* used these microsatellite markers set detected two panmictic groups of this species, one constituted by populations from the north of Spain, including Roncudo, Artabrian Gulf, Ortigueira, Andrín and Cape of Ajo, and another group conformed by barnacles from Safi, Guincho, Cíes Islands and Brest. These differences could be explained by oceanic currents which connect populations of each group, and/or the temperature of the water which is warmer from Artabrian Gulf to Cantabrian Sea.

Expression assays let test six reference genes: albumin, HSP70, HSP90, actin, β-actin, and histone H3. Of these genes, two of them, HSP70 and albumin showed stable expression levels in all analysed tissues and were validated to include them as reference genes in different expression assays.

Expression analyses of five genes related to muscularity and integrity of peduncle from both phenotypes were performed in different tissues belonged to barnacle peduncle using HSP70 as a reference gene. This assays demonstrated that cuticle tissue and peduncular muscle from sun phenotype showed higher levels of four genes (guanine nucleotide-binding protein, chitin-based cuticle attachment to epithelium, cuticular protein 47Ee, and cuticular protein 11B) than in shadow phenotype, except for cuticular protein RR-1 which showed the inverse profile.

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ANNEXES

9.1. Annex 1.

List of accession numbers from Genbank for all analysed species. Numbers in parentheses indicate the number of analised sequences belonged to each region.

Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
Acartiidae	*Acartia negligens*			AB753553-AB753554 (2)
	Acartia tonsa	EU274426-EU274430, JF308954-JF308973 (25)		
Alpheidae	*Alpheus lottini*	AY074920 (1)		
Ampithoidae	*Ampithoe longimana*	AY004840-AY004853 (14)	AY004840-AY004853 (14)	AY859548-AY859549 (2)
Anaspididae	*Anaspides tasmaniae*			JQ740818 (1)
Argulidae	*Argulus foliaceus*	JQ740818 (1)	JQ740818 (1)	DQ201289-DQ201300 (13)
Artemiidae	*Artemia franciscana*	DQ069923-DQ069924, DQ084190-DQ084191, DQ201289-DQ201301, FJ004925-FJ004942, GU252102-GU252107, GU323289-GU323317 (70)		
	Artemia parthenogenetica	DQ201271-DQ201274, DQ201278-DQ201284, DQ201288, FJ004943-FJ004944, HM138654-HM138655 (16)		DQ201271-DQ201284, DQ201288 (12)
	Artemia persimilis	DQ069925, DQ084192, DQ201263-DQ201268, FJ004922-FJ004924 (11)		DQ201263,DQ2012635, DQ201267 (3)
	Artemia salina	DQ201302-DQ201309, FJ004946 (9)	M20198 (1)	DQ201302-DQ201309, FJ004946 (9)
	Artemia sinica	DQ069929-DQ069931, DQ084196-DQ084198, DQ201285-DQ201287, FJ004945 (10)		DQ201285-DQ201287 (3)
	Artemia sp. China HL-2005	DQ069928, DQ084195 (2)		
	Artemia sp. Kazakhstan HL-2005	DQ069927, DQ084194 (2)		DQ201269-DQ201270 (2)
	Artemia tibetiana	DQ201269-DQ201270 (2)		
	Artemia urmiana	DQ069926, DQ084193, DQ201275-DQ201277 (5)		DQ201275-DQ201277 (3)
	SHRRGBA Artemia sp.	M33097 (1)	M33097 (1)	
Atelecyclidae	*Erimacrus isenbeckii*	AB194055-AB194063 (9)	AB194055-AB194063 (9)	AB194055-AB194063 (9)
	Telmessus acutidens	AB278154 (1)	AB278154 (1)	AB278154 (1)
	Telmessus cheiragonus	AB211305, AB426496-AB426497 (3)	AB211305 (1)	AB211305 (1)
Balanidae	*Balanus roseus*	AB426498 (1)		
Bosminidae	*Bosmina fatalis*	AF482745 (1)	AF482745 (1)	AF482745 (1)
	Bosmina freyi	AF482735-AF482738, EU650754-EU650755, EU650761-EU650763 (9)	AF482735-AF482738, EU650754-EU650755, EU650761-EU650763 (9)	AF482735-AF482738, EU650754-EU650755, EU650761-EU650763 (9)
	Bosmina liederi	AF482739-AF482742, EU650757-EU650758 (6)	AF482739-AF482742, EU650757-EU650758 (6)	AF482739-AF482742, EU650757-EU650758 (6)
	Bosmina longirostris	AF482743-AF482744 (2)	AF482743-AF482744 (2)	AF482743-AF482744 (2)
	Bosmina sp. DJT-2008	EU650764-EU650779 (16)	EU650764-EU650779 (16)	EU650764-EU650779 (16)
	Eubosmina cf. longispina DJT-2008	EU650748-EU650753, EU650756, EU650759-EU650760 (9)	EU650748-EU650753, EU650756, EU650759-EU650760 (9)	EU650748-EU650753, EU650756, EU650759-EU650760 (9)

Annex 1. Cont.

Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
	Eubosmina coregoni	AF482756 (1)	AF482756 (1)	AF482756 (1)
	Eubosmina coregoni coregoni	AY264757 (1)	AY264757 (1)	AY264757 (1)
	Eubosmina coregoni thersites	AY264759 (1)	AY264759 (1)	AY264759 (1)
	Eubosmina crassicornis	AY264760 (1)	AY264760 (1)	AY264760 (1)
	Eubosmina hagmanni	AF482749-AF482750 (2)	AF482749-AF482750 (2)	AF482749-AF482750 (2)
	Eubosmina longicornis kessleri	AY264758, AY264762, AY264772-AY264773 (4)	AY264758, AY264762, AY264772-AY264773 (4)	AY264758, AY264762, AY264772-AY264773 (4)
	Eubosmina longicornis longicornis	AY264756 (1)	AY264756 (1)	AY264756 (1)
	Eubosmina longicornis reflexa	AY264761 (1)	AY264761 (1)	AY264761 (1)
	Eubosmina longispina	AF482752-AF482753, AF482755, AY264763-AY264771 (12)	AF482752-AF482753, AF482755, AY264763-AY264771 (12)	AF482752-AF482753, AF482755, AY264763-AY264771 (12)
	Eubosmina maritima	AF482754 (1)	AF482754 (1)	AF482754 (1)
	Eubosmina oriens	AF482746-AF482748 (3)	AF482746-AF482748 (3)	AF482746-AF482748 (3)
	Eubosmina tubicen	AF482751 (1)	AF482751 (1)	AF482751 (1)
Branchipodidae	*Branchipodopsis affinis*	AJ421826 (1)	AJ421826 (1)	AJ421826 (1)
	Branchipodopsis relictus	AJ307675 (1)	AJ307675 (1)	AJ307675 (1)
	Branchipodopsis wolfi	AJ307676 (1)	AJ307676 (1)	AJ307676 (1)
	Branchipus schaefferi	AJ307677, AJ421827 (2)	AJ307677, AJ421827 (2)	AJ307677, AJ421827 (2)
	Branchipus visnyai	AJ307678 (1)	AJ307678 (1)	AJ307678 (1)
	Tanymastigites perrieri	AJ238066, AJ307679 (2)	AJ307679 (1)	AJ307679 (1)
Calanidae	*Calanus hyperboreus*			JQ819840 (1)
	Calanus jashnovi	AB297703, AB332362-AB332365 (5)		AB332383-AB332386, AB297711 (5)
	Calanus pacificus	AB297704, AF315016 (2)	AF315016 (1)	AB297712, AF315016 (2)
	Calanus sinicus	AB297697-AB297702, AB332345-AB332361 (23)		AB297705-AB297710, AB332366-AB332382, AB753669-AB753679 (34)
	Neocalanus cristatus		AB526958-AB526963 (6)	AB526958-AB526963 (6)
	Neocalanus flemingeri		AB526939-AB526957 (18)	AB526939-AB526957 (19)
	Neocalanus gracilis			AB753680-AB753683 (4)
	Neocalanus plumchrus		AB526964-AB526971 (8)	AB526964-AB526971 (8)
	Neocalanus robustior			AB753684 (1)
Caligidae	*Caligus sp. 1 JYW-2010*	HM545886 (1)	HM545886 (1)	HM545886 (1)
	Caligus sp. 2 JYW-2010	HM545889 (1)	HM545889 (1)	HM545889 (1)
	Lepeophtheirus salmonis	AF043980-AF043981, EU929084 (3)	EU929084 (1)	EU929084 (1)
Calliopiidae	*Apherusa glacialis*	FJ422962 (1)		FJ422962 (1)
Cambaridae	*Procambarus clarkii*	AF198585-AF198590, EU118284,	GQ369797 (1)	AF198591-AF198596, GQ369797 (7)
Cancridae	*Cancer magister*	EF035126 (1)	EF035126 (1)	EF035126 (1)
	Cancer productus	EF035124 (1)	EF035124 (1)	EF035124 (1)
Carcinidae	*Carcinus maenas*	AM410549, EF035109 (2)	EF035109 (1)	EF035109 (1)

Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
Centropagidae	*Centropages abdominalis*	AB200184 (1)		AB200200 (1)
	Centropages bradyi			AY335898 (1)
	Centropages gracilis			AB753558 (1)
	Centropages typicus			GU125729 (1)
Chthamalidae	*Chthamalus challengeri*	JF312392-JF312641 (250)		
	Chthamalus malayensis	JF312388-JF312389 (2)		
	Chthamalus montagui	EU699254-EU699277 (24)	EU699254-EU699277 (24)	
	Chthamalus moro	JF312390-JF312391 (2)		
	Chthamalus stellatus	EU699278-EU699308 (31)	EU699278-EU699308 (31)	
Clausocalanidae	*Clausocalanus arcuicornis*			AY265915 (1)
	Clausocalanus brevipes			AY265918 (1)
	Clausocalanus farrani			AB753580, AY265919 (2)
	Clausocalanus furcatus			AY265920 (1)
	Clausocalanus ingens			AY265921 (1)
	Clausocalanus jobei			AY265922 (1)
	Clausocalanus laticeps			AY265923 (1)
	Clausocalanus lividus			AB753581-AB753587, AY265924 (8)
	Clausocalanus mastigophorus			AY265925 (1)
	Clausocalanus minor			AB753588-AB753595, AY265926 (9)
	Clausocalanus parapergens			AY265916 (1)
	Clausocalanus paululus			AY265927 (1)
	Clausocalanus pergens			AY265917 (1)
	Drepanopus forcipatus			AY265928 (1)
Cletodidae	*Cletocamptus deitersi*	AF315008, AF315021-AF315033 (14)	AF315008, AF315021-AF315033 (14)	AF315008, AF315021-AF315033 (14)
	Cletocamptus helobius	AF315017-AF315020 (4)	AF315017-AF315020 (4)	AF315017-AF315020 (4)
Crangonyctidae	*Crangonyx islandicus*	JN258055-JN258095 (41)		
Cyclopettidae	*Paracyclopina nana*	FJ214952 (1)	FJ214952 (1)	FJ214952 (1)
Cyclopidae	*Acanthocyclops robustus*			EF114363 (1)
	Cyclops insignis	EF532821 (1)	EF532821 (1)	EF532821 (1)
	Cyclops kolensis	EF532820 (1)	EF532820 (1)	EF532820 (1)
	Mesocyclops aspericornis			GQ848501 (1)
	Mesocyclops darwini			GQ848497 (1)
	Mesocyclops edax			GQ848491-GQ848493 (3)
	Mesocyclops leuckarti			GQ848499 (1)
	Mesocyclops longisetus			GQ848494 (1)
	Mesocyclops longisetus curvatus			EF114364, GQ848495 (2)
	Mesocyclops major			GQ848498 (1)
	Mesocyclops meridianus			GQ848496 (1)

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Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
	Mesocyclops ogunnus			EF114366, GQ848502 (2)
	Mesocyclops pehpeiensis			GQ848500 (1)
	Mesocyclops thermocyclopoides			EF581893 (1)
	Metacyclops mendocinus			EF114365 (1)
	Thermocyclops crassus			GQ848489 (1)
	Thermocyclops decipiens			EF114367, GQ848490 (2)
	Thermocyclops inversus			EF114368 (1)
	Thermocyclops minutus			EF114369 (1)
Daphniidae	*Daphnia cucullata*	AY730402, FJ943903-FJ943934 (33)	AY730402 (1)	AY730402, HM161681-HM161687 (8)
	Daphnia dentifera	AB641909-AB641953, AB641973, AY730383-AY730384, AY730387, AY730389-AY730391, JX446621 (53)	AY730383-AY730384, AY730387, AY730389-AY730391 (6)	AB642049-AB642072, AY730383 (30)
	Daphnia galeata	AB641954-AB641971, AB641974, AY730377-AY730382, AY730388, AY730396-AY730401, FJ943870-FJ943902 (65)	AY730377-AY730382, AY730388, AY730396-AY730401 (13)	AB642073-AB642090, AY730377-AY730382, AY730388, AY730396-AY730401, HM161688-AY161704 (48)
	Daphnia longispina	AY730385-AY730386, AY730393-AY730395, AY730404, FJ943802-FJ943869 (74)	AY730385-AY730386, AY730393-AY730395, AY730404 (6)	AY730385-AY730386, AY730393-AY730395, AY730404 (6)
	Daphnia sp. Ygt27b_Iwt32a	AB641972 (1)		
	Daphnia thorata	AY730392 (1)	AY730392 (1)	AY730392 (1)
	Daphnia umbra	AY730403, FJ943800-FJ943801 (3)	AY730403 (1)	AY730403 (1)
Darwinulidae	*Vestalenula marmonieri*	GU324444 (1)	GU324444 (1)	GU324444, JX069229 (2)
	Vestalenula matildae	GU324443 (1)	GU324443 (1)	GU324443 (1)
Diaptomidae	*Acanthodiaptomus pacificus*	AB494176-AB494225 (50)	AB494176-AB494225 (50)	AB494176-AB494225 (50)
	Aglaodiaptomus leptopus	AY275451, EU582648 (2)	AY275451, EU582648 (2)	AY275451, EU582648 (2)
	Argyrodiaptomus furcatus	DQ248319 (1)		DQ248319 (1)
	Hesperodiaptomus arcticus	EU977322 (1)	EU977322 (1)	EU977322 (1)
	Hesperodiaptomus californiensis	EU977323 (1)	EU977323 (1)	EU977323 (1)
	Hesperodiaptomus eiseni	EU977321 (1)	EU977321 (1)	EU977321 (1)
	Hesperodiaptomus environmental sample	KC215983 (1)		
	Hesperodiaptomus franciscanus	EU977326 (1)	EU977326 (1)	EU977326 (1)
	Hesperodiaptomus hirsutus	EU977319 (1)	EU977319 (1)	EU977319 (1)
	Hesperodiaptomus kenai	EU977318 (1)	EU977318 (1)	EU977318 (1)
	Hesperodiaptomus nevadensis	EU977324 (1)	EU977324 (1)	EU977324 (1)
	Hesperodiaptomus shoshone	EU977320 (1)	EU977320 (1)	EU977320 (1)
	Hesperodiaptomus victoriaensis	EU977325 (1)	EU977325 (1)	EU977325 (1)
	Mastigodiaptomus nesus	AY275452, EU582649 (2)	AY275452, EU582649 (2)	AY275452, EU582649 (2)

Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
	Notodiaptomus conifer	DQ248320 (1)		DQ248320 (1)
	Notodiaptomus iheringi	DQ248321 (1)		DQ248321 (1)
	Sinodiaptomus sarsi	AB454174-AB454175 (2)	AB454174-AB454175 (2)	AB454174-AB454175 (2)
	Sinodiaptomus valkanovi	AB454171-AB454173, AB494228-AB494233, AB494235-AB494239 (13)	AB454171-AB454173, AB494228-AB494233, AB494235-AB494239 (13)	AB454171-AB454173, AB494228-AB494233, AB494235-AB494239 (13)
	Skistodiaptomus carolinensis	AY275453, EU582650-EU582651 (3)	AY275453, EU582650-EU582651 (3)	AY275453, EU582650-EU582651 (3)
	Skistodiaptomus mississippiensis	AY275454, EU582652-EU582653 (3)	AY275454, EU582652-EU582653 (3)	AY275454, EU582652-EU582653 (3)
	Skistodiaptomus oregonensis	AY275455, EU582654-EU582667 (15)	AY275455, EU582654-EU582667 (15)	AY275455, EU582654-EU582667 (15)
	Skistodiaptomus pallidus	AY275456, EU582668-EU582676 (10)	AY275456, EU582668-EU582676 (10)	AY275456, EU582668-EU582676 (10)
	Skistodiaptomus pygmaeus	AY275457, EU582677-EU582683 (8)	AY275457, EU582677-EU582683 (8)	AY275457, EU582677-EU582683 (8)
	Skistodiaptomus reighardi	AY275458, EU582684-EU582685 (3)	AY275458, EU582684-EU582685 (3)	AY275458, EU582684-EU582685 (3)
Epacteriscidae	*Enantiosis galapagensis*	HM48128 (1)		
Ergasilidae	*Ergasilus anchoratus*	DQ328766 (1)	DQ328766 (1)	DQ328766 (1)
	Ergasilus briani	DQ328762 (1)	DQ328762 (1)	DQ328762 (1)
	Ergasilus danjiangs	DQ328764 (1)	DQ328764 (1)	DQ328764 (1)
	Ergasilus hypomesi	DQ328765 (1)	DQ328765 (1)	DQ328765 (1)
	Ergasilus tumidus	DQ328763 (1)	DQ328763 (1)	DQ328763 (1)
	Pseudergasilus parasiluri	AY297732 (1)	AY297732 (1)	AY297732 (1)
	Sinergasilus major	AY297729-AY297731, DQ251664-DQ251693, DQ466196-DQ466197 (35)	AY297729-AY297731, DQ251664-DQ251693, DQ466196-DQ466197 (35)	AY297729-AY297731, DQ251664-DQ251693, DQ466196-DQ466197 (35)
	Sinergasilus polycolpus	AY297725-AY297726, DQ251694-DQ251723 (32)	AY297725-AY297726, DQ251694-DQ251723 (32)	AY297725-AY297726, DQ251694-DQ251723 (32)
	Sinergasilus undulatus	AY297727-AY297728, DQ251634-DQ251663 (32)	AY297727-AY297728, DQ251634-DQ251663 (32)	AY297727-AY297728, DQ251634-DQ251663 (32)
Euphausiidae	*Euphausia lucens*	GQ890562-GQ890564 (3)		
	Euphausia recurva	GQ890565-GQ890566 (2)		
	Euphausia vallentini	GQ890567-GQ890569 (3)		
Gammaridae	*Dikerogammarus bispinosus*	DQ222403 (1)		
	Dikerogammarus villosus	DQ222402 (1)		
	Gammarus minus	EU286097-EU286273 (177)		
	Gammarus tigrinus	DQ300256-DQ300260 (5)		
	Gammarus wilkitzkii	FJ422963 (1)		FJ422963 (1)
Geryonidae	*Chaceon quinquedens*	AY123200 (1)		AY123199-AY123200 (2)
Gnathiidae	*Elaphognathia cornigera*			AB519154-AB519156 (3)
	Elaphognathia discolor			AB519159 (1)
	Elaphognathia nunomurai			AB519157, AB547450 (2)
	Elaphognathia rangifer			AB519158 (1)

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Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
Harpacticidae	*Tigriopus brevicornis*	AM083335-AM083342 (8)		
	Tigriopus californicus	AY599492 (1)	AY599492 (1)	AY599492 (1)
	Tigriopus japonicus	EU054307, EU057568-EU057580 (14)	EU054307, EU057568-EU057580 (14)	EU054307, EU057568-EU057580 (14)
	Tigriopus sp. JSL-2005	EU520441 (1)	EU520441 (1)	EU520441 (1)
Haustoriidae	*Diporeia hoyi*	EU807701-EU807708 (8)		EU807701-EU807708 (8)
Leptocheliidae	*Leptochelia dubia*	AY033479-AY033480 (2)		
	Leptochelia forresti	AY033481-AY033482 (2)		
	Leptochelia rapax	AY033483-AY033485 (3)		
Lernaeidae	*Lamproglena chinensis*	DQ328767 (1)	DQ328767 (1)	
Lernaeopodidae	*Clavellopsis sp. JYW-2010*	HM545892 (1)	HM545892 (1)	HM545892 (1)
Leucosiidae	*Philyra pisum*	AB426491 (1)		
Limnadiidae	*Eulimnadia texana*	AY859574 (1)	AY859574 (1)	AY859574 (1)
Lithodidae	*Lithodes aequispinus*	AB236928, AB426493 (2)	AB236928 (1)	AB236928 (1)
	Lithodes confundens	HM021008 (1)		
	Lithodes ferox	HM021009-HM021011 (3)		
	Lithodes murrayi	HM021012-HM021014 (3)		
	Lithodes santolla	HM021015 (1)		
	Neolithodes asperrimus	HM021016-HM021020 (5)		
	Neolithodes duhameli	HM021021-HM021023 (3)		
	Paralithodes brevipes	AB211306, AB426494 (2)	AB211306 (1)	AB211306 (1)
	Paralithodes camtschaticus	AB194389-AB194392, AB194395 (5)	AB194389-AB194392 (4)	AB194389-AB194392 (4)
	Paralithodes platypus	AB188153, AB426492-AB426494 (4)	AB194393-AB194394 (2)	AB194393-AB194394 (2)
	Paralomis aculeata	HM020983-HM020985 (3)		
	Paralomis africana	HM020986 (1)		
	Paralomis anamerae	HM020987 (1)		
	Paralomis birsteini	HM020988 (1)		
	Paralomis elongata	HM020989 (1)		
	Paralomis erinacea	HM020990-HM020996 (7)		
	Paralomis formosa	HM020997-HM021001 (5)		
	Paralomis granulosa	HM021002-HM021004 (3)		
	Paralomis spinosissima	HM021005-HM021007 (3)		
Lysianassidae	*Onisimus glacialis*	FJ422964 (1)		FJ422964 (1)
	Onisimus nanseni	FJ422965 (1)		FJ422965 (1)
Macrocyprididae	*Macromckenziea aff. giambonini*	GU576984 (1)	GU576984 (1)	GU576984 (1)
	Macroscapha aff. walterae	GU577104-GU577105 (2)	GU577104-GU577105 (2)	GU577104-GU577105 (2)
	Macroscapha falcis	GU577010-GU577041 (32)	GU577010-GU577041 (32)	GU577010-GU577041 (32)
	Macroscapha inaequata	GU576985-GU576995 (11)	GU576985-GU576995 (11)	GU576985-GU576995 (11)
	Macroscapha opaca	GU577002-GU577009, GU577042-GU577056, GU577070-GU577103 (57)	GU577002-GU577009, GU577042-GU577056, GU577070-GU577103 (57)	GU577002-GU577009, GU577042-GU577056, GU577070-GU577103 (57)

Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
	Macroscapha solecavai	GU577057-GU577069 (13)	GU577057-GU577069 (13)	GU577057-GU577069 (13)
	Macroscapha turbida	GU576996-GU577001 (6)	GU576996-GU577001 (6)	GU576996-GU577001 (6)
	Macroscapha walterae	GU577106-GU577111 (6)	GU577106-GU577111 (6)	GU577106-GU577111 (6)
Majidae	*Chionoecetes bairdi*	AB188156, AB193501-AB193503, AY234856 (5)	AB193501-AB193503 (3)	AB193501-AB193503 (3)
	Chionoecetes japonicus	AB193504-AB193506, HQ909101 (4)	AB193504-AB193506, HQ909101 (4)	AB193504-AB193506, HQ909101 (4)
	Chionoecetes japonicus pacificus	AB501212 (1)	AB501212 (1)	AB501212 (1)
	Chionoecetes opilio	AB193499-AB193500, HQ909100 (3)	AB193499-AB193500, HQ909100 (3)	AB193499-AB193500, HQ909100 (3)
	Chionoecetes tanneri	AB546598 (1)	AB546598 (1)	AB546598 (1)
Melitidae	*Melita plumulosa*	JN790073-JN790100 (28)		
Mysidae	*Americamysis bahia*	AF169228, AF228803-AF228805 (4)	AF228803-AF228805 (3)	AF228803-AF228805 (3)
	Heteromysis sp. JM-2004			AY859578 (1)
	Neomysis integer	AJ549194 (1)		
	Neomysis sp. KIZ1	AB426490 (1)		
Mytilicolidae	*Mytilicola intestinalis*		HM775198-HM775203, HM775206 (7)	HM775198-HM775203, HM775206 (7)
	Mytilicola orientalis		HM775204-HM775205 (2)	HM775204-HM775205 (2)
Nebaliidae	*Nebalia sp. JM-2004*			AY859589-AY859590 (2)
Niphargidae	*Niphargus frasassianus*	GU973224-GU973250, GU973339, GU973341, GU973343-GU973348, GU973350-GU973355, GU973364-GU973369, GU973371-GU973399, GU973401-GU973410 (86)		GU973224-GU973250, GU973339, GU973341, GU973343-GU973348, GU973350-GU973355, GU973364-GU973369, GU973371-GU973399, GU973401-GU973410 (86)
	Niphargus ictus	GU973208, GU973215, GU973221, GU973223, GU973251-GU973268, GU973270, GU973273-GU973338, GU973340, GU973342, GU973349, GU973356-GU973363, GU973370, GU973400 (102)		GU973208, GU973215, GU973221, GU973223, GU973251-GU973268, GU973270, GU973273-GU973338, GU973340, GU973342, GU973349, GU973356-GU973363, GU973370, GU973400 (102)
	Niphargus ladmiraulti	GU973472-GU973475 (4)		GU973472-GU973475 (4)
	Niphargus montanarius	GU973207, GU973209-GU973214, GU973216-GU973220, GU973222 (13)		GU973207, GU973209-GU973214, GU973216-GU973220, GU973222 (13)
	Niphargus plateaui	GU973476-GU973480 (5)		GU973476-GU973480 (5)
	Niphargus schellenbergi	GU973481-GU973503 (23)		GU973481-GU973503 (23)
	Niphargus sp. Clade 4 JFF-2010	GU973269, GU973271-GU973272 (3)		GU973269, GU973271-GU973272 (3)
Palaemonidae	*Exopalaemon carinicauda*	GQ369794 (1)	GQ369794 (1)	GQ369794 (1)
	Exopalaemon cf. modestus	EU373484, GQ369793 (2)	GQ369793 (1)	GQ369793 (1)
	Macrobrachium nipponense	EU118285, EU346851, GQ369796 (3)	GQ36979 (1)	GQ369796 (1)
	Macrobrachium rosenbergii	EU118286, EU373480-EU373481, GQ369795, HM590579, HM804252 (6)	GQ369795, HM590579, HM804252 (3)	EU373482, GQ369795, HM590579, HM804252, F502465-F502520 (60)

Annex 1. Cont.

Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
Palinuridae	*Jasus edwardsii*	AB492048-AB492051 (4)		
	Panulirus argus			AY210832 (1)
	Panulirus brunneiflagellum	AB426512 (1)		
	Panulirus cygnus	AB492043-AB492047 (5)		
	Panulirus femoristriga	AB426511 (1)		
	Panulirus japonicus	AB426502-AB426508, AF253525 (8)		
	Panulirus longipes bispinosus	AB426509 (1)		
	Panulirus longipes longipes	AB426510 (1)		
Pandalidae	*Pandalus danae*			EF035131 (1)
	Pandalus eous		AB193475-AB193477 (3)	AB193475-AB193477 (3)
	Pandalus goniurus		EF035129 (1)	EF035129 (1)
	Pandalus hypsinotus		AB193479-AB193480, EF035130 (3)	AB193479-AB193480, EF035130 (3)
	Pandalus platyceros		EF035133 (1)	EF035133 (1)
Paracalanidae	*Paracalanus parvus*	AB426513-AB426518 (6)		
Penaeidae	*Farfantepenaeus notialis*	AF463511 (1)		
	Fenneropenaeus indicus	AY074919 (1)		
	Fenneropenaeus merguiensis	AY315657, AY331585-AY331590 (7)		
	Fenneropenaeus penicillatus	FR715679 (1)		
	Fenneropenaeus silasi	AY315658 (1)		
	Litopenaeus schmitti	AF463510 (1)		
	Litopenaeus setiferus	AY074918 (1)		
	Litopenaeus stylirostris	AF463508 (1)		
	Litopenaeus vannamei	AF463509, EU118282-EU118283, GQ392035 (4)		
	Marsupenaeus japonicus	AF463512, EU113302, EU118281 (3)		
	Melicertus latisulcatus	EU118290 (1)		
	Metapenaeopsis dalei	EU118289 (1)		
	Metapenaeus ensis	EU118287 (1)		
	Penaeus japonicus	AF253523-AF253524 (2)		
	Penaeus monodon	AY074921, EU118288 (2)		
	Penaeus semisulcatus	AY315659 (1)		
	Penaeus vannamei	AF124597 (1)	AF121132, AF124597 (2)	AF124597 (1)
Pollicipedidae	*Pollicipes elegans*	FR869941-FR869969, GQ472664 (30)	FR869941-FR869969, GQ472664 (30)	FR869941-FR869969, GQ472664 (30)
	Pollicipes pollicipes	FR869888-FR869940, GQ472641-GQ472648 (61)	FR869888-FR869940, GQ472641-GQ472648 (61)	FR869888-FR869940, GQ472641-GQ472648 (61)
	Pollicipes polymerus	FR869970-FR869985, GQ472656-GQ472663 (24)	FR869970-FR869985, GQ472656-GQ472663 (24)	FR869970-FR869985, GQ472656-GQ472663 (24)
	Pollicipes sp. JQ-2009	GQ472649-GQ472655 (7)	GQ472649-GQ472655 (7)	GQ472649-GQ472655 (7)

Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
Pontellidae	*Anomalocera patersoni*	AB200194 (1)		AB200209 (1)
	Calanopia elliptica	AB200195 (1)		AB200197 (1)
	Calanopia minor	AB200196 (1)		
	Labidocera acuta	AB200176 (1)		AB200208, HM439435 (2)
	Labidocera bataviae	AB200174 (1)		AB200204 (1)
	Labidocera detruncata	AB200173 (1)		AB200201, AB753559 (2)
	Labidocera japonica	AB200178 (1)		AB200207 (1)
	Labidocera jollae			AY335899 (1)
	Labidocera minuta	AB200175 (1)		AB200206 (1)
	Labidocera rotunda	AB200177 (1)		AB200210 (1)
	Pontella chierchiae	AB200180 (1)		AB200211 (1)
	Pontella fera	AB200183 (1)		AB200203 (1)
	Pontella princeps	AB200182 (1)		AB200205 (1)
	Pontella rostraticauda	AB200179 (1)		AB200212 (1)
	Pontella securifer	AB200181 (1)		AB200202 (1)
	Pontellina morii	AB200193 (1)		AB200198 (1)
	Pontellina plumata	AB200192 (1)		AB200199, AB753560 (2)
Pseudodiaptomidae	*Pseudodiaptomus inopinus*	AY500279 (1)	AY500279 (1)	AY500279 (1)
	Pseudodiaptomus koreanus	AY499003-AY499004, AY499009, AY496260 (4)	AY496260, AY499003-AY499004, AY499009 (4)	AY496260, AY499003-AY499004, AY499009 (4)
	Pseudodiaptomus marinus	AY496261 (1)	AY496261 (1)	AY496261 (1)
	Pseudodiaptomus nihonkaiensis	AY499005 (1)	AY499005 (1)	AY499005 (1)
	Pseudodiaptomus poplesia	AY499006-AY499008 (3)	AY499006-AY499008 (3)	AY499006-AY499008 (3)
Pseudocyclopidae	*Pseudocyclops cf. juanibali Cactus Forest*	JF933927-JF933928 (2)		
	Pseudocyclops cf. juanibali Deep Grietas	JF933921-JF933922 (2)		
	Pseudocyclops juanibali	JF933915-JF933920 (6)		
	Pseudocyclops saenzi	JF933923-JF933926 (4)		
Pseudothelphusidae	*Epilobocera sinuatifrons*	FN395370-FN395607 (238)	FN395370-FN395607 (238)	FN395370-FN395607 (238)
Ridgewayiidae	*Ridgewayia delfine*	HM481277-HM481279 (3)		
	Ridgewayia tortuga	HM481283-HM481285 (3)		
	Ridgewayia tunela	HM481280-HM481282 (3)		
Sacculinidae	*Polyascus gregaria*	GQ328921 (1)		GQ328922 (1)
	Sacculina carcini	AF109345-AF109346 (2)		
	Sacculina granifera	AF109348 (1)		
	Sacculina oblonga	AF109349 (1)		
	Sacculina sp. 'Western Port'	AF109347 (1)		
Scalpellidae	*Capitulum mitella*	AB426499 (1)		
Scyllaridae	*Ibacus ciliatus*	AB426500 (1)		

Annex 1. Cont.

Family	Species	ITS1 accesion numbers	5.8S rDNA accesion numbers	ITS2 accesion numbers
Sesarmidae	*Holometopus dehaani*	AF316352, AF316395 (2)	AF316395 (1)	AF315680, AF316395 (2)
	Parasesarma plicatum			JF836813-JF836814 (2)
	Sesarma dolphinum	FN395809-FN396039 (231)	FN395809-FN396039 (231)	FN395809-FN396039 (231)
	Sesarma fossarum	FN395309-FN395315 (7)	FN395309-FN395315 (7)	FN395309-FN395315 (7)
	Sesarma meridies	FN396040-FN396100 (61)	FN396040-FN396100 (61)	FN396040-FN396100 (61)
	Sesarma verleyi	FN395306-FN395308 (3)	FN395306-FN395308 (3)	FN395306-FN395308 (3)
	Sesarma windsor	FN395608-FN395808 (201)	FN395608-FN395808 (201)	FN395608-FN395808 (201)
Solenoceridae	*Solenocera crassicornis*	EU118283 (1)		
Squillidae	*Squilla empusa*			AY210841 (1)
Streptocephalidae	*Streptocephalus bowmani*	AY519846 (1)		
	Streptocephalus dorothae	AY519842 (1)		
	Streptocephalus dumonti	AY519847 (1)		
	Streptocephalus guzmani	AY519845 (1)		
	Streptocephalus mackini	AY519844 (1)		
	Streptocephalus moorei	AY519843 (1)		
	Streptocephalus namibiensis	AY519841 (1)		
	Streptocephalus papillatus	AY519839 (1)		
	Streptocephalus proboscideus	AY519840 (1)		
	Streptocephalus purcelli	AY519851 (1)		
	Streptocephalus sealii	AY519849 (1)		
	Streptocephalus texanus	AY519848 (1)		
	Streptocephalus trifideus	AY519850 (1)		
Varunidae	*Eriocheir formosa*	AF287996-AF288000, AF316329, AF316375, AF517681, AY181976 (9)	AF316375 (1)	AF315653-AF315654, AF316375 (3)
	Eriocheir hepuensis	AF316330-AF316333, AF316376-AF316379 (8)	AF316376-AF316378 (3)	AF315655-AF315657, AF316376-AF316378 (3)
	Eriocheir japonica	AF316334-AF316339, AF316379-AF316383, AF517680, AY181977 (13)	AF316379-AF316383 (5)	AF315658-AF315664, AF316379-AF316383 (12)
	Eriocheir leptognathus	AF253519, AF315347, AF316340, AF316374, AF316384-AF316385 (6)	AF316374, AF316384-AF316385 (3)	AF315665-AF315667, AF316374, AF316384-AF316385 (6)
	Eriocheir rectus	AF316341-AF316342, AF316386-AF316387 (4)	AF316386-AF316387 (2)	AF315668-AF315669, AF316386-AF316387 (4)
	Eriocheir sinensis	AF253518, AF279270, AF316343-AF316351, AF316388-AF316394, AY181978, EU161067, EU373478-EU373479, EU373485-EU373499, HQ534050, HQ534052, HQ534054-HQ534056, HQ534058, HQ534060-HQ534061, HQ534063-HQ534064 (47)	AF316388-AF316394, EU373478-EU373479, EU373485-EU373490, EU373492-EU373499, HQ534052, HQ534055-HQ534056, HQ534061, HQ534064 (29)	AF315670-AF315679, AF316388-AF316394, EU373478-EU373479, EU373485-EU373499, HQ534051-HQ534053, HQ534055-HQ534057, HQ534059, HQ534061, HQ534064 (47)
	Gaetice depressus			AY859575 (1)

9.2. Annex 2.

Compensatory base changes detected for ITS1, 5.8S rDNA and ITS2 regions in analysed crustacean species. Alternative base pairs (CBCs) detected in a specific paired pair and position in the family alignment (Position) are indicated.

Species	ITS1		5.8S rDNA		ITS2	
	CBC	Position	CBC	Position	CBC	Position
Ampithoe longimana	U···A<=>A···U	146···158				
	U···A<=>A···U	148···156				
Branchipus schaefferi					G···C<=>A···U	31···43
Calanus sinicus					G···C<=>A···U	14···29
					G···C<=>A···U	106···133
Cletocamptus deitersi	A···U<=>G···C	3···57			A···U<=>G···C	129···237
	A···U<=>U···A	17···41			A···U<=>C···G	184···196
	G···C<=>U···G	283···373			A···U<=>U···A	185···195
	A···U<=>C···G	307···329			U···A<=>G···C	248···284
	C···G<=>A···U<=>U···G	313···323			U···A<=>A···U	249···283
	G···U<=>U···A	450···493			A···U<=>G···C	255···278
	U···A<=>C···G	455···488				
	U···A<=>A···U	462···482				
	A···U<=>G···C	467···474				
	C···G<=>U···A	467···476				
Crangonyx islandicus	C···G<=>U···A	249···307				
Mesocyclops longisetus					G···U<=>C···G<=>A···U	276···288
Daphnia longispina					U···A<=>C···G	301···680
					G···C<=>U···A	457···524
					U···A<=>C···G	458···523
Skistodiaptomus pallidus	C···G<=>U···A	152···193				
Acanthodiaptomus pacificus	G···C<=>A···U	156···190	G···C<=>A···U	113···131	G···C<=>A···U	21···50
	A···U<=>U···G	158···189	A···U<=>G···C	115···129	G···C<=>A···U	22···46
	C···G<=>G···U	159···188			C···G<=>U···A	241···268
	A···U<=>G···C	237···258			C···G<=>U···A	242···266
					G···C<=>A···U	244···264
Euphausia recurva	A···U<=>U···A	265···276				
Gammarus minus	U···G<=>G···C	86···164				
Tigriopus japonicus	A···U<=>G···C	30···52			A···U<=>G···C	15···48
	A···U<=>U···A	32···42			U···A<=>C···G	66···88
	C···G<=>A···U	213···232				
	G···C<=>A···U	215···230				
	G···C<=>A···U	263···307				

Annex 2. Cont.

Species	ITS1		5.8S rDNA		ITS2	
	CBC	Position	CBC	Position	CBC	Position
	G···C<=>A···U	272···305				
	C···G<=>G···C	283···295				
	G···C<=>A···U	284···293				
	G···C<=>A···U	362···452				
	G···C<=>A···U	577···619				
Macrobrachium nipponense	G···U<=>U···A	123···176				
	G···U<=>U···A	140···159				
	A···U<=>C···G	1252···1510				
Macrobrachium rosenbergii	C···G<=>G···C	1096···1141				
Jasus edwardsii	A···U<=>C···G	125···198				
Paracalanus parvus	A···U<=>G···C	47···72				
Marsupenaeus japonicus	U···G<=>G···C	159···192				
Pollicipes elegans					U···A<=>C···G	195···214
Pollicipes pollicipes					G···C<=>A···U	49···266
Labidocera acuta					U···A<=>C···G	208···287
Pseudocyclops cf. juanibali	U···G<=>C···G<=>A···U	150···193				
	G···C<=>A···U	161···178				
	A···U<=>G···C	216···280				
Pseudodiaptomus koreanus					C···G<=>U···A	60···82
Epilobocera sinuatifrons			C···G<=>U···A	34···44	C···G<=>U···A	65···227
			G···C<=>A···U	71···111	G···C<=>U···A	99···169
					G···C<=>A···U	106···161
					A···U<=>G···C	112···157
					U···A<=>A···U	127···147
					G···C<=>A···U	256···405
					A···U<=>U···G	311···319
					U···A<=>C···G	315···334
					G···C<=>A···U	329···348
					C···G<=>U···A	337···359
Sesarma dolphinum					G···C<=>U···G	4···1057
					C···G<=>U···A	6···1055
					U···A<=>C···G	20···315

Annex 2. Cont.

Species	ITS1		5.8S rDNA		ITS2	
	CBC	Position	CBC	Position	CBC	Position
					G···C<=>A···U	39···167
					U···A<=>C···G	87···138
					C···G<=>U···A	194···216
					U···A<=>C···G	224···315
					C···G<=>U···A	224···316
					U···G<=>A···U	327···417
					G···C<=>A···U	608···716
					A···U<=>G···C	1008···1049
					G···C<=>A···U	1009···1045
Sesarma windsor					U···G<=>G···C	4···1057
					C···G<=>U···A	463···597
					A···U<=>G···C	519···559
					G···C<=>A···U	524···556
					U···A<=>C···G	624···642
Eriocheir japonica					G···C<=>A···U	210···301
					G···C<=>A···U	530···569
					G···C<=>A···U	533···564
					G···C<=>C···G	534···563
					A···U<=>G···C	535···561
Eriocheir sinensis	U···A<=>C···G	418···461			A···U<=>G···C	120···615
	U···A<=>C···G	419···460			G···C<=>A···U	210···301
	A···U<=>G···C	611···636			G···C<=>A···U	373···597
					A···U<=>G···C	504···583
					A···U<=>G···C	530···569
					A···U<=>G···C	533···564
					C···G<=>G···C	534···563
					C···G<=>A···U<=>U···G	546···555

9.3. Annex 3.

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MICROSATELLITE LETTERS

Characterization of 15 polymorphic microsatellite *loci* in gooseneck barnacle *Pollicipes pollicipes* (Gmelin, 1789), and cross-amplification in other *Pollicipes* species

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Abstract Gooseneck barnacles *Pollicipes pollicipes* are a highly exploited fishery causing its demise in some areas of the NW coast of Spain. Barnacles were used to develop and characterize microsatellite primer *loci*. After 64 individuals analyzed, 15 *loci* were polymorphic with number of alleles ranging from 2 to 20 and the observed heterozygosity between 0 and 1. In addition, cross-specific amplifications in the congeneric species *P. elegans*, *P. polymerus* and *P. caboverdensis* showed positive results.

Keywords *Pollicipes pollicipes* · Gooseneck barnacle · Microsatellite · Cross-specific amplification

The gooseneck barnacle *Pollicipes pollicipes* (Gmelin, 1789) is a sessile species which distributes from North coast of France to North coast of Africa. This species is commercially important in the European market, mainly Spain and Portugal, and, frequently, populations are over-exploited, occurring cases of population extinction in areas of the NW coast of Spain. Few genetic studies exist for *P. pollicipes*, and only one based on mitochondrial markers revealed no genetic differences among populations of *P. pollicipes*. To solve the lack of population genetic information, the development of other informative markers, as microsatellites, would help to elucidate the population genetic structure of *P. pollicipes*.

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Specimens were obtained from natural banks of the Cíes Islands (north-western Spain: 42.2210356, -8.9041741), Safi (Morocco: 32.2930773, -9.2366425), Brest (France: 48.408408, -4.4996006), and Guincho (Portugal: 38.7291285, -9.4659165) for species *P. pollicipes*, from the Cabo Verde Islands (15.120142,-23.6051721) for *P. caboverdensis*, from Lobos de Afuera Island (Peru: -6.928333, -80.710556) in the case of *P. elegans*, and from Olympic National Park (USA: 48.117806, -124.673788) for *P. polymerus*. Genomic DNA from 64 individuals belonging to these species and populations was extracted using the kit Genomic DNA from tissue, NucleoSpin Tissue (Macherey-Magel GmbH and Co.) following the manufacturer's instructions. Microsatellite markers (Table 1) were obtained from an expressed sequence tags library (Meusemann et al. 2010) using Tandem Repeats Finder (Benson 1999) with values of alignment parameters for match, mismatch and indels of 2, 3 and 5, respectively, minimum alignment score to report repeat of 30 and maximum period size of 500. Primers were designed using Primer3 (Rozen and Skaletsky 2000) and each reverse primer was labelled with one fluorescent dye, FAM or HEX. Microsatellite markers were amplified by polymerase chain reaction (PCR) in a MyCycler thermal cycler (BIO-RAD) using Type-it Microsatellite PCR Kit (Qiagen) following manufacturer's conditions with slight modifications. Specifically, PCR reactions were performed in a 12.5 µl final volume with 15 ng of genomic DNA per reaction. Thermal cycler protocol included an initial DNA polymerase activation step during 5 min at 95 °C, following by 30 cycles of 30 s at 95 °C, 90 s at different annealing temperature (Table 1) and 30 s at 72 °C; and a final extension of 30 min at 60 °C. Microsatellite fragments were sequenced using a 3130xl genetic analyzer (Applied Biosystems) in Servicios de Apoyo a la Investigación (Universidade da Coruña) and analyzed with Genemapper v3.7 software (Applied Biosystems). GenAlEx

Annex 3. Cont.

Table 1 Characterization of 15 microsatellite *loci* introducing *locus* name, EST accession number (EST), repeat motif, primer sequences, annealing temperature (Ta) and allele size range (bp)

Locus	EST	Motif	Primer (5′–3′)	T_a	Range
Pol001	FN246803	GTG	F: GACATGGCGGATATCAAG	57	417–450
			R: GAACTGAATGACGCTGTC		
Pol003	FN246779	AGC	F: AAGAACAAGACTCCCAAGC	57	210–304
			R: GCACGAGTTTCTTCACCT		
Pol004	FN246748	GAC	F: ACGAGTTGCTGGTTGACGAC	57	151–184
			R: GAGAGGGCGCAGCAAAAG		
Pol005	FN246719	CGG	F: ACTGTACGACGCAGGAA	57	182–239
			R: GCTAGTGGTCGCAGGA		
Pol008	FN246665	GGA	F: CGCAAAAGCACGTCTGCCCA	48	516–561
			R: AGGGAGACAGCTCACACACGCA		
Pol011	FN244203	AAG	F: GCGACATCATGGCTGAC	57	484–520
			R: ACCTCCTTGGGCGTGA		
Pol013	FN246659	TCG	F: ACCTTACACAACACTGACTGAG	57	400–403
			R: TGCACGTAATCCAGCTGCA		
Pol014	FN246655	CTG	F: GATGGGTCACACGGTCA	57	237–240
			R: CTTCCCTTCACGCACCT		
Pol019	FN244825	GCC	F: CCCGACCAAATCATCACT	57	505–511
			R: TCTGGAAGACAGTGCTGA		
Pol025	FN247045	ATG	F: GGTGTCTGCCATTGAACAGG	57	226–232
			R: TGCCTCATCATCACTGCCAA		
Pol043	FN244226	GGC	F: GATGATCCGCACGGCTTT	57	276–279
			R: CCTTCTTCTGGTTCGTCTTGA		
Pol044	FN244192	CGA	F: CCGAGAAGTTCAAGACGCCGGA	57	91–136
			R: CTTCAACGCCCGTCTCGTCGAT		
Pol114	FN247251	GCG	F: AGCGGCCCGAGTTTGTCGAGTA	57	154–208
			R: AACTATCTTCTGGCCAGCTCCCCC		
Pol115	FN247250	AGC	F: GCGCCAAGAGATGCACCAGTCT	57	457–460
			R: TGCTCAAAGTCGCTTCCGCTGG		
Pol118	FN245700	CGG	F: TCCGCTCCAGCACATTTCCACG	57	263–287
			R: CGGGCCCGCGTAACACTTTCAA		

(Peakall and Smouse 2012) and FreeNA (Chapuis and Estoup 2007) software were used for estimating population parameters and testing null alleles, respectively.

Microsatellite markers amplified in 16 individuals of four populations of *P. pollicipes* with different amplification success. The number of alleles detected for each *locus* ranged between 2 and 20. Only some of these markers showed deviation of Hardy–Weinberg equilibrium (H–W) after Bonferroni's correction due to deficit of heterozygotes. These H–W deviations are possibly due to high frequencies of null alleles detected in those *loci* and being *loci* Pol014, Pol019, Pol043 and Pol118 which presented of H–W deviation in different populations. Multiple alleles showed by these four *loci* appeared in homozygosis (Ho = 0) despite heterozygosis is significantly different than 0 and their frequencies of null alleles are the highest of all analyzed *loci* (Table 2). The half analyzed microsatellite markers presented private alleles in some of four populations. Cross-specific amplifications of these microsatellite *loci* performed in other three species of Pollicipedidae family

Annex 3. Cont.

Table 2 Statistical assessment of 15 polymorphic microsatellite *loci* in each of the four population including number of alleles presented in each *locus* (NA) and frequency of null alleles in each *locus* (between parentheses), observed heterozigosity (Ho) and expected heterozigosity (He)

Locus	All populations			Cíes Islands		Safi		Brest		Guincho	
	NA	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He
Pol001	5 (0.04862)	0.125	0.148	0.063	0.061	0.000	0.000	0.125	0.119	0.313	0.363
Pol003	20 (0.16378)	0.484	0.737	0.688	0.826	0.438	0.623	0.250	0.619	0.571	0.699
Pol004	4 (0.20993)	0.063	0.300	0.063	0.170	0.125	0.430	0.063	0.463	0.000	0.000
Pol005	13 (0.03630)	0.667	0.785	0.438	0.369	0.929	0.645	0.667	0.727	0.667	0.583
Pol008	6 (0.00000)	0.754	0.603	0.875	0.643	0.429	0.452	0.909	0.657	0.813	0.617
Pol011	7 (0.00001)	0.237	0.220	0.125	0.119	0.267	0.238	0.333	0.300	0.231	0.210
Pol013	2 (0.00001)	0.484	0.446	0.625	0.469	0.250	0.219	0.250	0.492	0.813	0.482
Pol014	2 (0.32145)	0.000*	0.464	0.000*	0.492	0.000*	0.219	0.000*	0.391	0.000*	0.469
Pol019	3 (0.28272)	0.000*	0.348	0.000*	0.430	0.000*	0.227	0.000*	0.219	0.000*	0.430
Pol025	3 (0.00000)	0.403	0.342	0.250	0.219	0.500	0.398	0.500	0.406	0.375	0.320
Pol043	2 (0.30484)	0.016	0.446	0.000	0.000	0.000*	0.375	0.063	0.482	0.000	0.000
Pol044	5 (0.00000)	1.000	0.739	1.000	0.746	1.000	0.576	1.000	0.629	1.000	0.557
Pol114	10 (0.00000)	0.969	0.824	1.000	0.652	0.875	0.760	1.000	0.729	1.000	0.699
Pol115	2 (0.00006)	0.031	0.031	0.063	0.061	0.000	0.000	0.000	0.000	0.063	0.061
Pol118	4 (0.23945)	0.066	0.350	0.063	0.174	0.188	0.451	0.000*	0.556	0.000	0.000

* Significant frequencies after Bonferroni correction

Table 3 Cross-especies amplification in different congeneric barnacles

Locus	*P. elegans*	*P. polymerus*	*P. caboverdensis*
Pol001	–	–	–
Pol003	–	–	–
Pol004	+	+	–
Pol005	+	+	+
Pol008	+	+	–
Pol011	–	–	–
Pol013	–	+	+
Pol014	+	+	+
Pol018	+	+	+
Pol019	+	–	–
Pol025	+	+	+
Pol043	–	+	+
Pol044	+	+	+
Pol114	–	+	+
Pol115	–	+	+
Pol118	–	+	+

showed positive amplification for four to six markers in each other barnacle species (Table 3).

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