Regional Studies in Marine Science
Available online 20 February 2021, 1016881
Accepted:
https://doi.org/10.1016/j.rsma.2021.101688

Mitochondrial DNA analysis reveals gene drift and structuring in the declining European piddock *Pholas dactylus* (L., 1758) confirming high vulnerability.

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Abstract

Pholas dactylus is a historically valuable species with a relevant role in both environmental and biotechnological fields. It has become scarce in Europe due to habitat destruction and human overuse. This species is currently undergoing steep population declines, which have caused local extinction and/or distribution range contraction. Six different localities were sampled between the southern central region of the Bay of Biscay (Spain) and the Black Sea (Bulgaria and Romania) with the aim of describing for the first time its genetic variation patterns and assisting its conservation. Analyses using the mitochondrial Cytochrome Oxidase I gene revealed a high number of unique haplotypes in the Atlantic and Black Sea areas and significant genetic structuring (F_{ST}=0.15495 p<0.001, Φ_{ST}=0.36501 p<0.001). Significant differences were found between the regions since higher haplotype and nucleotide diversities

were found in the Bay of Biscay (D*h*=0.913, π=0.97%) than in the Black Sea (D*h*=0.732, π=0.30%) and three different genetic units were discovered based on significant Φ_{CT} values (western Bay of Biscay, Villaviciosa (the easternmost locality sampled within the Bay of Biscay) and the Black Sea) (Φ_{CT}=0.41076 p<0.05). Globally, it seems that after different origins, gene drift has been acting on the species in its European geographical distribution. Results from this study reinforce the need for more efforts on obtaining data for this species and for a careful protection of its habitats.

Keywords: conservation genetics, population genetics, Bivalvia, mitochondrial DNA, gene drift, threatened species, scientific diving.

1. INTRODUCTION

Pholas dactylus Linnaeus 1758, vernacularly known as the common piddock is a bivalve mollusc belonging to order Myida, superfamily Pholadoidea, which comprises bivalve species with special adaptations for burrowing into soft rock or wood (Figure 1). The *Pholas* genus presents extracellular luminescence and glows bluish-green in the dark; the luminescent glands lie in the syphons and mantle cavity, into which the luminous material is secreted (Wilbur and Yonge, 1968). In Ancient times, Pliny the Elder mentioned the luminescence in the mouths of people who ate *Pholas* and of such importance was this phenomenon that he even declared that the first king of Scotland had won his throne by consuming these clams (Bage, 1904).

Pholas dactylus occurs along the Eastern Atlantic coast from Norway in the north, going through the Iberian Peninsula and Morocco, to Cape Verde Islands in the south, as well as in the Mediterranean and Black Sea (Hill, 2006; Micu, 2007). *Pholas* drills its flask-shaped burrows in soft rocks (limestone, sandstone, chalk, calcarenite, shale, marl, clay) or even peat and waterlogged wood (Arias and Richter, 2012; Gil De Sola et al., 2012) located across the shore from the lowest intertidal (spring low tide) to the lower subtidal down to 10m deep, large colonies being frequently found around 5m deep. Due to its cryptic lifestyle, there is little knowledge about the species. The adult form is sessile and does not have dispersal potential, as it cannot leave the burrow nor can it re-burrow if dislodged, so recruitment by migration of adults is impossible (Pinn et al., 2005; Smith et al., 2011; Arias and Richter, 2012; Gil De Sola et al., 2012). The larvae are planktotrophic, with a larval stage of 45 days before the larva is competent to settle (Knight, 1984).

The drilling activity of *P. dactylus* engineers its own hard substrate habitat, causing an alteration of its physical and spatial structure and thus being considered as an ecosystem engineer species (Jones et al., 1994, 1997; Pinn et al., 2008). The concept of ecosystem engineering explains processes that involve species and their environment, are not directly trophic or competitive and result in the creation, maintenance, or modification of habitats. Ecosystem engineers can be autogenic, modifying the environment through their own physical biostructures (dead or living tissues) or allogenic, modifying the environment through their behaviour and activities (Commito and Rusignuolo, 2000; Norkko et al., 2006; Spooner and Vaughn, 2006), as is *P. dactylus*. *Pholas* activity creates a network of burrows and differentially accelerates erosion, increasing topographic complexity, modifying the availability and accessibility to different resources, which leads to an increase in species abundance and diversity within the habitat. Frequently, the galleries excavated by *P. dactylus* provide shelter

to other species and/or their broods. In addition to its ecological importance, the species has an extensive record at the archaeological and cultural level (Lovell, 1884; Pinn et al., 2005; Gutierrez Zugasti, 2009), as well as possible applications as a bioindicator species and as source of the protein pholasin, which can be used as a probe of oxygen free radicals in living cells (Nourooz-Zadeh et al., 2006).

Historically, *P. dactylus* had a wide distribution range, but it has become rare in Europe recently due to the destruction and pollution of its habitat along with overexploitation by humans for food and fish bait (Michelson, 1978; Pinn et al., 2008; Arias and Richter, 2012). The species is highly protected nowadays and it is included in Annex II of the Convention on the Conservation of European Wildlife and Natural Habitats (Berne Convention) and in Annex II of the Protocol on Special Protection Areas and Biological Diversity of the Mediterranean of the Barcelona Convention (Ministerio de Medio Ambiente y Medio Rural y Marino, 2011).

In the Black Sea the most significant pressure has been chemical pollution and especially eutrophication as a result of nutrient enrichment (N, P and organic matter), most acutely experienced in 1970-1980s in the north-western Black Sea where there is high riverine input. *Pholas dactylus* cannot survive in anoxic or hypoxic conditions caused by eutrophication. Since the 1990s this pressure has been reduced due to tighter controls on pollution in the catchment of the Danube and other rivers which enter the north-western Black Sea. Whilst this pressure is now reduced, it is still posing a threat especially for non-EU countries surrounding the Black Sea, which are not bound by agreements like the Water Framework Directive (WFD). Basin-wide decline of the habitat at present is due to beam-trawling and coastal protection works, causing habitat destruction, smothering and siltation. It is recognized as rare and protected in Ukraine and Romania (Anistratenko, 1999; Micu, 2007), and although not mentioned in the

Black Sea Red Data Book (Dumont, 1999) it was subsequently included in the inventory of aquatic and semi-aquatic Red List species, endangered in at least one country around the Black Sea in Annex 5 of the Black Sea Transboundary Analysis (Black Sea Economic Recovery Project, 2007). As ecosystem engineers, *Pholas dactylus* and *Barnea candida* create in the Black Sea a specific EUNIS level 5 habitat "A3.3 Infralittoral soft rock with Pholadidae". This habitat type was assessed against the IUCN criteria as Endangered for the Black Sea region, within the project "Establishment of a European IUCN Red List of Habitats"(Gubbay et al., 2016). In the Bay of Biscay, again anthropogenic pressure has also been identified as the main cause of the *Pholas* disappearances (Arias and Richter, 2012). Despite this, a progressive increase of environmental protection at extended areas in Asturias as Sites of Community Importance (SCIs) and Special Protection Areas for birds (SPAs) under the Habitats (92/43/CE) and Birds (2009/147/CE) Directives have been taking place in the last years.

The main role of genetic approaches in the management and conservation of marine invertebrates is the identification of species and groups of individuals belonging to differentiated, disconnected genetic stocks, providing indirect measures of connectivity (Thorpe et al., 2000). Connectivity among populations shapes the genetic structure of species and determines the dynamics of metapopulation systems, how genetic diversity arises and is maintained within species, and the adaptability and resilience of populations to human pressures and environmental changes (Botsford et al., 2001), being crucial for an effective management of biological resources. Understanding the distribution of genetic variability is key for environmental resources management and conservation biology of marine species (Moritz, 1994; Palumbi, 2003; Cowen et al., 2006). Population connectivity plays a crucial role in local and metapopulation dynamics, genetic structure and population resilience, e.g., in response to human exploitation (Hastings and Harrison, 1994; Cowen et al., 2007; Weersing

and Toonen, 2009; Puckett and Eggleston, 2012). Defining connectivity patterns for marine organisms is a challenging task since factors that affect connectivity (life history traits, habitat, hydrological regime, occurrence of geological/topographical boundaries, layout of coastline, etc.) act at very different geographic and temporal scales (Villamor et al., 2014). Most marine species release planktonic larvae which disperse over days up to months with the currents and thereby constitute the primary source of the dispersal capacity (Mileikowsky, 1971; Ward et al., 1994; Gilg and Hilbish, 2003). Direct labelling and tracking of larvae is seldom feasible, so genetic data are widely used for the indirect inference of population connectivity (Hellberg et al., 2002; Thorrold et al., 2002; Palumbi, 2003; Broquet and Petit, 2009; Cowen and Sponaugle, 2009; Lowe and Allendorf, 2010).

High levels of genetic differentiation have been often found in marine invertebrates, remarkably in corals and sponges, in which case may be related to their common biological characteristics like sessile life, great evolutionary age, limited ability to disperse and low homoeostatic capability (Solé-Cava and Thorpe, 1991). Previous research has shown that there is commonly an inverse relation between genetic connectivity among separated populations of a certain species and the extent to which said geographically separated populations have diverged (Burton and Feldman, 1982). It appears that species whose populations can maintain genetic exchanges via long-ranging propagules (eggs, seeds, planktotrophic larvae, adults) show little population differentiation over very large distances. Populations of species with low dispersal capacity, fragmented distribution and small stocks are much more vulnerable to overfishing or environmental changes (Thorpe et al., 2000). An additional difficulty is the high incidence of cryptic speciation in marine invertebrates, even in commercially important and comparatively well-studied species (Thorpe et al., 2000; Pogson, 2016).

The Cytochrome Oxidase subunit I (COI) gene has been very useful in population structure and phylogenetic studies since maternal inheritance, high copy number, relatively rapid mutation rate, and lack of recombination are all advantageous features for these type of studies (Palumbi, 2003). The COI gene has been used to properly identify species and therefore to reveal possible cases of cryptic speciation (Hebert et al., 2003; Szuster-ciesielska and Tustanowska-stachura, 2003; Plazzi and Passamonti, 2010; Jose and Mahadevan, 2016; Miralles et al., 2016). Moreover, COI has been demonstrated as a useful genetic marker to obtain information related to populations' genetic structure in many marine invertebrates (Calderón and Turon, 2010; Campo et al., 2010; Muñoz-Colmenero et al., 2015; Fourdrilis et al., 2016; Deli et al., 2017). It has been argued that to fully gauge haplotype variation at the species level, an strongly taxon-specific approach is necessary although typical sample sizes for molecular biodiversity assessment using DNA barcodes (COI) range from 5 to 10 individuals per species (Phillips et al., 2019). When working on endangered populations, the samples are usually difficult to obtain but genetic diversity, even if sample sizes are less than ideal, is still a relevant data (Pruett & Winker, 2008).

Processes related to dispersion, which ultimately determines patterns of connectivity, are highly linked to the biology and ecology of each species, but they are also contingent on the evolutionary history of the group and the geological history of the inhabited area. More taxon-specific analyses are therefore needed to better understand how dispersion and connectivity of marine species are shaped through time and geographic space, and their evolutionary and ecological consequences. Currently, there is not available genetic data about patterns of genetic variation in *P. dactylus*, although the species is already protected. It seems essential to provide more scientific support for the establishment of effective measures and conservation policies for the sustainable management of this species in its different

distribution areas. In this work, we study the spatial genetic variation patterns for *P. dactylus* in two disjunct areas of its distribution range (Bay of Biscay and Black Sea) to characterize populations and to define units of management/conservation.

2. MATERIALS AND METHODS

2.1. Sample collection and biometric analysis

Based on previous studies about the distribution of *P. dactylus*, four localities from the southern area of the Bay of Biscay and two from the Western Black Sea were selected: Tapia de Casariego, Zeluán, Peñarrubia and Villaviciosa in the Asturias coast (Perez, 2003; Arias and Richter, 2012) Costinești in Romania (Micu, 2018) and Byala in Bulgaria (Gubbay et al., 2016) (Figure 2). Samplings were authorized by competent authorities (i.e.: General Administrations of Maritime Fishing of the Principality of Asturias). Some of these sampling sites fall within the limits of protected sites under the Natura 2000 European Ecological Network (Habitats and Birds Directives): Villaviciosa is a Site of Community Importance (SCI) in Asturias and Zeluán is Special Protection Area for birds (SPA), while in the Black Sea Costinești is within both a marine SCI and a larger SPA.

In each locality, characteristic drilled holes indicative of the presence of *P. dactylus* were found. The burrows containing living piddocks were excavated around, using hammer and chisel to extract the individuals. In all the sampling places, the rocks in which the individuals were found were relatively soft and fragile, either due to its composition or the conditions in which it was found. In Tapia, it was a very weathered Cambro-Ordovician quartzite. The substrate present in Zeluán was Triassic shale, a grain-sized rock very delicate; the red colour being due to a high iron content. The Peñarrubia substrate was a Jurassic limestone formed by calcite mineral, one of the forms of calcium carbonate, probably of biogenic origin and fine

grain size. Finally, the Villaviciosa sample corresponds to a very weathered shale with a lower iron content than that of Zeluán. However, it is difficult to specify the rock type and age since it is coming from an artificial seawall built before 2001. In Costinești the substrate was paleokarstic Sarmatian limestone with Quaternary inclusions of hard red clay with gypsum, with *Pholas* drilling its burrows into these inclusions (Micu, 2018). In Byala the *Pholas* burrows occurred in light gray marly limestones and gray calcareous marls of the Cretaceous–Tertiary (K/T) boundary with high cosmogenic Cr and Fe content associated with the K/T Iridium anomaly (Stoykova et al., 2000; Kostov et al., 2013).

The samples were immediately fixed 96% in ethanol. Each sample was labelled and measured in the Natural Resources genetics lab from the University of Oviedo. Measurements were made of maximum length and width of the shell, thickness of the individuals and length of the third valve in dorsal position (the latter served to verify that it existed a constant relationship with the length of the other valves) using a conventional calliper (Figure 1). Later, the clams were dissected and stored in an individual container filled with 96% alcohol at room temperature. The Black Sea samples were prepared in the same way and sent to Oviedo stored in containers with 96% ethanol. As they were collected on a stormy day, most of the samples had very damaged or missing shells, and so measurements were not taken.

2.2. DNA extraction and Cytochrome Oxidase I (COI) amplification

A portion of muscle was taken from each individual's foot from which genomic DNA was extracted using the E.Z.N.A Mollusc DNA Kit (Omega Bio-tek, Norcross, USA) following the instructions from the manufacturer. Once the DNA was extracted, it was stored at -20°C until its later use.

The amplification of Cytochrome Oxidase I (COI) gene was conducted by PCR using the primers jgHCO2198 and jgLCO1490 (Geller et al., 2013). A total PCR volume of 40 µl was used as follows: primers at 1µM each, MgCl2 at 2.5mM, dNTPs at 250µM, Green GoTaq ® Flexi Buffer (Promega Corporation, Wisconsin, USA) at 1x and GoTaq G2 Flexi Polymerase (Promega Corporation, Wisconsin, USA) at 0.03 U/µl. A Verity Blue thermocycler (Applied Biosystems, California, USA) was used, carrying out a denaturation phase at 95 °C for 1 minute, followed by the hybridization phase at 49 °C for 1 minute and finally the extension phase at 72 °C for 1 minute. In total 35 cycles were made, with a final extension phase of 5 minutes at 72 °C. The PCR products were visualized using electrophoresis on a 2% agarose gel stained with SimplySafe TM (EURx, Gdańsk, Poland). The samples were sent to Macrogen Spain to be sequenced using Sanger's method (Sanger et al., 1977).

2.3. Genetic variation analyses

BioEdit 7.0.5.3 (Hall, 2001) was used to visually check all the sequences and manually edit them when necessary. Subsequently, BLAST was used (Hall, 2001) to verify that the genetic identity of the samples corresponded to *P. dactylus*, considering 98% identity as cut-off limit (Madden, 2013). The MUSCLE algorithm (Edgar, 2004) was used to align the sequences. Finally, the program DnaSP 6.11.1 (Rozas et al., 2016) allowed to obtain diversity data.

The Network 5 program was used for obtaining a haplotype network using the median-joining model (Bandelt and Peter Forster, 1999; Fluxus Technology Ltd., 2015). The Arlequin 3.5 software (Excoffier, 2010), was used to study population parameters based on genetic data. Comparisons between localities were made using the pairwise fixation index (F_{ST}), which analyses the differences in haplotype frequencies, and the Φ_{ST} index, which, in addition to haplotype frequencies, also considers the molecular differences between samples. AMOVA

tests were performed using both indexes to determine molecular differences within the localities, among them, and between the groups of populations that were defined. This software also provided information about our samples' past demography and its current dynamics through neutrality tests such as Tajima's D (Tajima, 1989) and Fu's F (Fu and Li, 1993). These last tests were only performed for the regions where a larger population size (N) could give useful preliminary information about these parameters (Domingues et al., 2007).

Using both MEGA X (Koichiro et al., 2011) and Jmodeltest2 (Guindon and Gascuel, 2003; Darriba et al., 2012) we predicted the nucleotide substitution model followed by the samples as they evolved over time. The model was then used to draw a Bayesian Skyline tree using the software BEAST (Bayesian Evolutionary Analysis Sampling Trees) (Suchard et al., 2018). Its complementary software Tracer was used to do the Skyline reconstruction analysis (Drummond et al., 2011), which aims to give an estimate of the effective population size through time. We used a generation time of 12 years (*P. dactylus* average lifespan is 14 years and the larvae need up to two years to reach adulthood and be able to produce offspring), mitochondrial mutation rate as $5.3x10^{-8}$ per site per year (taken from the bivalve *Anadara tuberculosa*, (Diringer et al., 2019)).

2.4. Statistical analysis

To carry out statistical analyses (normality tests, comparisons of means and analysis of distributions) (Kruskal and Wallis, 1952; Shapiro and Wilk, 1965) the Excel 2016 (Microsoft, 2016) and RCommander 2.4 (Fox, 2005, 2016) programs were used.

3. RESULTS

Thirty two samples were obtained from the southern central area of the Bay of Biscay (13 from Peñarrubia, 5 from Tapia, 7 from Villaviciosa and 7 from Zeluán) and twenty seven individuals were collected from the Black Sea region (15 from Costinești and 12 from Byala (Figure 2, Table 1)). The *P. dactylus* samples from Asturias cover a wide range of sizes (30.5 - 99.7 mm in length), the largest individuals being those from Zeluán (mean value=82.4 ± 17.8 mm) and the smallest ones those from Peñarrubia (44.2 ± 9.4 mm) (Table 1). The rest of morphometric measurements conducted in this work, width and length of the third valve, were correlated with the length (R = 0.912 p value = 3.36E-13, R = 0.758 p value = 3.95E-10, respectively) and therefore do not contribute with new information.

After sequencing and editing, an alignment of a consensus fragment of 518 base pairs in size was obtained for 59 samples. The n-BLAST tool from the NCBI was employed to confirm species genetic identity of each haplotype. Sample P06 was identified as *Barnea candida* with an identity of >98%. The rest of the sequences were identified as *P. dactylus* with more than 99% of identity, except in the case of the sample P07, from which a low quality sequence (an identity of less than 98%) was obtained, and was consequently discarded and not used in further analysis.

Nineteen different haplotypes were identified in the Bay of Biscay samples (GenBank accession numbers MN623228-MN623246), resulting from 22 variable sites. The sequences presented six singletons (unique mutations present in only one individual). The haplotype diversity (D*h*) in the Asturias samples was 0.913, and the nucleotide diversity (π) was 0.97% (Table 1). The two protected sites Villaviciosa (SCI) and Zeluán (SPA) showed higher levels of haplotype diversity (Table 1, Figure 3). In the Black Sea samples, the number of haplotypes

was lower (6 haplotypes from 7 variable sites, GenBank accession numbers MT157397-MT157402). As such, the haplotype and nucleotide diversities were lower (D*h*=0.732, π=0.30%) and significantly different when compared with the Bay of Biscay samples using a Welch two sample T-test ($p<0.05$). Also in the Black Sea we saw no correlation of haplotype and nucleotide diversities with environmental protection, as the levels at the protected site Costinești (RO) (D*h*=0.629, π=0.26%) were actually lower than at the unprotected site Byala (BG) (D*h*=0.818, π=0.34%) (Table 1, Figure 3).

A global pattern of significant genetic structuring was found in this work (F_{ST}=0.15495 $p<0.001$, Φ_{ST}=0.36501 $p<0.001$). No significant genetic differentiation was found within the Bay of Biscay, or within the Black sea localities, using either F_{ST} or Φ_{ST} (after Bonferroni corrections) (Figure 4). However, significant differentiation among the Black Sea and Bay of Biscay localities was found in all the pairwise comparisons ($p<0.0023$) (Figure 4). A three-group structure was found as the most probable supra-population structuring when using AMOVA tests based on both distance-based and allele/haplotype-based metrics (western of the Bay of Biscay (Tapia, Zeluán, Peñarrubia); Villaviciosa (the easternmost locality sampled within the Bay of Biscay) and the Black Sea) (Φ_{SC}=0.0.0377 $p>0.05$ and Φ_{CT}=0.41076 $p<0.05$).

Haplotypes analyses using Network revealed BSeaPdR07 in the Black Sea and AstPd04 haplotypes in Bay of Biscay as the most common haplotypes by areas (Figure 5). The Bay of Biscay AstPd04 haplotype is similar to the UK reference KX713491.1, while there were no shared haplotypes between the Bay of Biscay and Black Sea coasts (Figure 5). Furthermore, the samples from Villaviciosa do not present any of the most common haplotypes and instead possess a multitude of different and less frequent haplotypes (Figure 5). The Bay of Biscay haplotypes distribution showed a star-like pattern typical from population expansion

processes whereas the Black sea samples revealed a more reticulate pattern with a few main representative haplotypes shared between the Bulgarian and Romanian samples suggesting a more stable population (Figure 5). A central area of the network diagram showed some connection among Bay of Biscay, Black Sea and the reference haplotypes (e.g: AY070141.1, also previously reported with a UK origin) (Figure 5). The Bayesian Skyline tree was done for the all the samples globally due to low number of samples by localities (Figure 6A). All the Black Sea haplotypes appeared clustered together away from all the others, except BSeaPdB08, that clusters with the Atlantic AstPdP08-AY070141.1 haplotype (Figure 6A). The Skyline plot (Figure 6B) shows a sudden reduction in the past by four degrees of magnitude on the effective population size of the species as a whole, followed by a quick recover. The Tajima D (-1.689, p= 0.0213) and the Fu statistics (-8.598, p= 0.0012), were negative and significant for Asturias which could be suggesting a recent selective sweep, population expansion after a recent bottleneck and/or genetic hitchhiking. Meanwhile, in the Black Sea these statistics were not significant (D=-0.568, p=0.3186; F=-0.652, p=0.3560), which suggests an absence of selective processes.

4. DISCUSSION

Useful information on past and recent demographics of a species and its populations can be inferred and interpreted from genetic data. Sea-level changes in the Pleistocene of Europe often led to the fragmentation of marine populations, creating a dynamic of spatial and demographic expansion and contraction over time (Provan and Bennett, 2008). In the last decades, cases of human-related range expansion have increasingly been reported (Rogers and Harpending, 1992; Grant and Bowen, 1998) and the natural patterns of biodiversity have been altered by artificial translocation (Carlton and Geller, 1993; Ruiz et al., 2000; Molnar et

al., 2008), making it difficult to decipher both past demographic history and contemporary genetic structure of marine species. Here, mitochondrial genetic data for the species *P. dactylus* is reported for the first time for two areas of its geographical distribution.

High values of haplotype diversity (>50%) and nucleotide diversity (>0.5%) have been detected in the Asturias samples, in the southern central Bay of Biscay, which could suggest a large or stable population with a long evolutionary history, as well as a possible secondary contact between differentiated lineages (Grant and Bowen, 1998). It has been claimed, when studying other marine invertebrates such as stalked barnacles, that the southern area of the Bay of Biscay was probably one of the glacial refuges from which norwestern Atlantic species recolonized non-available areas (Campo et al., 2010). We found common genetic variants in the Atlantic since Asturias and UK (where only two haplotypes were previously described) shared haplotypes. Some of the Bay of Biscay samples in study (Zeluán and Peñarrubia and also Villaviciosa) are only a few kms away of the two largest Asturian shipping harbours, Gijon and Aviles with high maritime traffic with different Atlantic and Mediterranean areas (Miralles et al., 2018). It is possible that some propagules from distant locations could be arriving via shipping. In any case, genetic data rejects the existence of a single population or panmictic genetic stock, and therefore a single management unit, although the use of greater sample sizes and perhaps nuclear variable markers could be advisable to confirm this results.

In the Bay of Biscay, the most frequent haplotypes are shared in Tapia, Zeluán and Peñarrubia, and not in Villaviciosa. Despite this, an 84% of the haplotypes found are specific or unique for each Asturias locality although most of them appeared in very low frequencies. The data suggests Villaviciosa samples as a “peculiar unit”. It might be the result of some posterior colonization event from another location of the Bay of Biscay/Atlantic area. This last scenario

353 is supported by the planktonic lifestyle of *P. dactylus* larvae, the greater age of the samples in
354 other locations (as inferred by larger sizes), and the fact that the Villaviciosa seawall where
355 the samples were found is a relatively recent artificial construction finished in 1930 (Morales
356 Mato, 1987) and with recent restorations prior to 2001. Moreover, the Cantabrian coast is an
357 area of special biogeographical interest because the existence of a marked longitudinal
358 gradient related to the sea surface temperature (SST) that results in colder areas to the west
359 of the Cape Peñas (Asturias) (to Galicia) than to the east (to Basque Country and where we
360 found Villaviciosa) (Anadon et al., 2014). This has been associated to significant changes in the
361 marine species distributions (Anadon et al., 2014; Muñoz-Colmenero et al. 2015, Semeraro et
362 al., 2016).

363 Preliminary results obtained with the tests for the Tajima D and Fu's F statistics seem to
364 suggest the possibility of the two regions evolving in different ways although low samples sizes
365 invite to be cautious in these statements. In the case of the Bay of Biscay, the indexes pointed
366 out to an expanding population after a bottleneck event and now under the effects of genetic
367 drift (Tajima, 1989; Chiu et al., 2013). Lately, extensive areas of the Asturias coast, including
368 some of our sampling sites, have been designated as SCIs and SPAs under the Habitats
369 (92/43/CE) and Birds (2009/147/CE) Directives. In some way, this could be favouring survival
370 and recovery of the *P. dactylus* populations.

371 A single management unit has been detected within the Black Sea (Bulgaria and Romania).
372 Haplotypes are shared by most of the samples from both populations, and neither F_{ST} nor Φ_{ST}
373 values revealed significant genetic differences between the two Black Sea populations in
374 Costinești (RO) and Byala (BG). However, the Black sea samples show significant lower levels
375 of genetic variation, with high D*h* (> 50%) and low π (< 50%), which may suggest bottleneck

events followed by rapid population growth and accumulation of mutations within the area (Grant and Bowen, 1998). During the late Pliocene-early Quaternary the co-evolution of the Mediterranean and Black seas is dominated by major changes in water (lake and sea) levels resulting in a pulsating system of connected and isolated basins. The Black Sea achieved its current marine status only *ca.* 7,000 years ago (Krijgsman et al., 2019). Before its post-glacial flooding with marine water at the onset of the Chernomorian, it was ascertained that the Black Sea, together with the Marmara Sea, was a lacustrine basin (Neoeuxinian sea-lake stage) completely secluded from the Mediterranean Sea (Büyükmeriç, 2016; Krijgsman et al., 2019). This fact rules out any chance that any marine refugia existed (even if euryhaline) within the Black Sea during the last glaciation, a theoretical claim put forth for other areas but not applicable to the Black Sea. The settling of the modern Black Sea by *P. dactylus* is thus a geologically young phenomenon that took place less than 7,000 years ago, from the Aegean Sea *via* the Marmara Sea. A similar post-glacial timing of Black Sea colonization, facilitated by the reopening of the connection between the Black Sea and the Mediterranean Sea about 7000 years ago, has been recently confirmed for the mussel *Mytilus galloprovincialis* and the black scorpionfish *Scorpaena porcus* (Boissin et al., 2016; Paterno et al., 2019). In spite of the present-day connection of the Black Sea with the ocean via the Mediterranean, genetic diversity is always noticeably lower in the Black Sea, even for highly mobile species of migratory fish (Dudu et al., 2008; Wilson and Eigenmann Veraguth, 2010), a notion which is reinforced by our results.

The vulnerability of the sessile adults to episodic events (catastrophic erosion by storms, smothering by sediments) and the chronic erosion of the already limited available substrate suggests that this species must rely heavily on good regional connectivity among populations, to attain any level of sustainability and resilience. This implies dependence on an influx of

propagules from more or less distant source populations. Taking into consideration the maximum pelagic larval duration (PLD) of 45 days for the planktotrophic larva of *P. dactylus* (Knight, 1984) and the known distribution of the species in the Black Sea, the only viable sources of larvae for the Costinești (RO) and Byala (BG) populations are each other, plus the populations on the western coast of Crimea (Tarhankut to Sevastopol) (Micu, 2018). Long range transport across the open sea by the Black Sea Rim Current and medium cyclonic gyres (Sevastopol Gyre, Kaliakra Gyre) is possible as shown by trajectories of Lagrangian drifters. A full round trip around the Black Sea takes 3 to 6 months, the distance between Sevastopol and Cape Kaliakra taking less than 30 days, thus within the PLD of 45 days for the planktotrophic larva of *Pholas dactylus* (Christensen et al., 2015). A recent study on genetic and physical connectivity of the seagrass *Zostera noltei* (Jahnke et al., 2016) has demonstrated that rare long-distance dispersal is possible in the Black Sea. Dispersal alongshore by coastal currents is more beneficial to the larva; at it may find and use patches of suitable habitat as stepping stones, thus allowing for a more frequent success of a staged dispersion over a wider range. Modelling of dispersion by coastal currents in the Black Sea, for a PLD of 50 days and release in May (*Pholas* reproduces in spring and autumn, at 19°C) for the years 1993, 2001 and 2005 (Christensen et al., 2015) shows that connectivity between Costinești (RO) and Byala (BG) populations is indeed supported, although it does not happen every year. This agrees well with our genetic findings from the present study which suggest very brief isolation or a weak restriction to gene flow.

In the marine environment, historical and evolutionary processes as well as biological, physical and ecological factors strongly contribute in shaping species distribution at large biogeographical scale and thus determine different, species-specific connectivity patterns. Indeed, from a marine conservation perspective, the implementation of networks of MPAs

should rely on the knowledge of connectivity patterns of a representative panel of species, with a variety of life history traits living in the selected areas (Melià et al., 2016). There are no shared haplotypes between the populations of the Black Sea and Bay of Biscay although we detected a Black Sea haplotype (BSeaPdB08) genetically proximate to Atlantic haplotypes. We must consider that probably there could be isolation by distance (IBD) in *P. dactylus*, which may explain the genetic distances between the samples of the Bay of Biscay and the Black Sea. IBD has previously been reported between Atlantic and Mediterranean populations (Domingues et al., 2007; Patarnello et al., 2007; Castilho et al., 2017), between populations in several regions of the Mediterranean (De Matthaeis et al., 2000; Lo Brutto et al., 2013), and between Mediterranean and Black Sea populations (Durand et al., 2013). Samplings in intermediate locations would be needed to assess many more relevant features in *P. dactylus* as it has been done previously for other marine invertebrate species (e.g.: Frattini et al. 2016).

The COI genetic marker has been of great utility in this work as a successful barcoding tool but it has also been able to show (with a limited number of samples), a predictable, but so far never studied, genetic structuring pattern that exist within the geographical distribution area of *P. dactylus*. The COI marker is not perfect and probably is an unsuitable marker for the study of recent historical events (Hurst and Jiggins, 2005; Phillips et al. 2019) or can be affected by non-neutral evolution (but see Berry, 2006). In any case, first data being reported in this work already suggests the idea of different origins and probably significant gene drift events on the species in its European geographical distribution. This may lead to local extinction and/or distribution range contraction of this species. To increase sampling intensity and geographical coverage and to develop new genetic tools for this species seem to be mandatory for the case of the endangered, protected and poorly studied *P. dactylus*. The use of nuclear genetic markers with higher mutation rates such as microsatellites or SNPs, would provide clues about

most recent evolution processes for the species and help to understand its demography which therefore can help us in our understanding of past, recent and future environmental history of the marine realms. Despite this, large samples sizes are always needed to capture without bias genetic diversity when using highly variable nuclear genetic tools (minimum of 20-30 individuals by population) (Pruett and Winker, 2008; Flesch et al., 2018; Sunde et al., 2020) and this is not always available. That means that mitochondrial DNA could still be a useful resource to fully unveil *P. dactylus*'s.

5. FUNDING

This work was funded through a grant from the Asturias Government to Research Groups FC-GRUPIN-IDI/2018/000201. The present study is a contribution of the Biotechnology Institute of Asturias (IUBA) and Marine Observatory of Asturias (OMA).

6. COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. ACKNOWLEDGEMENTS

The authors would like to thanks to the General Directorate of Marine Fisheries in the Principality of Asturias for providing us with sampling permits. Irene Fernandez (Zoology, UNIOVI) helped us with the Villaviciosa sampling.

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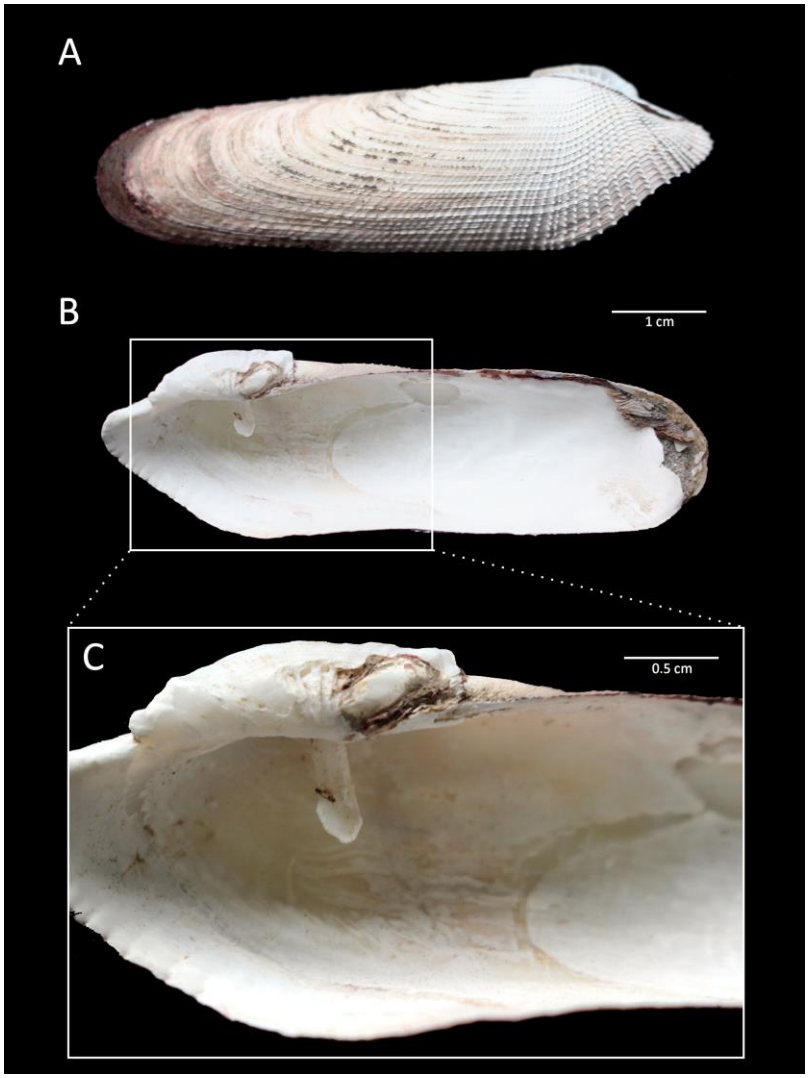
Tables

Table 1. Genetic data from the *P. dactylus* samples from southern central area of the Bay of Biscay and Black Sea by localities. N= number of samples, Nh= number of haplotypes, Nhs= number of unique haplotypes, Dh= haplotype diversity, π= nucleotide diversity.

Region	Locality	Coordinates	Code	N	Size range (mm)	Nh	Nhs	Dh (±SD)	π (±SD)
Bay of Biscay	Tapia	43º 34' N, 6º 56' W	T	5	47.50-68.80	6	2	0.900 (±0.161)	0.01100 (±0.00380)
	Zeluán	43º 35' N, 5º 55' W	Z	6	50.55-99.77	6	5	1.000 (±0.096)	0.01399 (±0.00363)
	Peñarrubia	43º 33' N, 5º 37' W	P	12	30.50-63.10	6	3	0.758 (±0.122)	0.00667 (±0.00175)
	Villaviciosa	43º 31' N, 5º 23' W	V	7	32.60-63.55	7	6	1.000 (±0.076)	0.00912 (±0.00133)
	Asturias	-	-	*30*	*30.5-99.77*	*19*	*19*	*0.913 (±0.040)*	*0.00967 (±0.00145)*
Black Sea	Byala (BG)	42º 52' N, 27º 53' E	B	12	-	5	1	0.818 (±0.070)	0.00343 (±0.00087)
	Costinești (RO)	43º 57' N, 28º 38' E	R	15	-	5	1	0.629 (±0.125)	0.00264 (±0.00077)
	Black Sea	-	-	*27*	-	*6*	*6*	*0.732 (±0.061)*	*0.00301 (±0.00054)*
Global values		-	-	*57*	-	*25*	-	*0.919 (±0.020)*	*0.00898 (±0.00084)*

773 # Figures



774

775 Figure 1. Shell of *Pholas dactylus*. A) External view of right valve; B) Internal view of right valve;
776 C) enlarged view of the same showing the flattened spoon-shaped process (apophysis).

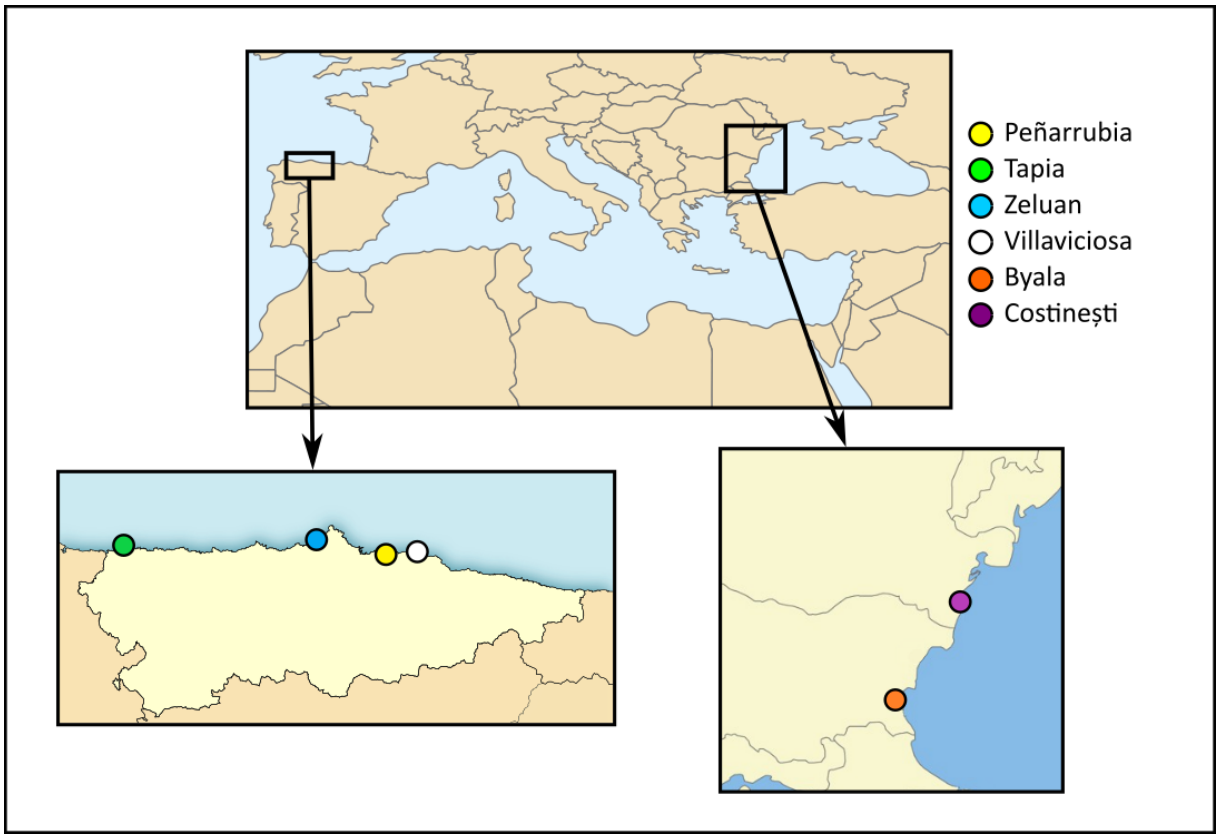
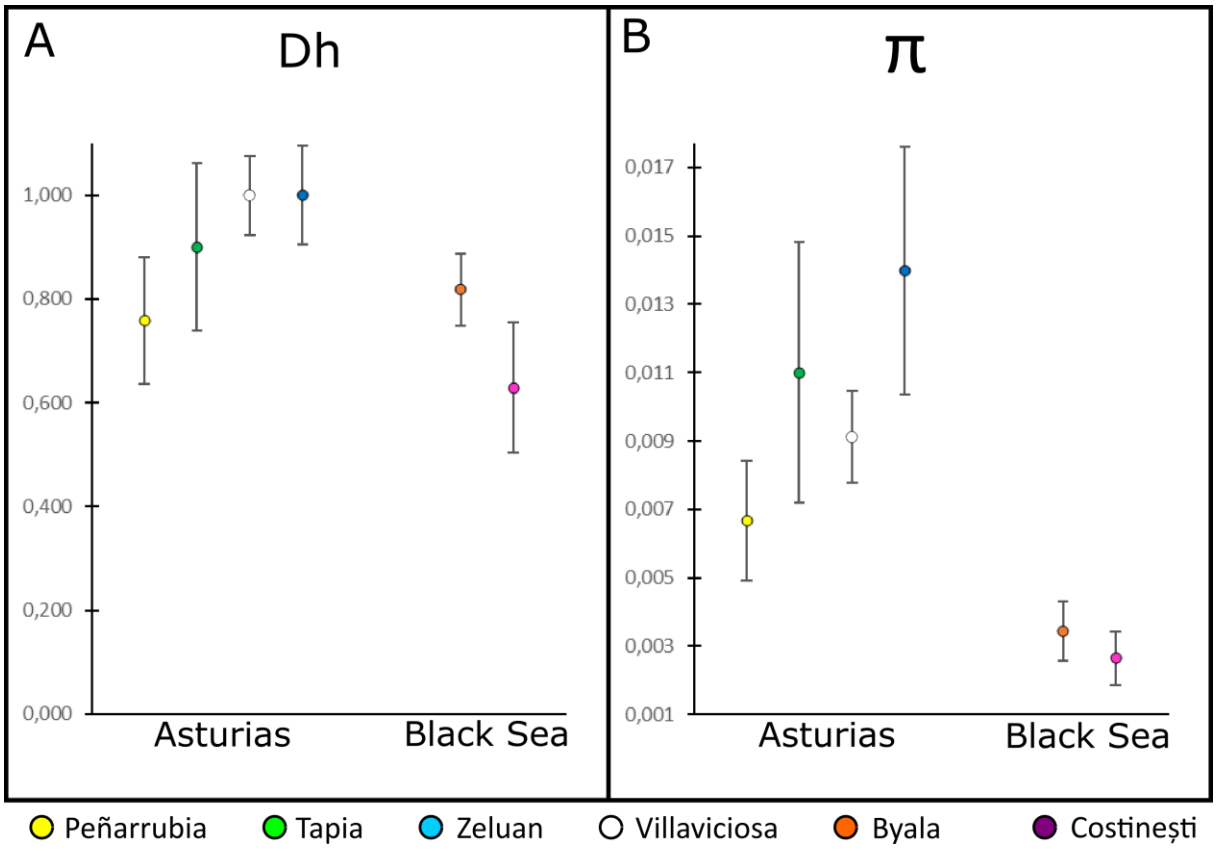


Figure 2. Location of sampling sites for the species *P. dactylus* in Asturias, southern central area of the Bay of Biscay, and the Black Sea.



780

781 Figure 3. Haplotype (D*h*) and nucleotide (π) diversities from the *P. dactylus* samples from the

782 southern central area of the Bay of Biscay and the Black Sea.

783

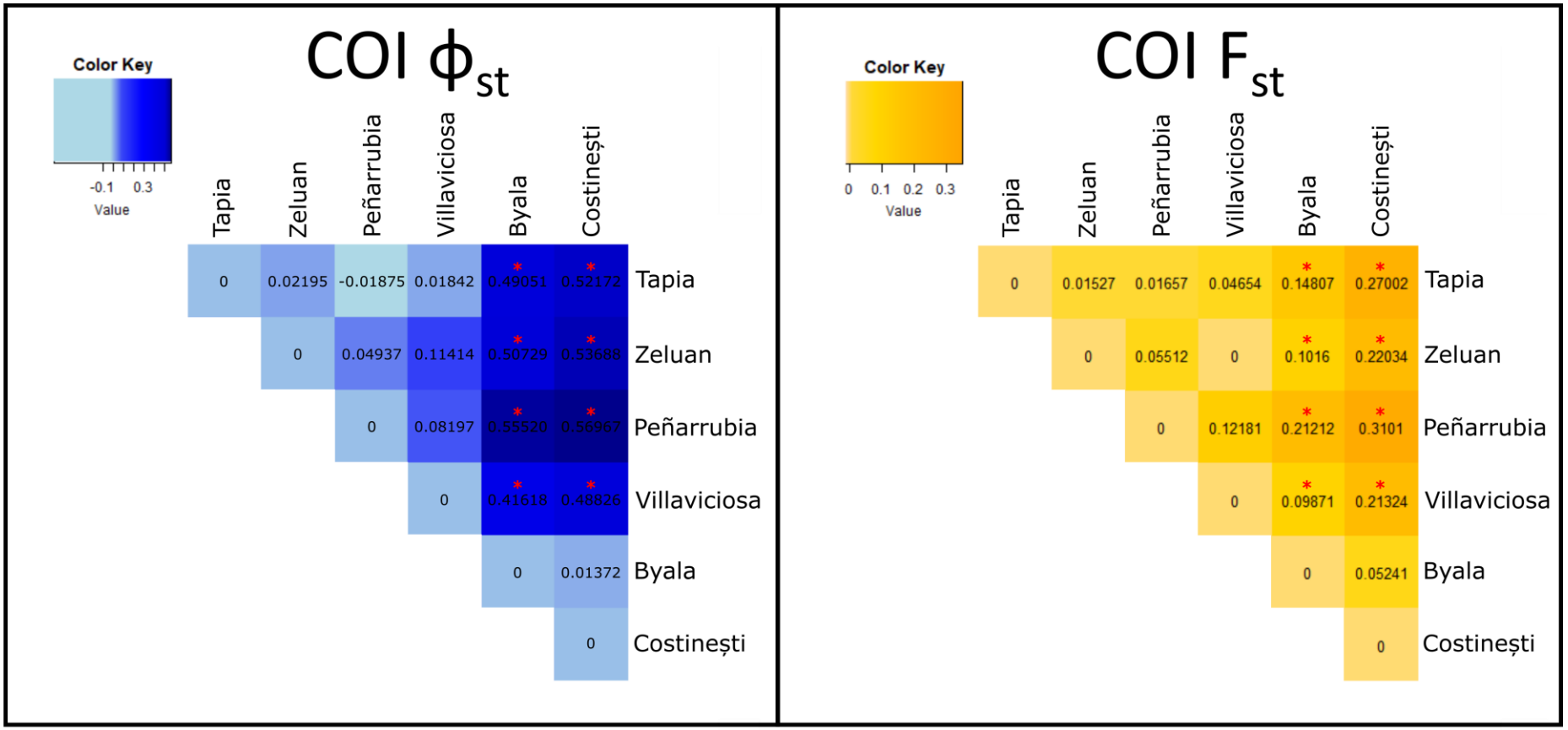
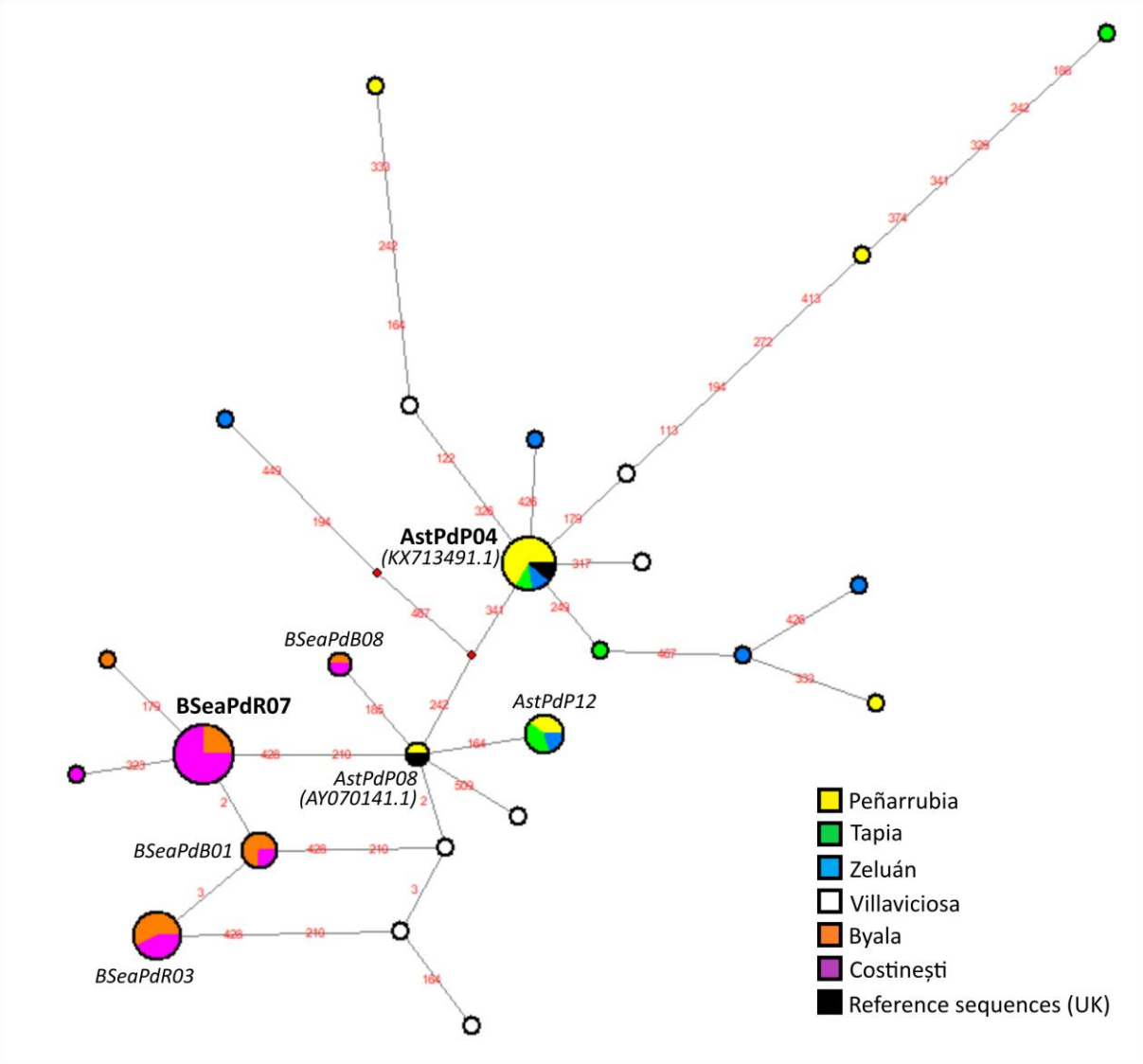
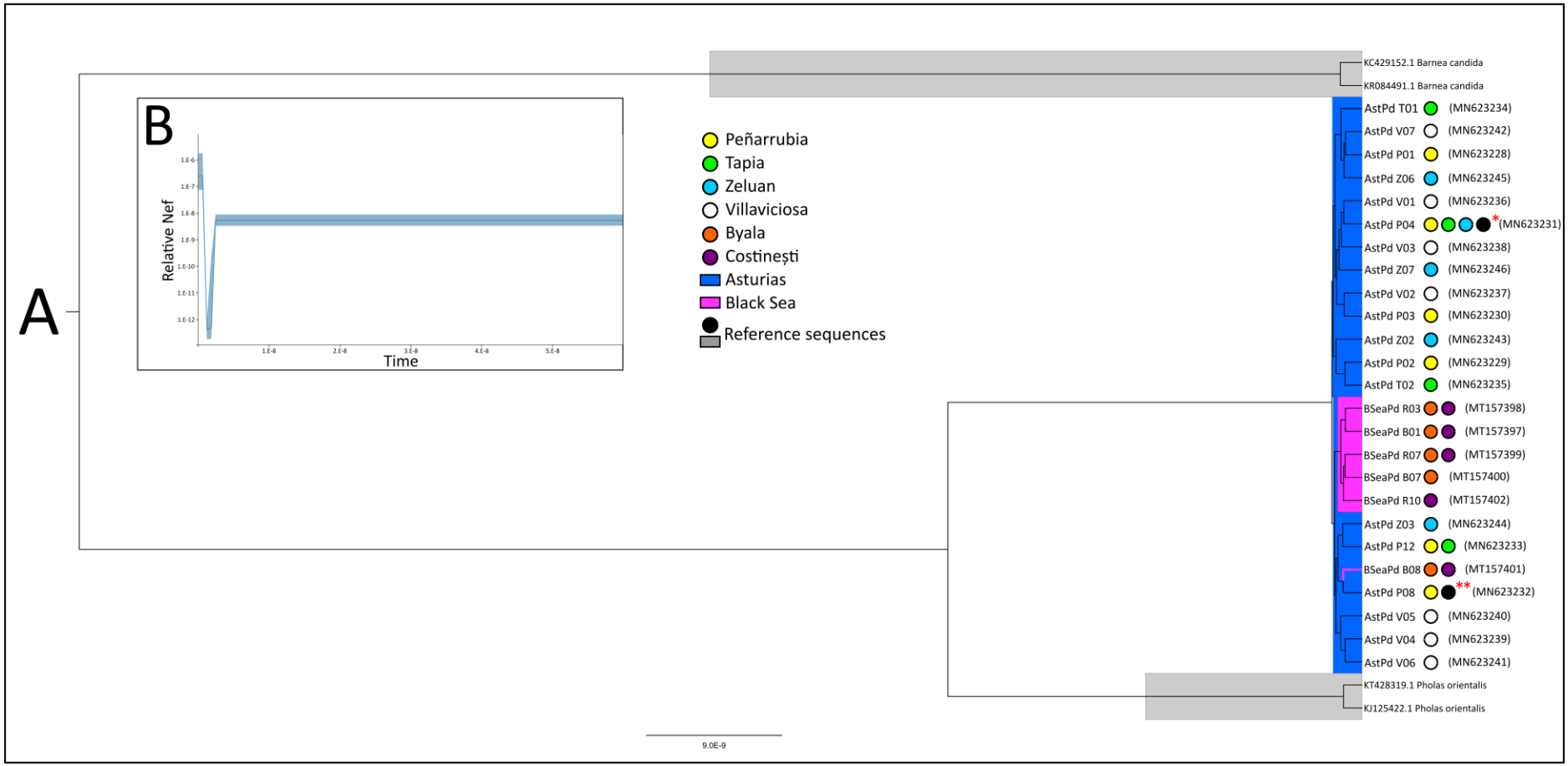


Figure 4. Heatmap representing the pairwise Cytochrome Oxidase I gene Φ_{ST} values among localities for the species *P. dactylus*. The darker the colour, the higher the value. Asterisks indicate significant p-values ($p<0.05$) (in red significant after a Bonferroni correction).



787

788 Figure 5. The *P. dactylus* haplotype network from the samples collected in Asturias, Bay of
789 Biscay, and the Black Sea. Node size is proportional to the number of samples in which the
790 haplotype was observed, with the colour portions relating to the proportion of samples from
791 each locality in which the haplotype was present. Numbers in red indicate the number of
792 mutations needed to get from one haplotype to another. Red rhombuses represent
793 hypothetical nodes where sequences should branch.



794

795 Figure 6. A) Bayesian Skyline tree for the *P. dactylus* haplotypes from southern central area of the Bay of Biscay and Black Sea. The colours indicate
796 in which sampling sites the haplotypes were present. Accession numbers for each haplotype in Genbank are between parentheses. *Reference
797 sequence KX713491.1 **Reference sequence AY070141.1 B) Bayesian Skyline plot of the global *P. dactylus* samples along with reference
798 sequences from GenBank.