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Mitochondrial DNA variation in the caramote prawn *Penaeus (Melicertus) kerathurus* across a transition zone in the Mediterranean Sea

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Abstract In this study we analysed mitochondrial DNA variation in *Penaeus kerathurus* prawns collected from seven locations along a transect across the Siculo–Tunisian region in order to verify if any population structuring exists over a limited geographical scale and to delineate the putative transition zone with sufficient accuracy. Partial DNA sequences of COI and 16S genes were analysed. In contrast to the highly conservative 16S gene, the COI sequences exhibited sufficient diversity for population analysis. The COI gene revealed low levels of haplotype and nucleotide diversities. The size of the annual landings of this commercial species suggests large population sizes. Hence, the low genetic diversity detected in this study could indicate a possible reduction in effective population sizes in the past. We detected significant genetic differentiation between eastern and western populations likely due to restricted gene flow across the Siculo–Tunisian boundary. We discuss the different evolutionary forces that may have shaped the genetic variation and suggest that the genetic divide is probably maintained by present-day dispersal limitation.

Keywords Siculo–Tunisian strait · mtDNA · Genetic transition · Bottleneck

Introduction

In marine environments, where effective physical barriers are difficult to occur, most species spend part of their life cycle in open waters as free-moving gametes, larvae or adults. The expected pattern is that species with high dispersal capabilities (i.e. planktotrophic larvae and continuous environment) have little genetic structure and high gene flow (Lacson 1992; Ovenden et al. 1992; Russo et al. 1994; Uthicke and Benzie 2003). Nevertheless, broad-scale surveys of genetic variation within some marine species have shown that many are genetically more structured than expected (Avise 2000; Hellberg 1996; Lemaire et al. 2005), because a variety of factors including biological, ecological, physiological, physical and geological factors might contribute to the shaping of the population structure of marine species through space and time. In this regard, population genetics and phylogeographic studies are necessary in order to examine the spatial and temporal scales at which populations are genetically structured and can help us understand how speciation takes place in the open sea.

Between 5.96 and 5.33 Myr ago, isolation of the Mediterranean Sea from the Atlantic Ocean led to the Messinian salinity crisis during which sea-levels dropped considerably, reducing the Mediterranean Sea into hypersaline lakes (Krijgsman et al. 1999). Most of the species comprising the present-day fauna have colonized the Mediterranean due to

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the opening of Gibraltar strait at the end of Miocene. During the Pleistocene-era, glacial episodes involved cyclical environmental variations with associated marine regressions and transgressions directly affecting population distributions and demographics (Hewitt 1996; Avise 2000). The large climatic fluctuations during the Quaternary have led to the assumption that vicariance is the most likely model of speciation promoting genetic discontinuities across geographical ranges (McMillan and Palumbi 1995; Cunningham and Collins 1998; Benzie 2000). However, other contemporary factors that limit effective genetic dispersal, including oceanographic currents, isolation-by-distance, habitat discontinuities, larval behaviour and local adaptation (Johnson and Black 1995; Benzi and Williams 1997; Palumbi et al. 1997; Schmidt and Rand 1999; Riginos and Nachman 2001), may also play a pivotal role. These factors may be important singly or in combination, and their relative contributions are hard to disentangle. The predominant mechanisms leading to population differentiation are however, not always clear (Palumbi 1994).

Places where genetic transitions occur on a limited geographical scale may provide opportunities to study these mechanisms because one expects to find the various forces that may reduce gene flow. A number of studies have hypothesized the existence of a genetic transition in the Mediterranean around the Siculo–Tunisian (S–T) strait for several species (e.g. bivalves, Quesada et al. 1995; Nikula and Vainola 2003; several fish species, Borsa et al. 1997; sea bass, Bahri-Sfar et al. 2000 and seagrass, Arnaud-Haond et al. 2007). Almost all these authors surveyed populations from very distant localities and did not specifically concentrate their sampling efforts on the regions surrounding the S–T strait. This raises the following question: do the differentiation they depicted between eastern and western Mediterranean populations actually due to the S–T barrier itself? The Mediterranean has sometimes been referred to as a 'sea of seas' because of its division into different subbasins, each with its own distinct characteristics, including partially enclosed gyral current systems and likely different ecological conditions. Thus distinct hydrographic and ecological conditions may be sufficient to reduce gene flow even on a relatively small geographic scale within seemingly continuous populations (see for example Waples 1987; Planes et al. 1995; Magoulas et al. 2006). Furthermore, precision in delineating transition zones depends on sampling schemes. For instance, the genetic boundary between northeastern Atlantic and the western Mediterranean populations of the mussel *Mytilus galloprovincialis* has been suspected to coincide with the Gibraltar strait (Borsa et al. 1997). However, appropriate sampling in the same species showed that the genetic discontinuity actually corresponds in position with the Almeria–Oran oceanographic front some 400 km eastward the strait (Quesada et al. 1995).

The caramote prawn *Penaeus* (*Melicertus*) *kerathurus* (Forskäl 1775) is an ecologically and economically important penaeid species. It is widespread in the Mediterranean and ranges from the south coast of England to Angola in the Eastern Atlantic. The benthic adults inhabit nearshore and offshore waters to a depth of about 80 m and prefer muddy or sandy-mud flats. In summer, adults migrate to reproduce in coastal areas and spawn in offshore waters. After a planktonic larval stage (about 4 weeks) post-larvae move into shallow waters, where they enter the juvenile stage until they reach 5–8 cm in length and then join the adult population (Garcia and Le Reste 1981). This species is extensively fished; the annual global capture production is around 6 million kg (FAO 2006). Despite its economic and ecological importance, little is known on the different aspects of *P. kerathurus* biology or genetics and almost no data are available on the stock structure (see Benzie 2000 for review). A recent study based on allozymes showed low genetic diversity and suggested population structuring over a relatively short geographical scale (Zitari-Chatti et al. 2008).

The present study sampled *P. kerathurus* from seven localities along a transect (from Naples, Italy to Djerba, Tunisia) of about 1,500 km of the species range in order to obtain a fine-scale spatial coverage of the S–T region. We analysed sequence variation of two mtDNA genes in order to verify if any population structuring exists over a limited geographical scale and to localize the putative transition zone with sufficient accuracy.

Materials and methods

Sampling

Eighty-four individuals were collected from inshore sites at seven locations ranging from North to South Tunisia and one location in Italy (Naples) (Fig. 1). These samples belong to two regions: the western region represented by Naples, Tabarka and Kalatlandalos locations and the eastern region represented by the four remaining sample locations. All samples were obtained from commercial fleet. Ten or more individuals were analysed per site (Table 1). Muscle tissue was extracted from fresh shrimp and immediately frozen at −80°C until processed. All individuals, except the Naples specimens, were surveyed in a previous allozyme study (Zitari-Chatti et al. 2008).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted using the Nucleospin kit following the instructions of the supplier (Clontech, Maountain view, USA). The concentration of extracted DNA

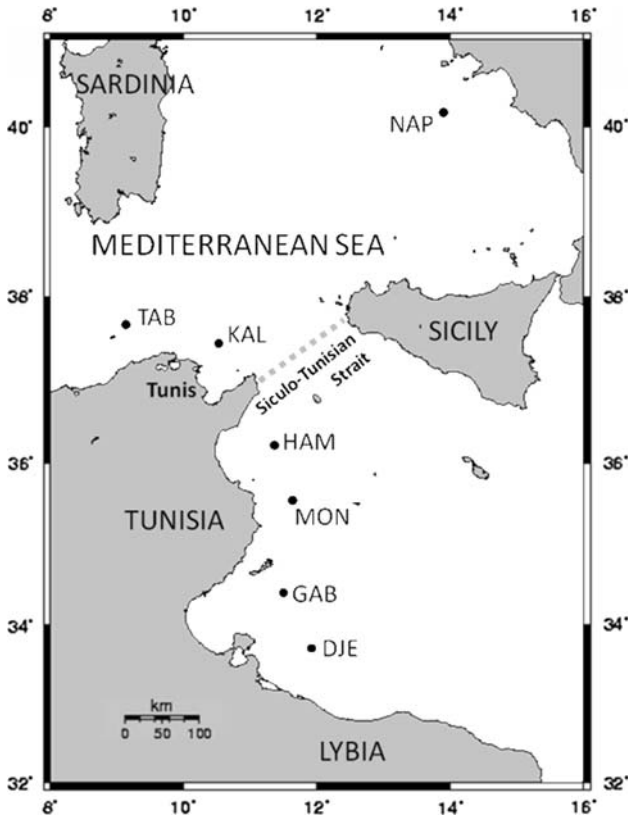

Fig. 1 Geographic location of the seven samples of *Penaeus kerathurus*. For the full names of locations see Table 1

was spectrophotometrically estimated. The lowest DNA concentration was estimated to be about 30 ng μl^{-1}. PCR amplification of a ~562 bp fragment of the 16S rRNA gene (16S) and of a ~374 bp fragment of the mitochondrial cytochrome oxidase *c* subunit I gene (COI) were carried out using respectively, the primers reported in Simon et al. (1994), 16Sar (5′-CGCCTGTTTATCAAAAACAT-3′) and 16Sbr (5′-CCGGTCTGAACTCAGATCACGT-3′), and our primers derived from *P. kerathurus* partial COI gene sequence (Lavery et al. 2004) COI1 (5′-GGGTTCG TAGTCTGAGCACACC-3′) and COI2 (5′-TTAGGGTT AAGGGTTAAGCCGG-3′). The reaction volume (50 µl) for 16S contained 3 µl of undiluted DNA extract, 2.5 mM $MgCl_2$, 0.12 µM from each primer, 400 µM of dNTPs, 1 U of Taq polymerase and ddH_2O. The PCR mix and volume for COI were similar except that 4 mM of $MgCl_2$, 0.16 µM from each primer, 480 µM of dNTPs were used. The cycling profile for the two genes was one step at 95°C for 3 min followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final 5 min extension step at 72°C. The size and the quality of PCR products were visualised on 1% agarose gels. PCR products were cleaned with the GeneElute™ PCR DNA purification kit (Sigma, St Louis, USA) and directly sequenced using the BigDye™ terminator cycle sequencing chemistry, following the manufacturer's protocol (Applied Biosystems, Foster City, USA). The sequences were recorded with an ABI 97 3100 automated sequencer (Perkin-Elmer, Waltham, USA).

Data analysis

All sequences were aligned using the multiple-alignment programme CLUSTAL W (Thompson et al. 1994) with adjustments made by eye. The data sets for both COI and 16S genes were analysed independently. The alignment of COI sequences was confirmed by translating the aligned DNA sequences into amino acid. Identification of the COI and 16S fragments was confirmed by comparing our sequences with those for *P. kerathurus* published by Lavery et al. (2004). Data analyses were performed using ARLEQUIN 3.0 (Excoffier et al. 2005) except where noted. The nucleotide composition, number of transitions/transversions, number of haplotypes and haplotype diversity (h) and nucleotide diversity (π) values (Nei 1987) were calculated. The total number of nucleotide differences and percent sequence divergence values were calculated between each pair of haplotypes.

Table 1 Genetic diversity of COI and 16S sequences for *P. kerathurus* populations

Population	COI					16S			
	N	nh(id)	np	*h*(SD)	π(SD)	nh(id)	np	*h*(SD)	π(SD)
Naples (NAP)	9	2(1,7)	1	0.389 (0.164)	0.0013 (0.0015)	1(1)	0	0	0
Tabarka (TAB)	10	2(1,6)	1	0.200 (0.154)	0.0007 (0.0010)	2(1,2)	1	0.355 (0.159)	0.0007 (0.0009)
Kalatlandalos (KAL)	14	2(1,6)	1	0.143 (0.119)	0.0005 (0.0008)	2(1,3)	1	0.143 (0.119)	0.0003 (0.0005)
Hammamet (HAM)	8	2(1,2)	1	0.571 (0.094)	0.0019 (0.0019)	1(1)	0	0	0
Monastir (MON)	17	2(1,2)	1	0.515 (0.059)	0.0017 (0.0017)	1(1)	0	0	0
Gabes (GAB)	13	4(1,2,3,5)	3	0.718 (0.089)	0.0033 (0.0027)	1(1)	0	0	0
Djerba (DJE)	13	4(1,2,3,4)	3	0.603 (0.130)	0.0030 (0.0025)	1(1)	0	0	0
Total	84	7	6	0.546 (0.047)	0.0022 (0.0019)	3	2	0.0703 (0.0383)	0.00015 (0.00035)

Sample size (*N*), haplotype number (nh) and identity (id), number of polymorphic sites (np), haplotype diversity (h), nucleotide diversity (π) and standard deviation (SD)

To assess the geographic structuring of collections, we calculated the pairwise genetic distances (F_{ST}) and their significance by performing 10,100 permutations among the individuals between populations. We also performed a hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) pooling the populations in western and eastern Mediterranean groups. The statistical significances of the F_{ST} values were tested by executing 16,002 permutations. The sequential Bonferroni test (Rice 1989) was used to correct for multiple tests.

To determine whether there was a relationship between genetic distance and geographical distance, we used the Mantel test (in GENETIX version 4.03; Belkhir et al. 2001) on matrices of standardized pairwise F_{ST} values [$F_{ST}/(1 - F_{ST})$] and pairwise geographical distances. The geographical distance between each pair of populations was estimated by measuring the linear map distance.

Relationships among haplotypes were analysed in a minimum-spanning network estimated with MINSPNET programme implemented in ARLEQUIN, using the total number of nucleotide differences. This method provides a 95% plausible set for all sequences type linkages within an unrooted tree.

Results

Gene and haplotype diversities

Nucleotide sequences of the COI and 16S fragments were determined for 84 *P. kerathurus* individuals (Table 1). Final truncated sequences analysed were 296 and 454 bp for COI and 16S, respectively. The nucleotide composition of the COI sequences averaged 23% C, 33% T, 27% A and 17% G. There were five transitions and one transversion and no indels. A total of seven different haplotypes was found (GenBank Accession numbers EU430763-EU430769), their respective frequencies in each population are described in Fig. 2. The seven haplotypes differed from one another by 1–4 mutations (mean = 2.19, SD = 0.90), and had pairwise sequence divergence values ranging from 0.34 to 1.37%. All the mutations resulted in synonymous substitutions. The overall π–value was 0.0022 (SD = 0.0019) and the within population π–values ranged from 0.0005 to 0.0033 (Table 1). All populations showed low values of haplotype diversity (mean 0.546 ± 0.047) and low values of nucleotide diversity (mean 0.0022 ± 0.0019) (Table 1).

For 16S sequences, base composition averaged 12% C, 34% T, 34% A and 20% G. There were only three haplotypes differing by 1–2 mutations (GenBank Accession numbers EU430760-EU430762), one haplotype (haplotype no. 1 present in 96% of the individuals analysed) being shared by all seven populations while the other two

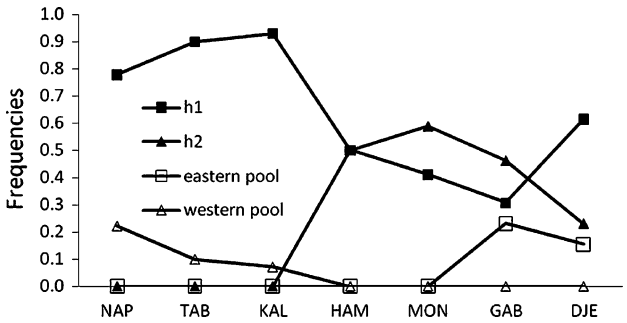

Fig. 2 Mitochondrial COI haplotype frequencies in the seven samples of *P. kerathurus*. Less frequent haplotypes present in eastern (h3, h4 and h5) and western (h6 and h7) samples were combined, respectively under the 'eastern pool' and 'western pool'

haplotypes occurred only in Tabarka (h2: two individuals) and Kalatlandalos (h3: one individual) from the western Mediterranean basin. All populations showed very low values of haplotype and nucleotide diversities (Table 1). Because of the very low variation in 16S sequences, population genetics analyses were performed on COI sequences only.

Population structure

All pairwise F_{ST} values within each Mediterranean region were very low (Table 2). However, all inter-regions pairwise comparisons yielded high values, with four of the 12 comparisons remaining significant after Bonferroni's correction. The hierarchical AMOVA analysis revealed that 29% ($P = 0.02$) of the genetic variance was found among groups and 70.5% ($P < 0.001$) within populations (Table 3). However, the variance component explained by comparisons among populations within groups (0.5%) was low and not significant ($P = 0.35$).

Table 2 Pairwise F_{ST} values between populations of *P. kerathurus* based on cytochrome *c* oxidase subunit I (COI) sequences

	NAP	*TAB*	*KAL*	HAM	MON	GAB
NAP						
TAB	0.0226					
KAL	0.0671	−0.0879				
HAM	0.2182	0.3182	0.3943			
MON	0.3211	0.3979*	0.4515*	−0.0837		
GAB	0.2556	0.3420*	0.4053*	−0.0523	−0.0214	
DJE	0.0405	0.0851	0.1359	−0.0187	0.0876	0.0463

Samples from the western Mediterranean region are indicated in italics

The eastern Mediterranean region is represented by populations on the eastern side of the Siculo–Tunisian strait and the western region is represented by populations on the western side

* Significant value after the Bonferroni's procedure

Table 3 Hierarchical analysis of molecular variance (AMOVA) for the COI sequences of *P. kerathurus*

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*-value
Among groups	1	3.987	0.0933 Va	29.05	F_{CT}: 0.2905	0.02
Among populations within groups	5	1.231	0.0016 Vb	0.53	F_{SC}: 0.0074	0.35
Within populations	77	17.425	0.2263 Vc	70.43	F_{ST}: 0.2957	<0.001
Total	83	22.643	0.3213			

Analyses are presented pooling populations in western and eastern Mediterranean groups

The eastern group corresponds to populations on the eastern side of the Siculo–Tunisian strait and the western group corresponds to populations on the western side

Va, Vb and Vc are the associate covariance components. F_{CT}, F_{SC} and F_{ST} are the *F*-statistics

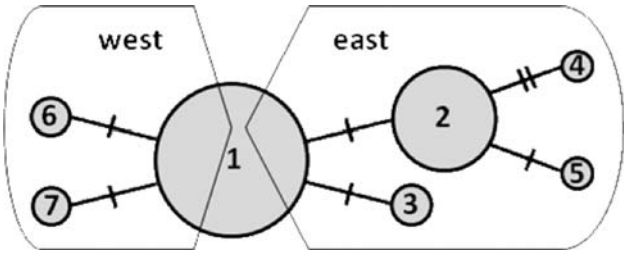

Fig. 3 Minimum-spanning network of the seven *P. kerathurus* COI haplotypes (*1–7*). The *sizes of circles* are proportional to Haplotype frequency. *Perpendicular tick marks* on the lines joining haplotypes represent the number of nucleotide substitutions

There was no evidence of isolation by distance between the populations studied. The Mantel test detected no relationship between genetic and geographical distances ($r = 0.019$, $P = 0.4$). The statistical parsimony procedure yielded one network with unambiguous connections (Fig. 3). All COI haplotypes were closely connected and clustered according to their main area of occurrence. The high frequency and wide geographical distribution of haplotype 1 indicate that it is likely the ancestral haplotype.

Discussion

Partial sequence for the 16S rRNA mitochondrial gene revealed very low variation between all *P. kerathurus* individuals analysed with a sequence divergence nearly equal to zero. This is consistent with previous observations on other penaeid species (Quan et al. 2001, 2004; Chu et al. 2003; De Francisco and Galetti 2005; Kumar et al. 2007) and contrasts with the relatively high diversity of this gene observed in many invertebrate marine species (Shearer et al. 2002 and references therein). 16S rRNA gene is known to occur in the conserved portion of the mtDNA (Meyer 1994) and has a low rate of evolution which makes it inaccurate for studying population differentiation but useful, as it has been shown in several cases, in characterizing cryptic decapod species (the spiny lobster *Panulirus argus*, Sarver et al. 1998; the pink shrimp *Farfantepenaeus subtilis*, Maggioni et al. 2001; the giant freshwater prawn *Macrobrachium rosenbergii*; De Bruyn et al. 2004 and the kuruma shrimp *Penaeus japonicus*, Tsoi et al. 2005). The homogeneity of the *P. kerathurus* populations seen in our study is probably related to the conserved nature of this gene in penaeids. In contrast to the situation with the 16S rRNA gene, the COI region sequences exhibited sufficient nucleotide diversity for population analysis. The sequence divergence values ranging from 0 to 1.37% were similar to those reported in various studies on other penaeid species investigated using COI gene sequencing (Baldwin et al. 1998; McMillen-Jackson and Bert 2003; Quan et al. 2001, 2004; De Francisco and Galetti 2005; Gusmão et al. 2006).

The main finding of this study was the population structuring across the distribution area studied. Two groups were identified on either side of the Siculo–Tunisian (S–T) strait, an eastern group and a western group. Populations within each group appeared to be panmictic and there was no evidence for an isolation-by-distance scheme of differentiation at the scale studied. Both groups showed group-specific haplotypes and restricted gene exchange (Fig. 2). For instance, the haplotype 2 was found in all eastern populations with moderate to high frequencies and absent in western populations. Furthermore, in the highly conservative 16S rRNA gene, we found two haplotypes, although with low frequencies, only in western populations. These genetic characteristics strongly affected the population pairwise F_{ST} values and the AMOVA analyses. Thus, the F_{ST} values between populations belonging to different groups were very high and significant for four comparisons. All intra-region comparisons were not significantly different from zero.

This genetic differentiation between eastern and western groups could be explained by hydrographic regimes in the region. In benthic species with long pelagic larval stages like *P. kerathurus*, water currents are assumed to play an important role in shaping the structure of genetic polymorphism. The water circulation along the North African coasts, characterized by a unidirectional east–south–east

flow of currents (coming from the Atlantic) which leave the Tunisian coasts at the entry of the S–T strait, might therefore, constitute a physical barrier to larval dispersal. This may be plausible in our case, as gene flow could only be promoted by larvae drifting with currents, and also in the case of other species with similar life cycles in this region and in other regions where marine physical barriers were also suspected. For instance, genetic discontinuities have been noticed for bivalves (Quesada et al. 1995; Nikula and Vainola 2003) and seagrass (Arnaud-Haond et al. 2007) around the S–T strait; for sponge (Duran et al. 2004a, b), urchin (Duran et al. 2004c) and lobster (Triantafyllidis et al. 2005) around the strait of Gibraltar and for crab (Lavery et al. 1996) and prawn (Duda and Palumbi 1999) in the Indo-west Pacific. However, the same geographical regions do not necessarily restrict gene flow of other species with similar dispersal capabilities (Arculeo et al. 2003; Bargelloni et al. 2003, 2005; Zardoya et al. 2004; Patarnello et al. 2007 and references therein). We unfortunately cannot obtain a realistic estimate of gene flow by the use of indirect measure extrapolated from haplotype frequencies because our populations may not be at migration-drift equilibrium (Whitlock and McCauley 1999), as assumed by the model upon which the Wright's (1931) equation [$F_{ST} \approx 1/(2Nm + 1)$] is based to estimate the absolute number of effective female migrants per generation (Nm). Even if we apply the Wright's equation, the Nm-value between eastern and western groups (about 2) indicated that larval transportation on either side of the S–T strait may not be negligible. A common rule of thumb holds that Nm-values above 1 are sufficient to prevent the accumulation of fixed haplotype differences (Slatkin and Barton 1989). Moreover, substantial migration has been suggested for example in the European hake and the sea bass across the Almeria–Oran front (Naciri et al. 1999; Cimmaruta et al. 2005), which is a much more important oceanographic discontinuity than the S–T strait (Tintore et al. 1988; Pinardi and Masetti 2000). Therefore, water currents seem to be insufficient by themselves to explain the differentiation observed for certain species as genetic homogeneity is found in the same areas for other species with similar dispersal abilities. Overall, while present-day currents appear to be a good primary template for the establishment of genetic structure as the present data support, forces other than purely passive hydrological mechanisms may be also implied, such as homing behaviour and larval retention (Jones et al. 1999, 2005; Swearer et al. 1999) or selection against immigrants (Allegrucci et al. 1994, 1997; Lemaire et al. 2000). No data on larval retention are available for our species and little is known about its ecology. It is likely that further ecological and behavioural studies will provide further clues as to the reasons for the observed pattern of genetic structure.

The mtDNA variation in *P. Kerathurus* is consistent to some extent with an ancient split between eastern and western Mediterranean populations as suggested by the phylogenetic relationships between haplotypes. The minimum-spanning network demonstrated that the haplotypes, despite being closely connected and separated by few numbers of mutational steps, clustered to their main area of occurrence (Fig. 3). This suggests that populations on both sides of the S–T strait might be derived from one recent maternal ancestor. The overall mean nucleotide divergence between the two basins is 0.0025 ± 0.0017. Assuming a divergence rate for the COI gene of approximately 3% per million years (calibrated by the rise of the isthmus of Panama, Baldwin et al. 1998), the approximate divergence time is estimated to be about 82,000 years ($\pm$55,000), which suggests that the current populations of *P. kerathurus* might have diverged from a common lineage about 82,000 years ago. This timescale, which coincides with the later Pleistocene global glacial period, should be interpreted with caution since molecular clocks are known to be imprecise and sequence divergence rates may vary even within the same species (Zhang and Ryder 1995). During this period, climate fluctuations produced episodes of habitat fragmentation and reduced connectivity between eastern and western Mediterranean. The sea level recurrently dropped below the present-day level, reducing the width and depth of the S–T passage (Thiede 1978). Some genetic studies on other species showed that the S–T strait is an important genetic boundary between the eastern and western Mediterranean basins (Bahri-Sfar et al. 2000; Nikula and Vainola 2003; Arnaud-haond et al. 2007) and have highlighted the importance of such environmental fluctuations as an evolutionary force shaping population structure in this region. Concordance in the geographical positions of significant gene-tree partitions across multiple co-distributed species indicates that the same historical biogeographical factors likely influenced intraspecific patterns of genetic differentiation in these species.

We observed low mean values of haplotype and nucleotide diversities that are much lower than those reported for crustaceans ($h > 0.8$ and $\pi > 0.01$; see for examples Lavery et al. 1996; Stamatis et al. 2004; Inoue et al. 2007). Moreover, the haplotype relationship network is characterized by a nearly star genealogy centered on one geographically widespread haplotype. This phylogenetic pattern is commonly interpreted as a signature of a recent population expansion following a population bottleneck (Slatkin and Hudson 1991). Our results suggest that *P. kerathurus* populations may have undergone an historical bottleneck. A possible decline of population sizes can also explain the low genetic diversity but it is an unlikely cause because the population sizes are supposed to be large as it can be inferred from the catch sizes (the *P. kerathurus*

landings in the studied area averaged 2,000 tons per year; Ben Abdallah et al. 2003). It is noteworthy that genetic diversity in populations from the western region was approximately 50% of that found in the eastern ones.

Ultimately, our study shows genetic differentiation between the eastern and western Mediterranean populations and suggests that divergent entities may meet in a contact zone between the gulf of Tunis and the gulf of Hammamet. The question is: how could such genetic disjunction be maintained? Is the presumed physical barrier (i.e. S–T strait) sufficient to limit gene flow? Is there any genetically determined barrier, resulting from population vicariance? Or do both barriers contribute in conjunction? If our estimate is realistic, the elapsed time since the divergence between eastern and western groups (around 82,000 years) seems insufficient for the establishment of reproductive isolating mechanisms. However, recent evidence from molecular genetics suggests diversification and speciation of some Indo-west Pacific marine taxa occurred as late as the Pleistocene (McMillan and Palumbi 1995; Palumbi 1996). We cannot conclude if this timescale is an underestimate or simply reflects very recent disjunction; the use of more rapidly evolving molecular markers such as microsatellites and/or mtDNA control region could help resolve this issue.

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