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Phylogeography of the marine isopod *Stenosoma nadejda* (Rezig, 1989) in North African Atlantic and western Mediterranean coasts reveals complex differentiation patterns and a new species

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The transition zone between the Mediterranean and Atlantic basins has been extensively addressed in phylogeographical studies of marine species. However, biases exist towards the analysis of highly dispersive species, and there is a higher sampling effort in European coasts compared to North Africa. This may be hindering a detailed understanding of the historical and contemporary processes that shaped patterns of population genetic structure in the region. In the present study, we investigated the phylogeographical and phylogenetic patterns of mitochondrial cytochrome *c* oxidase subunit I sequences from a species with direct development and low dispersal abilities, *Stenosoma nadejda* (Rezig, 1989). The study area included 13 localities along the Atlantic and Mediterranean North African coasts, as well as the Alboran Sea. A new *Stenosoma* species, from the coasts of Algeria and Alboran Island, was discovered. For *S. nadejda*, phylogeographical analyses revealed three distinct clades: one in the Iberian Atlantic plus the Alboran Sea, one in the western Mediterranean, and another in the Atlantic coast of Africa. Haplotypes from the Alboran Island were more related to those from the western Mediterranean coast (east of the Almeria–Oran Front). Given the strong differentiation, it is probable that this species survived in multiple glacial refugia during the Pleistocenic glaciations. *Biological Journal of the Linnean Society*, 2011, **104**, 419–431.

ADDITIONAL KEYWORDS: Alboran Sea – Alboran Island – direct development.

INTRODUCTION

The Mediterranean Sea is one of the most important biodiversity hotspots (Coll *et al*., 2010). The causes of such diversity are probably linked to the great ecological heterogeneity and the complex geological and climatic events that predominated throughout the history of this basin (Bianchi & Morri, 2000). It is generally recognized that the Mediterranean, along with the Atlantic coasts of North Africa, were important glacial refugia for many marine organisms (Almada *et al*., 2001; Maggs *et al*., 2008). For these reasons, numerous studies have addressed the phylogeography of marine species in the Mediterranean and surrounding areas, aiming to match patterns of genetic differentiation with the history of the basin (Gysels *et al*., 2004; Huyse, Van Houdt & Volckaert, 2004; Charrier *et al*., 2006; López-Legentil & Turon,

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2006). However, the literature is largely biased either in relation to the geographical coverage or the type of organisms studied.

Regarding the geographical coverage, most studies have focused on the western part of the basin, often because of interest in the transitional area between Atlantic and Mediterranean genetic lineages, which, for most species, is located not at the Strait of Gibraltar, but eastwards at the Almeria–Oran oceanographic front (AOF) (Patarnello, Volckaert & Castilho, 2007). Even within this region, the sampling effort in the European margin (Spain, France, and Italy) far exceeds that in North Africa. Only a few studies have explored the Mediterranean coasts of Morocco or Tunisia (Daguin & Borsa, 1999; Naciri *et al*., 1999; Lemaire, Versini & Bonhomme, 2005; Calderón, Giribet & Turon, 2008; Shemesh *et al*., 2009), whereas the Algerian coast remains largely unknown. Interestingly, among the few studies in this area, some have uncovered high levels of genetic diversity. For example, when studying the endangered fish *Epinephelus marginatus* Lowe, 1834, Gilles *et al*. (2000) found a cryptic species from the Algerian coast. Also, a recent study of the *Tripterygion tripteronotus* (Risso, 1810) complex revealed the existence of two genetically divergent lineages: one inhabiting the northern and eastern Mediterranean basin and another restricted to Southern Spain and North Africa, from Morocco to Tunisia (Carreras-Carbonell, Pascual & MacPherson, 2007).

The other source of bias arises from the disproportionate focus on organisms with potentially high dispersal abilities, either as adults or at larvae/propagule stages. For example, 67% of the studies included in a recent review on the phylogeography of the Atlantic–Mediterranean transition zone dealt with vertebrates (of which 90% were fish) and the remaining organisms were mostly invertebrate species with long-lived larval stages (Patarnello *et al*., 2007). Thus, species with potentially low dispersal abilities are largely under-represented in the literature. Although dispersal abilities are not always good indicators of the genetic structure displayed by marine populations (Cunningham & Collins, 1998; Kelly & Palumbi, 2010), genetic signatures of historical events are more likely to be preserved in species with low dispersal capacity (Petit *et al*., 2003).

The distribution of the peracarid isopod genus *Stenosoma* Leach, 1814 (= *Synisoma* Collinge, 1917), in the north-east Atlantic and the Mediterranean, suggests that the diversification of this group occurred mostly within the transitional area between the two basins. From the 11 species described for the whole region, six appear to be restricted to small geographical areas in the south-west Mediterranean and/or adjacent Atlantic coasts, whereas the other four occur exclusively in the north-east Atlantic (two species) or throughout the whole Mediterranean (two species). *Stenosoma nadejda* (Rezig, 1989) is an intermediate case. Originally described from Tunisia (Rezig, 1989), it was later found to range throughout the Mediterranean coasts of Spain, including the Alboran Sea (Junoy & Castelló, 2003). Recently, it was recorded outside the Mediterranean, in the Gulf of Cadiz (Castelló & Carballo, 2001), and was later found to be abundant along the southern Portuguese coast (Pereira *et al*., 2006).

Using molecular data, Xavier *et al*. (2009) observed high levels of genetic structure in *S. nadejda* from south-west Iberia, discarding the hypothesis of a recent Atlantic colonization. However, some questions remained unanswered. Although this species has never been recorded from the Atlantic coasts of Africa, there were reasons to consider that it also occurred there. Not only had it passed unnoticed until 2001 in the much more explored coasts of Portugal and Spain, but early records of a similar species exist for a few sites in Morocco (Monod, 1925; Daguerre de Hureaux, 1968). However, these records precede the description of *S. nadejda* (in 1989), and refer to a species described from the Black Sea in the early 19th Century: *Stenosoma capito* (Rathke, 1837). Hence, the hypothesis of 'taxonomic confusion' cannot be discarded. A detailed discussion on this topic is provided in Xavier *et al*. (2009).

More importantly, being a species with direct development (no larval stage in its life-cycle), *S. nadejda* is among the group of motile marine organisms with the lowest capacity to disperse. Rafting in floating substrata has been suggested to be the main mode of dispersal for peracarid species, particularly in algae where these species are commonly found and on which they possibly feed (Thiel & Gutow, 2005b). In the case of *S. nadejda*, which shows no preference for particular algal substrata (Pereira *et al*., 2006), long distance passive dispersal on floating algae (e.g. for hundreds of kilometres) may be theoretically achieved (Fraser, Nikula & Waters, 2010). However, the true determinants of the dispersal distances associated with rafting are related to both the longevity of floating substrate and local current patterns (Thiel & Gutow, 2005a). Consequently, complex patterns of genetic differentiation are to be expected in the main portion of its range, the western Mediterranean, which includes the AOF. By placing an emphasis on the poorly-explored North African coast and based on the patterns of sequence variation of the mitochondrial cytochrome *c* oxidase subunit I gene, we test the hypothesis that the transitional area between the Atlantic and the Mediterranean played a crucial role on the diversification of *S. nadejda*. Because high levels of genetic variation and structure were already

found in populations of *S. nadejda* along the Atlantic coast of Iberia, the expectation is that further diversity and differentiation will be observed, especially east from the AOF.

MATERIAL AND METHODS

Sample collection, DNA extraction, and polymerase chain reaction (PCR) gene amplification

An extensive sampling effort was made throughout the Mediterranean coast of Spain, Northern Morocco, Algeria and Tunisia. A total of 101 individuals were collected from 13 localities (Fig. 1, Table 1). Individuals were collected among brown and red algae. Algae were collected on the lower intertidal during spring tides in the Atlantic and by snorkelling in Mediterranean localities. Algae were immediately washed with fresh water to extract all specimens. These were sorted in the field and stored in 95% ethanol. Genomic DNA was extracted from legs except when individuals were too small (in those cases the whole specimen was used) using the commercial kit Jetquick (Genomed).

A region of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified as described previously Xavier *et al*. (2009). Additionally, to investigate the levels of interspecific divergence, the complete sequence of the nuclear 28S rRNA was obtained for five individuals: two individuals from a highly divergent COI lineage (see Results) collected from Dellys and Tigzirt (Algeria), one *S. nadejda* from Rabat (Morocco), one *Stenosoma lancifer* (Miers, 1881) from Biarritz (France), and one *S. capito* from Lesbos (Greece). The 28S rRNA is a conserved gene that displays little intraspecific variation, but which proved to be a good discriminant of species from the genus *Stenosoma* (R. Xavier, unpubl. data). The primers described by Whiting (2002) were used with the PCR conditions: initial 4 min denaturation at 94 °C, followed by 24 cycles of 45 s at 94 °C, 45 s at 60–62 °C and 1 min at 72 °C. Final extension was achieved at 72 °C for 12 min. Platinum Taq (Invitrogen) was used for all PCR amplifications.

All PCR products were sequenced by a commercial company (High-Throughput Genomics Unit; Department of Genome Sciences of the University of Washington). Sequences were checked and edited using CODONCODE ALIGNER.

Estimates of genetic diversity

A total of 581 bp of COI from 101 individuals were aligned using CLUSTALW (Thompson, Higgins & Gibson, 1994) as implemented in BioEdit (Hall, 1999). Sequences were uploaded in DNASP (Librado & Rozas, 2009) and translated to amino acids to obtain the number of synonymous and nonsynonymous substitutions, as well as to search for stop codons that would be indicative of the presence of pseudogenes.

Uncorrected *p*-distances between taxa were calculated in MEGA, version 4 (Tamura *et al*., 2007), and were used to estimate genetic divergence between pairs of taxa. Indices of genetic diversity, namely

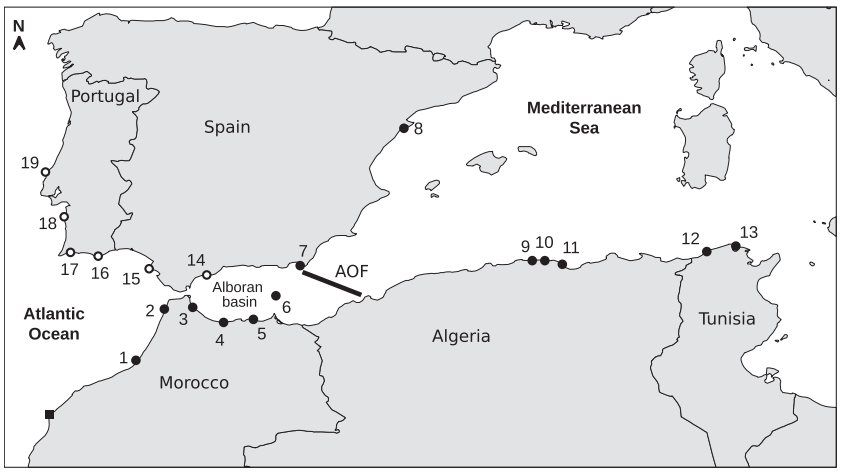


Figure 1. Map of sampling localities included in the present work. Localities indicated by black dots were sampled for the present study. Data from localities indicated by white dots were taken from Xavier *et al*. (2009). Localities: 1, Rabat; 2, Assilah; 3, Cap Mazari; 4, Cala Iris; 5, Al Hoceima Bay; 6, Alboran island; 7, Cabo de Gata; 8, Peñiscola; 9, Dellys; 10, Tighremt; 11, Tigzirt; 12, Cap Serrat; 13, Bizerte; 14, Marbella; 15, Chipiona; 16, Olhos d'Agua; 17, Porto de Mós; 18, Oliveirinha; 19, Baleal. The black square corresponds to the locality of Cape Ghir mentioned in the text. The black line corresponds to the approximate location of the Almeria–Oran oceanographic front *sensu* Patarnello *et al*. (2007).

Table 1. Estimates of genetic diversity for sampling sites and genetic clusters (IbAt, AfAt, Alb, WMed + AI) of *Stenosoma nadejda* and individuals from the highly differentiated clade (C_{10})

Localities	Country	*N*	*H*	*Hd*	π	Accession numbers
Stenosoma nadejda						
Rabat	Morocco	14	11	0.956	0.0087	JF915252-JF915254, JF915256-JF915261, JF915274-JF915278
Assilah	Morocco	13	13	1	0.0061	JF915255, JF915262-JF915273
Cala Iris	Morocco	10	6	0.806	0.0068	JF915207-JF915209 JF915211-JF915216, JF915218
Al Hoceima Bay	Morocco	10	9	0.978	0.0052	JF915198-JF915206, JF915219
Cap Mazari	Morocco	2	2	–	–	JF915217, JF915210
Cabo de Gata	Spain	6	4	0.800	0.0022	JF915283-JF915288
Alboran Island	Spain	3	3	–	–	JF915220-JF915222
Peñiscola	Spain	4	4	–	–	JF915279-JF915282
Tigzirt	Algeria	11	7	0.818	0.0024	JF915229-JF915233, JF915235-JF915239, JF915241
Tighremt	Algeria	2	2	–	–	JF915234, JF915240,
Cap Serrat	Tunisia	6	3	0.600	0.0011	JF915246-JF915251
Bizerte	Tunisia	10	5	0.800	0.0045	JF915223-JF915228, JF915242-JF915245
IbAt		61	34	0.924	0.0063	–
AfAt		27	24	0.989	0.0103	–
Alb		42	32	0.983	0.0114	–
WMed+AI		36	22	0.927	0.0076	–
C_{10}						
Dellys	Algeria	2	1	–	–	JF915297-JF915298
Tigzirt	Algeria	1	1	–	–	JF915291
Tighremt	Algeria	5	1	–	–	JF915292-JF915296
Alboran Island	Spain	2	2	–	–	JF915289-JF915290
Total		10	5	0.710	0.003	

N, number of individuals; *H*, number of haplotypes present; H_d, measure of haplotype diversity; π, nucleotide diversity.

haplotype diversity (H_D) and nucleotide diversity (π) were calculated for each locality, using ARLEQUIN, version 3.11 (Excoffier, Laval & Schneider, 2005). Four localities with less than five individuals or that displayed no polymorphism were excluded from the analyses (Cap Mazari, Alboran Island, Peñiscola, and Tighremt).

Sequences of 28S were aligned using the MAFFT algorithm (Katoh *et al*., 2005) and highly variable regions were eliminated from the analysis using GBLOCKS (Castresana, 2000). The final alignment was 1996 bp long. Uncorrected *p*-distances between taxa were also calculated in MEGA, version 4 (Tamura *et al*., 2007) and were used to estimate genetic divergence between pairs of taxa.

Estimates of genetic differentiation and population structure

Estimates of population structure and differentiation were computed for *S. nadejda*, using an extended dataset. This included COI sequences from 80 individuals of the current dataset (excluding locations with less than five specimens; see above), plus 75 individuals from six locations of the southern Iberian Peninsula previously reported by Xavier *et al*. (2009). Population structure was investigated by calculating F_{ST} measures between all pairs of localities using the pairwise differences distance method. Significance of pairwise F_{ST} values was tested by permuting haplotypes between localities under the null hypothesis of no differentiation. All analyses were performed using ARLEQUIN, version 3.11.

The genetic structure of populations was further investigated with BAPS, version 5 (Corander *et al*., 2004). This software uses Bayesian statistics to test mixture and to define clusters of populations, requiring no a priori knowledge on the geographical structure of populations (Corander, Sirén & Arjas, 2008). Parameters of population admixture and gene flow were estimated with BAPS, version 5 (Corander & Marttinen, 2006; Tang *et al*., 2009). Unlike the mixture analysis that clusters individuals by maximizing differences between clusters, admixture analysis group individuals in accordance with the ancestral origin of their alleles (Corander & Marttinen, 2006). The levels of gene flow were determined through the calculation of the posterior probability of the origin of

each individual over the clusters defined by the mixture model (Tang *et al*., 2009). Additionally, indices of molecular diversity (H_D and π) were calculated for each cluster.

Phylogenetic analysis

Phylogenetic analyses of the COI data set, including the sequences of 75 individuals taken from Xavier *et al*. (2009) were performed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) and only unique haplotypes were included in these analyses. The software JMODELTEST (Posada, 2008) was used to determine the best model of evolution. The model selected was TPM1uf + I + G. Because of the limited number of models included in the available software, the GTR + Γ + I model of evolution was used for ML and BI analysis. The MP tree was reconstructed using MEGA, version 4 (Tamura *et al*., 2007). For the MP analysis 1000 bootstraps were conducted to evaluate branch support. The ML tree was reconstructed using PHYML (Guindon & Gascuel, 2003). Branch support was estimated using 1000 bootstraps. The Bayesian tree was reconstructed using MRBAYES, version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Two separate analyses were run, with 1.7×10^7 generations each. Parameters were sampled every 1000 generations with the heating parameter set to 0.1. Majority-rule consensus trees were estimated combining results from duplicated analyses, after discarding the first 4250 samples as burn-in (corresponding to 25% of the total samples). All trees were rooted using two outgroups from the same genus (*S. capito* and *S. lancifer*) with sequences available in GenBank (accession numbers: FJ905097 and FJ905098, respectively). Haplotype genealogy was also investigated by building a network of haplotype using TCS, version 1.21 (Clement, Posada & Crandall, 2000) with a 95% statistical parsimony connection limit. All sequences were deposited in GenBank with accession numbers (JF915198-JF915303).

RESULTS

Estimates of genetic diversity

Genetic differentiation calculated from the alignment of 581 bp of the mtDNA gene COI revealed two highly divergent clusters: one with 91 individuals (C_{91}) and the other with ten individuals (C_{10}). The 1996 bp of the nuclear gene 28S also confirm the existence of these two divergent lineages (C_{91} and C_{10}). Comparison of COI sequences of C_{91} individuals sampled in Tunisia (from where *S. nadejda* was described) with those from Xavier *et al*. (2009) showed that both datasets correspond to the same species (*S. nadejda*; Rezig, 1989), thus confirming the taxonomic status of the individuals found along the Portuguese coast (see also the results of the phylogenetic analysis). For COI, genetic distances between *S. nadejda* and C_{10} were in the range 13.6–16.0%, which is within the interspecific genetic divergence observed between other *Stenosoma* species (Xavier *et al*., 2009). For the 28S gene, genetic divergence between *S. nadejda* and C_{10} was approximately 3.9%. Divergence of both *S. nadejda* and C_{10} relatively to *S. lancifer* was 9.1–9.6%. Genetic divergence between *S. nadejda* and *S. capito* was approximately 5.2%, whereas it was in the range 4.5–5.2% for C_{10} and *S. capito*. Divergence between *S. capito* and *S. lancifer* was approximately 8.0%.

Within *S. nadejda*, uncorrected *p*-distance between individuals was in the range 0–5.7%, exceeding the previously described estimate of a maximum of 2% (Xavier *et al*., 2009). Individuals from the Atlantic coasts of Morocco (Rabat and Assilah) were 2.9–5.7% divergent from individuals of the Alboran basin, and approximately 2.8–4.3% divergent from individuals of the remaining Mediterranean localities. Individuals from Algeria, Tunisia, and the Alboran Island were approximately 2.9–4.3% divergent from the remaining localities. A mean haplotype diversity of 0.98 and nucleotide diversity of 0.028 was obtained for *S. nadejda* (Table 1). A total of 65 haplotypes were observed, defined by 80 substitutions (only one is nonsynonymous) in 76 variable sites, of which 58 were parsimony-informative. Fifty-five haplotypes corresponded to single occurrences and only four were shared between localities. Haplotype sharing was observed in north-east Morocco (between Cap Mazari and Cala Iris), Algeria (between Tigzirt and Tighremt), and west Tunisia (between Cap Serrat and Bizerte).

The known distribution of C_{10} is currently restricted to the Algerian coast (Dellys, Tigzirt, and Tighremt) and the Alboran Island. Within C_{10} levels of divergence between individuals were in the range 0–0.5%, which is much lower than those obtained for *S. nadejda*. Observed haplotype and nucleotide diversities were 0.71 and 0.003, respectively (Table 1). Four haplotypes were defined by five substitutions, of which three were parsimony-informative. Only one substitution occurred at a nonsynonymous site. Of the four localities where C_{10} was found, only Dellys and Tigzirt (Algeria) shared one haplotype.

Estimates of genetic differentiation and population structure

The estimates of pairwise F_{ST} values (Table 2) revealed high levels of divergence between localities,

regardless of the region. Only Baleal and Oliveirinha (western Portugal) and Porto de Mós and Olhos d'Água (southern of Portugal) displayed non-significant F_{ST} values, as previously reported (Xavier *et al.*, 2009). Intermediate to large levels of divergence were found only between some neighbouring locations. The Bayesian analysis of population structure rendered a clear picture of the patterns of geographical structure by suggesting four major clusters: (1) Iberian Atlantic localities (IbAtl group); (2) African Atlantic localities (AfAtl group); (3) Alboran basin localities (Alb group); and (4) Western Mediterranean localities and the Alboran Island (WMed + AI group). The probability for populations being structured in four clusters was 1.0. Both admixture analysis and gene flow calculations indicated that there is no gene exchange between these clusters, with all individuals showing a null probability of being assigned to any other cluster than the one they were first assigned to by the mixture analysis. Values of H_D and π are presented in Table 1.

Phylogenetic analysis

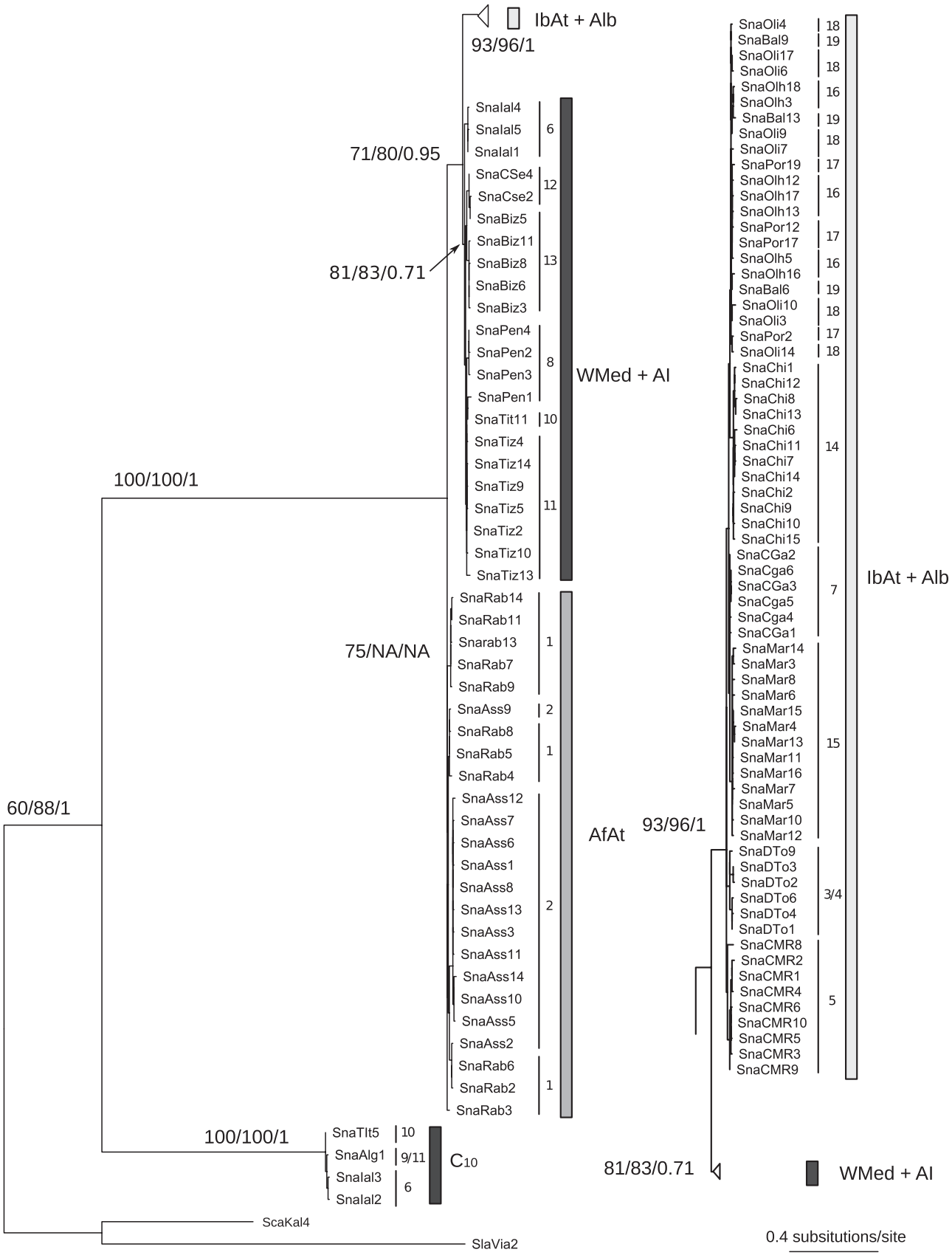
Most of the phylogenetic analyses showed four well supported groups (Fig. 2). All analyses suggested that *S. nadejda* and C_{10} are sister groups with good bootstrap support. Within *S. nadejda*, three major clades were obtained: (1) the AfAt (monophyletic in the MP analysis with bootstrap support of 75%), which is sister to a clade composed of two subclades; (2) the WMed + AI; and (3) the IbAt + Alb. The WMed + AI and IbAt + Alb clades appear as sister groups in all the analyses. Overall, MP, ML, and the Bayesian analyses were congruent with each other, except with respect to the monophyly of the AfAt group. Unlike MP, this group was estimated to be paraphyletic in ML and Bayesian analyses. This may be an artefact resulting from the minimal variation within groups compared to the long divergences between species (including the outgroups). The reconstruction of the haplotype network for all sequenced data retrieved four separate networks that could not be connected using the 95% parsimony connection limit (Fig. 3).

Table 2. Pairwise F_{ST} values between 14 locations, based on 581 bp of mitochondrial cytochrome *c* oxidase subunit I DNA

	Bal	Oli	PMo	Olh	Chip	Mar	CGa	CIr	AHo	Tiz	CSe	Biz	Ass
Oli	0.057												
PMo	0.481	0.280											
Olh	0.342	0.243	–0.001										
Chip	0.741	0.633	0.715	0.686									
Mar	0.730	0.653	0.708	0.689	0.670								
CGa	0.814	0.596	0.742	0.679	0.717	0.471							
CIr	0.784	0.711	0.754	0.734	0.749	0.579	0.659						
AHo	0.866	0.760	0.831	0.793	0.770	0.713	0.793	0.603					
Tiz	0.950	0.900	0.930	0.915	0.901	0.888	0.931	0.872	0.911				
CSe	0.974	0.912	0.952	0.929	0.914	0.893	0.953	0.858	0.916	0.766			
Biz	0.933	0.887	0.912	0.900	0.882	0.869	0.899	0.842	0.880	0.600	0.364		
Ass	0.925	0.893	0.908	0.902	0.899	0.888	0.902	0.872	0.894	0.860	0.876	0.854	
Rab	0.892	0.863	0.872	0.872	0.862	0.843	0.847	0.828	0.850	0.820	0.822	0.808	0.415

Pairs of localities with nonsignificant F_{ST} values are highlighted in black. Pairs of localities with intermediate F_{ST} values are highlighted in dark grey. Pairs of localities with large differences between them are highlighted in light grey. Pairs of localities highly divergent from each other are not highlighted. There are four phylogeographical groups: IbAtl group: Baleal (Bal), Oliveirinha (Oli), Porto de Mós (PMo), Olhos d'Água (Olh), and Chipiona (Chip); AfAt group: Assilah (Ass) and Rabat (Rab); Alb group: Marbella (Mar), Cabo de Gata (CGa), Calla Iris (Cir), and Al Hoceima Bay (AHo); WMed group: Tigzirt (Tiz), Cap Serrat (CSe), and Bizerte (Biz).

Figure 2. Bayesian phylogenetic tree representing unique haplotypes and the position of a highly differentiated clade (C10) and of the three main clades of *Stenosoma nadejda* (IbAt + Alb, Iberian Atlantic and Alboran basin clade; AfAt, African Atlantic clade; WMed + AI, western Mediterranean plus Alboran island clade). The tree was rooted with *Stenosoma capito* (ScaKal4) and *Stenosoma lancifer* (SlaVia2). Bootstrap values of nodes correspond to the maximum parsimony, maximum likelihood and to posterior probabilities given by the Bayesian analysis, respectively. Numbers in front of sample codes correspond to sampling localities in accordance with Figure 1.


IbAt + Alb
93/96/1
71/80/0.95
81/83/0.71
100/100/1
75/NA/NA
60/88/1
100/100/1
93/96/1
81/83/0.71
WMed + AI
AfAt
IbAt + Alb
C10
WMed + AI
ScaKal4
SlaVia2
0.4 subsitutions/site
SnaIal4
SnaIal5
SnaIal1
SnaCSe4
SnaCse2
SnaBiz5
SnaBiz11
SnaBiz8
SnaBiz6
SnaBiz3
SnaPen4
SnaPen2
SnaPen3
SnaPen1
SnaTit11
SnaTiz4
SnaTiz14
SnaTiz9
SnaTiz5
SnaTiz2
SnaTiz10
SnaTiz13
SnaRab14
SnaRab11
Snarab13
SnaRab7
SnaRab9
SnaAss9
SnaRab8
SnaRab5
SnaRab4
SnaAss12
SnaAss7
SnaAss6
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SnaAss8
SnaAss13
SnaAss3
SnaAss11
SnaAss14
SnaAss10
SnaAss5
SnaAss2
SnaRab6
SnaRab2
SnaRab3
SnaTIt5
SnaAlg1
SnaIal3
SnaIal2
SnaOli4
SnaBal9
SnaOli17
SnaOli6
SnaOlh18
SnaOlh3
SnaBal13
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SnaPor12
SnaPor17
SnaOlh5
SnaOlh16
SnaBal6
SnaOli10
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SnaPor2
SnaOli14
SnaChi1
SnaChi12
SnaChi8
SnaChi13
SnaChi6
SnaChi11
SnaChi7
SnaChi14
SnaChi2
SnaChi9
SnaChi10
SnaChi15
SnaCGa2
SnaCga6
SnaCGa3
SnaCga5
SnaCga4
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SnaDTo3
SnaDTo2
SnaDTo6
SnaDTo4
SnaDTo1
SnaCMR8
SnaCMR2
SnaCMR1
SnaCMR4
SnaCMR6
SnaCMR10
SnaCMR5
SnaCMR3
SnaCMR9

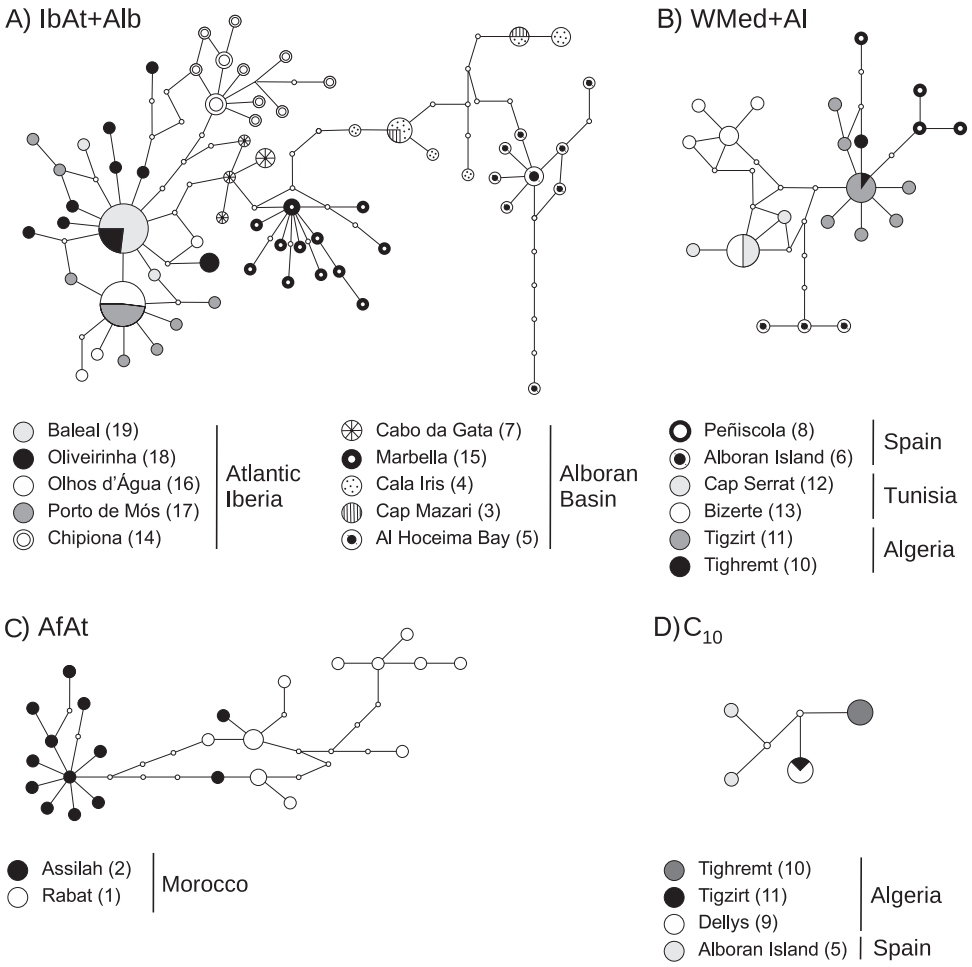

Figure 3. Haplotype networks (95% parsimony connection limit) for all sequenced data. Haplotype networks (A), (B), and (C) correspond to haplotype relationships between individuals of *Stenosoma nadejda* (IbAt + Alb group, AfAt group, and WMed + IA group). Haplotype network (D) represents the relationship between individuals from the highly differentiated clade (C10). Circle sizes are proportional to haplotype frequency and each circle is colour/pattern coded in accordance with the haplotype's presence in sampling locations. Non-observed haplotypes are represented by small white circles. Numbers in front of the names of localities correspond to their geographical position in accordance with Figure 1.

Not unexpectedly, one corresponded to C_{10}, whereas the remaining three coincided with the *S. nadejda* clades observed in the phylogenetic analyses: AfAt, WMed + AI, and IbAt + Alb.

DISCUSSION

By uncovering high levels of genetic diversity and strong phylogeographical structure within the north-west African distribution of *S. nadejda*, as well as by discovering a new species of the same genus from the Alboran Island and Algeria, the present study adds to the concept that the still poorly-explored shores of north-west Africa may harbour one or more hotspots of marine biodiversity. The present results further support the hypothesis that the diversification of the genus *Stenosoma* might have occurred in this region because a quarter of the Atlantic–Mediterranean species are restricted to this area.

The analyses of mtDNA sequence data of *S. nadejda* revealed strong genetic differentiation not only between Atlantic and Mediterranean localities, but also within the Atlantic. Moroccan haplotypes formed a highly differentiated clade relative to those from Iberian Atlantic localities, as well as to the ones from the Alboran basin. As expected, strong differentiation was also found across the AOF. However, the results also confirmed that the distribution of *S. nadejda* extends to both sides of this important geographical barrier. Contrasting patterns of geographical structure also emerged from the analysis of the Alboran localities. In particular, individuals from the Alboran

Island were found to be more related to those to the east of the AOF than with continental populations of the Alboran basin.

A new species of *Stenosoma* from the Alboran Island and Algerian coasts

Phylogenetic analyses revealed that some specimens from the Alboran Island and Algerian localities formed a highly differentiated clade (C_{10}). The uncovered levels of differentiation strongly suggested that an undescribed species is present in that region. Morphological analysis also confirmed that individuals belonging to this new species can be distinguished from *S. nadejda* on the basis of morphology alone, although they share many morphological traits (A.M. Santos & R. Xavier, unpubl. data).

Two other species of *Stenosoma* appear to be exclusive to the Mediterranean coast of North Africa: *Stenosoma carinatum* (Lucas, 1949) has so far been recorded from Algeria and Tunisia (Prunus & Pantoustier, 1976; Rezig, 1989) and *Stenosoma spinosum* (Amar, 1957) is only known from the south-eastern coast of Tunisia (Rezig, 1989). Hence, three out of 12 *Stenosoma* species (25%) currently known from the Atlantic–Mediterranean region are confined to the Mediterranean coast of North Africa. Additionally, two other species have relatively restricted distributions that overlap with the Alboran basin: *Stenosoma bellonae* (Daguerre de Hureaux, 1968) is known from Atlantic Moroccan coasts, but also from the northern Alboran Sea (Algeciras) and the Balearic islands, and *Stenosoma raquelae* (Hedo & Junoy, 1999) was recorded from the Strait of Gibraltar, Tarifa, and the Alboran Island (Junoy & Castelló, 2003). Although more geographical coverage is needed in other Mediterranean regions, the present data reinforce the idea that North African coasts, and in particular those of the Mediterranean, may have been of major importance for the diversification of the genus *Stenosoma*.

Complex patterns of genetic structure suggest a long history of vicariance in North African coasts during the Pleistocenic glaciations

Almost no haplotype sharing was observed between localities of *S. nadejda*, which may indicate that such strong geographical structure is a signature of an ancient evolutionary history. There are two main factors prone to maintain this kind of geographical structure: a low potential for dispersal, considered to be mainly achieved through rafting (Thiel & Gutow, 2005b), and the predominant patterns of water circulation typical from this region (Skliris & Beckers, 2009).

From the two main genetic breaks detected along the study area, one coincides with the AOF, which has been widely described as a major dispersal barrier for many marine organisms (e.g. Galarza *et al*., 2009). The other, separating the Northern portion of the Atlantic Moroccan coast from the Atlantic Iberia and the Alboran Sea has never been reported in the literature. Studies focused in this region always failed to detect any significant break (Pannacciulli, Bishop & Hawkins, 1997; Naciri *et al*., 1999; Lemaire *et al*., 2005; Atarhouch *et al*., 2007; Quinteiro, Rodríguez-Castro & Rey-Méndez, 2007; Campo *et al*., 2010). This may be explained by the high potential for dispersal of the species studied, either as adults, as is the case for *Dicentrarchus labrax* (Linnaeus, 1758) and *Sardina pilchardus* (Walbaum, 1792), or as planktonic larvae, in the case of *Chthamalus montagui* Southward, 1976 or *Pollicipes pollicipes* (Gmelin, 1789). One exception to this pattern comes from the study of Jaziri & Benazzou (2002) on North Atlantic populations of the Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819. A genetic break at Cape Ghir (central Morocco) was attributed to an abrupt change in the typical north–south direction of the coastal currents, towards the Canaries archipelago. However, Cape Ghir is located approximately 480 km south of the southernmost site included in the present study. Because no other physical or ecological barriers are evident across the northern portion of the Atlantic coast of Morocco, the most likely explanation for the observed genetic differentiation of *S. nadejda* populations in this region is that it is a consequence of historical isolation.

One hypothesis, as previously advanced by Maggs *et al*. (2008), is that the currently observed patterns result from a history of vicariance and allopatric divergence during glacial periods. Yet, the Atlantic Moroccan coast has seldom been proposed as a glacial refugium for marine organisms. Genetic evidence favouring this hypothesis is scarce, probably as a result of the bias towards the study of organisms with high dispersal abilities (see above). Nonetheless, even in the presence of high gene flow, signatures of putative refugia can be detected. For example, Campo *et al*. (2010) proposed a refugium in northwest Africa for *P. pollicipes*, based on the higher genetic (haplotypic) diversity of these populations compared to those from European waters.

Low gene flow within the Alboran basin and the biogeographical affinity of the Alboran island

The Alboran basin is a transitional zone between the Atlantic and the Mediterranean, forming an independent biogeographical unit (Bianchi, 2007). Although

the close phylogenetic relationship between the Iberian and Alboran localities is well supported in the present analyses, the data also suggest that there is no evidence of current gene flow between these regions. The Alboran basin is characterized by particularly complex hydrological and oceanographic patterns, such as two large-scale quasi-permanent gyres, tidal motions, mesoscale eddies, and upwelling (Skliris & Beckers, 2009), which might limit or enhance gene flow in a nonlinear manner. Indeed, high levels of genetic heterogeneity were observed within this relatively small area. For example, if gene flow east from Cala Iris appears to be compromised (F_{ST} values of 0.56 between Cala Iris and Al Hoceima, which are 60 km apart), the opposite appears to be happening towards the west because the two individuals collected at Cap Mazari (90 km apart) shared their haplotypes with Cala Iris. Moreover, large differences were also observed between the north and south margins, with F_{ST} in the range 0.58–0.79. Although F_{ST} values were generally high throughout the studied area, heterogeneity within the Alboran region contrasted with the patterns observed within each of the remaining regions.

Another important aspect concerning the complex phylogeographical patterns in the Alboran basin concerns the phylogeographical affinities of Cabo de Gata and the Alboran Island. Depending on the targeted species, populations from Cabo de Gata have been described in the literature as being either part of Mediterranean or Atlantic lineages. For example, in the case of the Mediterranean mussel (*M. galloprovincialis*), Cabo de Gata appears to have more affinity with Atlantic populations (Quesada, Beynon & Skibinski, 1995), whereas, in the case of the sea urchin, *Paracentrotus lividus* (Lamarck, 1816), this is more related to western Mediterranean populations (Calderón *et al*., 2008). For *S. nadejda*, our data suggest that the individuals from Cabo de Gata are more related to the Iberian Atlantic and Alboran basin clade. The instability on the geographical position and intensity of the AOF, which is driven by the seasonal intensity of the eastern Alboran gyre (Prieur & Sournia, 1994; Viudéz & Tintore, 1995), might be at the origin of the contrasting genetic affinities described for this region.

The closer affinity of the individuals from the Alboran Island with those from the western Mediterranean is more difficult to explain given that the former is located to the west of the AOF, and even more if it is considered that there appears to be no connection between the island and the remaining localities of the Alboran basin. The affinity between the Alboran Island and the western Mediterranean basin may result from a historical connection between them or from contemporary water circulation patterns (e.g. instability of the AOF). In a recent study, Alberto *et al*. (2008) observed a secondary contact zone for the seagrass *Cymodocea nodosa* (Ucria) Ascherson, in the Alboran basin. However, contrary to the expectations based on the predominant surface currents, gene flow was apparently unidirectional across the AOF, from the east to the west. Under such a scenario, it would be expected to find both Atlantic and Mediterranean haplotypes from *S. nadejda* in the Alboran Island. The absence of Atlantic haplotypes from our sample may be a result of its small size. Therefore, the hypothesis of a contact zone in Alboran cannot be discarded in light of the present data.

The biogeographical affinities of the Alboran island are far from being understood because, in many cases, its fauna do not resemble the nearby localities at the Spanish or African shores of the Alboran Sea (Carballo, Naranjo & García-Gómez, 1997). However, one of the most interesting findings of the present study is that the phylogenetic patterns uncovered for the new *Stenosoma* species agree with those observed for *S. nadejda* (i.e. no evidence of a genetic break between the Algerian coast and the Alboran Island). This type of pattern may be more common in other organisms with low dispersal abilities. For example, while studying the phylogeography of the ascidian *Cystodytes dellechiajei* (Della Valle, 1877), for which dispersal is presumably short-range, López-Legentil & Turon (2006) found that individuals from the Alboran Island belonged to the same clade as individuals east of the AOF.

Conclusions

Investigating the phylogeography of marine organisms with intrinsic dispersal limitations may uncover patterns of genetic structure that, otherwise, would pass unnoticed (Hurtado, Mateos & Santamaria, 2010; Baratti, Filippelli & Messana, 2011). There is a range of organisms suitable for this task, including (but not limited to) the peracarid crustaceans (isopods, amphipods, tanaids, mysids, cumaceans, etc.) and several molluscs that lay eggs from which crawl-away juveniles emerge. This is not to say that genetic structure will always be found for species with direct development because exceptions do exist (Hayes & Karl, 2008), although the chances of detecting such differences are much higher for this type of organism (Petit *et al*., 2003).

The observed patterns of genetic structure between populations of *S. nadejda* suggest that historical isolation occurred in the past history of this species. One possible explanation is that such pattern originated as a result of retraction into refugial areas during Pleistocenic glaciations. If this is the case, it will be important to determine whether the Atlantic coast of

Morocco is simply a marginal part of a much broader refugial area, the Macaronesia and North Africa (*sensu* Maggs *et al*., 2008) or if it deserves a separate status. Similar to Cape Ghir (Jaziri & Benazzou, 2002), other features prone to shape population structure of coastal species may come to light from a more detailed analysis of the genetic variation across the North African Atlantic coast.

The affinities of Alboran Island with the Western Mediterranean prompt for a more extensive survey of the island itself, and the North African coast eastwards of the AOF. The natural dispersal route for passive dispersers coming from the Atlantic (or the Alboran Sea) into the Mediterranean passes Alboran Island and then goes along the African coast, where a strong eastwards coastal current persists during most of the year (Millot, 1999). Unfortunately, the scarcity of data (either genetic or taxonomic) for Tunisia and, in particular, for Algeria, is still hampering our knowledge on the role of this region as a centre of diversification of Atlantic–Mediterranean species.

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