Invasion or invisibility: using genetic and distributional data to investigate the alien or indigenous status of the Atlantic populations of the peracarid isopod, *Stenosoma nadejda* (Rezig 1989)

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Abstract

The peracarid isopod, *Stenosoma nadejda* (Rezig 1989), until recently considered to be endemic of the Mediterranean region, was first reported in the Atlantic coast of southern Spain in 2001, and in 2006 abundant populations were discovered throughout the southwestern Portuguese coast. This fast expansion was intriguing because, as a direct brooder, this species has limited mechanisms for dispersal, such as rafting on seaweeds. Did *S. nadejda* recently extend its range into the Atlantic or was it overlooked in the past? We examined the patterns of genetic diversity and population differentiation accordingly by sequencing the cytochrome c oxidase subunit I mitochondrial gene from 75 individuals collected in five locations in Atlantic Iberia and one in the Mediterranean. Our results indicate that the newly discovered Atlantic populations of *S. nadejda* appear to be old and have long persisted on Atlantic shores rather than being a recent introduction. High levels of genetic diversity and geographic structure were uncovered in what was initially suspected to be an 'invasive' species. Recent changes in population dynamics may have made *S. nadejda* more conspicuous in the Atlantic shores, or a more comprehensive survey led to the recognition of this species where it was not expected.

Keywords: marine isopod, mtDNA, phylogeography, range expansion, *Stenosoma nadejda*

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Introduction

Studies of species' range shifts have increased dramatically in the last decade, due to concern over the effects of global warming (Helmuth *et al.* 2006). In most cases, large-scale surveys relied on historical data as the primary source of information for tracking distributional shifts (e.g. Stachowicz *et al.* 2002; Zacherl *et al.* 2003; Richardson & Schoeman 2004; Rivadeneira & Fernández 2005; Lima *et al.* 2007; Wethey & Woodin 2008). However, these data are often too scarce or absent for resolving the nature of most species range shifts. Human-mediated introductions are

ubiquitous in coastal ecosystems, and the rate of reported invasions of nonindigenous species has increased dramatically in the last century (Carlton 1989). Second, poorly explored faunas and unresolved taxonomies in many cases lead to doubtful inferences on the indigenous or invasive status of newly observed species [see, for example, the debate between Chapman & Carlton (1991) and Poore (1996)]. Cryptogenic species, that is, those that cannot be reliably assigned to either category, are remarkably common in marine ecosystems exposed to sustained anthropogenic influence (Carlton 1996). Last, but not least, ruling out human intervention on recent range expansions can be difficult, or even impossible, if the life history of the organisms and their environment are not fully understood.

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For most species, a widespread but disjunct geographic distribution is a sign of their invasive status in some regions (Chapman & Carlton 1991). Sometimes the mechanisms of introduction are recognizable, as are the exact or approximate dates of invasion. In these cases, genetic data are used to tackle the problem of the origin of invasion, especially if native populations are widespread in one or more oceanic basins (e.g. Martel *et al.* 2004; Blakeslee *et al.* 2008; Geller *et al.* 2008; Provan *et al.* 2008). But genetic markers are also useful to address problems of identity. Growing evidence indicates that the number of cryptic species, taxa that are morphologically similar but genetically distinct, is underestimated in the marine environment, particularly among invertebrates (Knowlton 2000). Not surprisingly, several studies focusing primarily on the origin of invasions ended up identifying cryptic species (e.g. Bastrop *et al.* 1998; Ó Foighil *et al.* 1998). Because published records of invasive species are not always accurate, genetic techniques should be used more often to verify the accuracy of putative invasions, not only for taxa that are difficult to identify but also when morphologically cryptic species may be present (Hart *et al.* 2006; McGlashan *et al.* 2008).

The genus *Stenosoma* Leach 1814 (=*Synisoma* Collinge 1917) consists of 13 species, of which 11 occur in the NE Atlantic and the Mediterranean and 2 in the N Pacific. Among the 'strictly' Mediterranean species, *Stenosoma nadejda* was described in the late 1980s from the Tunisian coast by Rezig (1989), who differentiated it from *Stenosoma capito* (Rathke 1837) on the basis of the shape and the traces of sutures in their pleotelson. *Stenosoma capito* was described much earlier, in the 19th century (Rathke 1837), and since then it has been found throughout the Mediterranean and the Black Sea. However, after Rezig's description of *S. nadejda*, a reassessment of Mediterranean *Stenosoma* revealed that most western basin (Spain, Algeria, Tunisia) records of *S. capito* were in fact *S. nadejda* (Rezig 1989; Rodríguez-Sánchez *et al.* 2001).

The first records of *S. nadejda* outside the Mediterranean were made by Castelló & Carballo (2001) who observed this species at several locations, from the Strait of Gibraltar up to Cadiz. Pereira *et al.* (2006), being unaware of Rezig's description of *S. nadejda*, reported an additional species of *Stenosoma* for the Portuguese coast, which was identified as *S. capito*. Reexamination of this material revealed that these specimens are all *S. nadejda* (A.M. Santos). Thus *S. nadejda* is currently widespread throughout southern and central Portugal, where it is one of the most abundant Idoteidae in intertidal rocky shores. In some sites, it is even more abundant than the autochthonous Atlantic species *Stenosoma lancifer* and *Stenosoma acuminatum* (Pereira *et al.* 2006).

A fast range expansion of *S. nadejda* into the Atlantic is intriguing because isopods, like all peracarid crustaceans, are direct brooders and dispersal is primarily achieved by rafting on seaweed. It is possible that *S. nadejda* was overlooked, despite the good knowledge about the intertidal fauna of the Iberian Atlantic coast (e.g. Saldanha 1974). It is also conceivable that it has been confounded with one of the autochthonous Atlantic species, even though *S. nadejda* bears a cephalic tubercle that easily distinguishes it from its Atlantic congenerics. Finally, because the taxonomy of the genus *Stenosoma* is not yet fully resolved, the hypothesis of a cryptic species could not be discarded without additional evidence. To understand which is the most probable explanation for the current distribution of *S. nadejda*, we analysed the genetic diversity, population structure and level of differentiation in six locations in southern Iberia, through sequencing of 75 individuals for the mitochondrial gene, cytochrome c oxidase subunit I (COI). One sampling site in the Mediterranean coast was chosen to confirm the identity of the species as *S. nadejda* and five Atlantic locations were sampled to represent the current known Iberian Atlantic distribution of the species (Fig. 1). Furthermore, we also obtained sequences for sympatric Atlantic (*S. acuminatum* and *S. lancifer*) and Mediterranean species (*S. capito*) to confirm the current taxonomic status of *S. nadejda*.

Materials and methods

Sample collection, mtDNA extraction, PCR amplification and sequencing

Specimens of *Stenosoma* were collected among the dominant algae and preserved in absolute ethanol. All individuals were identified according to the key in the study of Castellanos & Junoy (2005). DNA was extracted from legs and antennae using the tissue extraction kit NucleoSpin Tissue (Macherey-Nagel). A total of 658 bp from the COI was amplified by PCR using universal primers (Folmer *et al.* 1994). PCR conditions were the following: initial 5 min denaturation at 94 °C; 36 cycles of 45 s at 94 °C, followed by 30 s at 47 °C and 1 min at 72 °C; final 7 min extension at 72 °C. The total volume of reactions was 20 μL and Platinum Taq (Invitrogen, Carlsbad, CA, USA) was employed. PCR product purification and sequencing, using both forward and reverse PCR primers, were provided by a commercial company (High-Throughput Genomics Unit – HTGU, Department of Genome Sciences, University of Washington).

Sequence checking and editing was carried out using the CodonCode aligner (CodonCode, Dedham, MA, USA). All sequences were trimmed to 627 bp for the
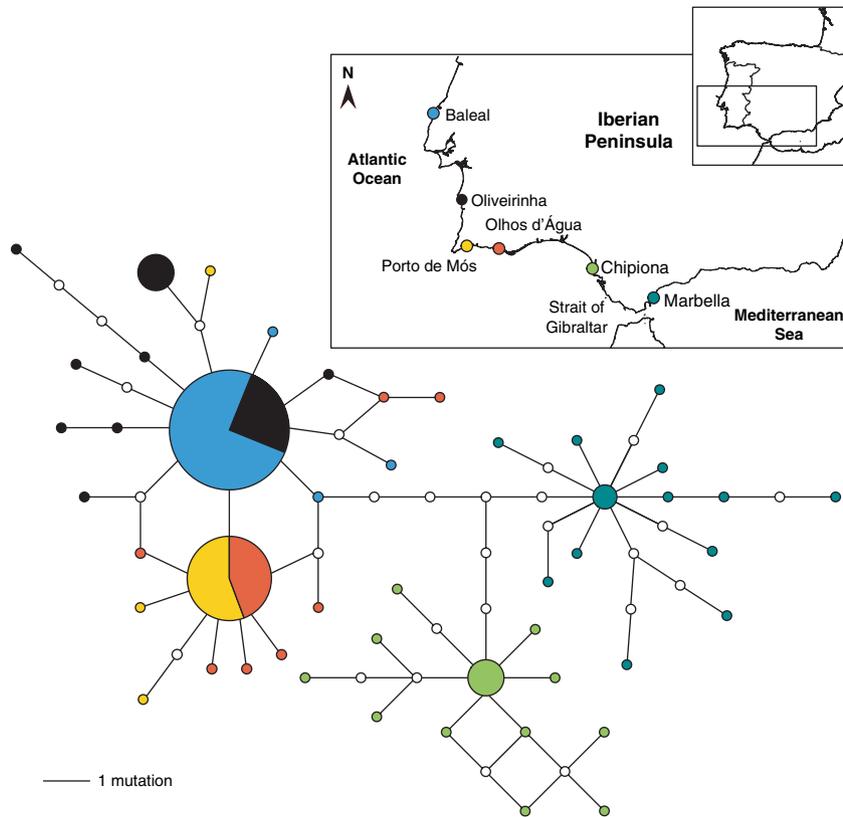
Fig. 1 Map of localities sampled for *Stenosoma nadejda* in SW Iberia and median joining network of haplotypes. Circle sizes are proportional to haplotype frequency and each circle is colour coded according to the haplotype's presence in sampling locations. Nonobserved haplotypes are represented by white circles.

final alignment. Sequences of *Stenosoma nadejda* were deposited in GenBank with accession numbers FJ905048–FJ905096. To assess interspecific divergence, one individual of *Stenosoma capito* from Lesbos (Greece), one *Stenosoma lancifer* and one *Stenosoma acuminatum*, both from Northern Portugal, were also sequenced and submitted to the GenBank with accession numbers FJ905097, FJ905098 and FJ905099 respectively.

Data analyses

For each sampling location, estimates of *S. nadejda*'s haplotype diversity (Hd), nucleotide diversity (π), neutrality tests, Tajima's D and Fu's FS, as well as Harpending's raggedness index (r) were obtained. The significance of Tajima's D and Fu's FS statistics were tested by generating random samples under the null hypothesis of selective neutrality and population equilibrium, using a coalescent simulation algorithm adapted from Hudson (1990). The *P*-value of the statistics was obtained as the proportion of random statistics less than or equal to the observed value.

Significance of r was tested using a parametric bootstrap approach (Schneider & Excoffier 1999). Geographic structure was evaluated from pairwise F_{st}s between sampling sites using pairwise differences as a distance measure. Significance of pairwise F_{st} values was tested by permuting haplotypes between locations under the null hypothesis of no differentiation. The geographic structure was further tested with AMOVA (Excoffier *et al.* 1992). All estimates and respective significance tests were obtained with Arlequin 3.11 (Excoffier *et al.* 2005).

To describe the genealogical relationships between haplotypes, a median joining network was built using the software package Network 4.5.0.2 (Bandelt *et al.* 1999). Information on the frequency and distribution of haplotypes was also depicted in the network by making circle size proportional to haplotype frequency in the total sample and colour coding them according to their frequency in each sampling location (Fig. 1). Uncorrected *P*-distances between taxa were calculated in MEGA (Tamura *et al.* 2007) and were used to estimate the intra- and interspecific genetic divergences.

Results

Genetic diversity and neutrality

The analysis of 627 bp of the mtDNA gene COI in 75 individuals of *Stenosoma nadejda* recovered 49 haplotypes defined by 54 substitutions in 53 variable sites (24 substitutions are parsimony informative). Most substitutions occurred in third positions, and five in first positions of which one was nonsynonymous. As no stop codons were present, there was no reason to believe that these results came from a nuclear pseudogene. Overall, haplotype (Hd = 0.954) and nucleotide (π = 0.009) diversities were high, but the COI genetic divergence between *S. nadejda* individuals was on average 1% (ranging from 0 to 2%) showing that they most likely belong to the same species (Lefébure *et al.* 2006), thus confirming the a priori identification based on morphology. Interspecific divergence within the genus *Stenosoma* is on average 17% (ranging from 16% to 18%), which is also concordant with the values found for crustaceans (Lefébure *et al.* 2006).

Forty-seven haplotypes were found to be restricted to a single location, of which 44 were single occurrences. Only the two most common haplotypes were shared between sites. Haplotype h33 was shared by the two sites in southern Portugal and h27 was shared by the two sites in western Portugal (Fig. 1). The highest values of genetic diversity were found in Marbella (Mediterranean Spain) and the lowest in Baleal (western coast of Portugal), the latter lying at the northernmost limit of the known distribution of the species. However, no westwards trend of declining diversity was observed: Oliveirinha, only 120 km southward of Baleal, had a high genetic diversity. Following the coastline southeastwards, diversity dropped at Porto de Mós and rose again at Olhos d'Água and Chipiona (Table 1).

Overall, significant deviations to neutrality were found for all locations, suggesting a signature of demographic expansion or of a selective sweep. Fu's FS was significantly negative for all sites except Porto de Mós, and Tajima's *D*, which has been shown to be rather conservative (e.g. Fu 1997; Ramos-Onsin & Rozas 2002), gave significantly negative values for Porto de Mós, Baleal and Marbella. According to the *r* index, all locations conformed to the model of sudden expansion (Table 1).

Given the nonsignificance of pairwise F_{st} values (see below) between Oliveirinha and Baleal (western Portugal–WP) and between Porto de Mós and Olhos d'Água (southern Portugal–SP), these sites can be considered samples from the same population. Therefore, parameters of genetic diversity and neutrality tests were recalculated by grouping those locations. Except for the rejection of the sudden expansion model for SP, all other estimates conformed to what has been described above. Neutrality tests were both significantly negative and the highest levels of genetic diversity still lie to the east (Table 1).

Geographic structure

Most pairwise F_{st} values were significantly different from zero (Table 2). The only exceptions were between Porto de Mós and Olhos d'Água in southern Portugal and between Oliveirinha and Baleal in western Portugal. Three levels of differentiation were observed. The lowest level of differentiation was observed between sites within the southern and western coasts of Portugal with nonsignificant F_{st} values of zero and 0.05 respectively. An intermediate level of differentiation was observed between sites of the southern and western coasts of Portugal with significant F_{st} values ranging from 0.24 (Oliveirinha–Olhos d'Água) to 0.45 (Baleal–Porto de Mós). The highest level of differentiation

Table 1 Estimates of genetic diversity and neutrality tests for six sampling sites of *Stenosoma nadejda* and for subregions SP (South Portugal: Olhos d'Água + Porto de Mós) and WP (West Portugal: Oliverinha + Baleal)

Sampling site	*N*	*h*	*S*	Hd	π	*D*	FS	*r*
Marbella	14	13	20	0.989	0.0053	−1.974*	−10.421*	0.041
Chipiona	15	13	14	0.971	0.0044	−1.421	−10.557*	0.048
Olhos d'Água	12	8	10	0.848	0.0033	−1.535	−3.871*	0.059
Porto de Mós	9	4	6	0.583	0.0021	−1.728*	−0.450	0.092
Oliveirinha	13	9	13	0.923	0.0043	−1.446	−3.926*	0.062
Baleal	12	4	4	0.455	0.0011	−1.747*	−1.489*	0.105
SP	21	11	15	0.738	0.0028	−2.105*	−6.581*	0.020*
WP	25	12	17	0.770	0.0029	−2.143*	−7.149*	0.060

N, number of individuals; *h*, number of haplotypes; *S*, number of segregating sites; Hd, haplotype diversity; π, nucleotide diversity; *D*, Tajima's test of neutrality; FS, Fu's test of neutrality; *r*, Harpending's raggedness index.
*Significant estimates for *P* < 0.05.

Table 2 Pairwise F_{st} values between six sampling sites from SW Iberia of *Stenosoma nadejda*, based on 627 bp of mtDNA COI

	Marbella	Chipiona	Olhos d'Água	Porto de Mós	Oliveirinha
Chipiona	0.627*	—	—	—	—
Olhos d'Água	0.700*	0.692*	—	—	—
Porto de Mós	0.715*	0.711*	−0.001	—	—
Oliveirinha	0.664*	0.642*	0.237*	0.271*	—
Baleal	0.731*	0.729*	0.328*	0.454*	0.052

*Significant values for $P < 0.05$.

involved comparisons between Spanish localities, Marbella and Chipiona, and between these and the Portuguese ones. Values of F_{st} ranged from 0.63 (Marbella–Chipiona) to 0.73 (Marbella–Baleal and Chipiona–Baleal).

Based on pairwise F_{st} values and their significances, we defined three regions: Marbella, Chipiona and Portugal. Within region geographic structure was observed in Portugal where two subregions, SP and WP, were defined. Concomitantly, geographic structures with three (Marbella, Chipiona and Portugal) and four groups (Marbella, Chipiona, WP and SP) were tested with AMOVA and a four-group structure was confirmed as all variance components were significant, while for the three-group structure the 'among groups' variance component was not (results not shown).

Genealogy of haplotypes

In the haplotype network depicted in Fig. 1, three haplogroups were evident, each one restricted to one of the three regions defined in the previous section (Marbella, Chipiona and Portugal). These haplogroups were separated by five to six mutations, showing similar branching patterns but different patterns of variation. While within haplogroups from Marbella and Chipiona no central haplotype could be distinguished by higher frequencies, the haplogroup from Portugal had two internal haplotypes with high frequency. Besides, the Portugal haplogroup was further subdivided into several variants around the two common internal haplotypes themselves separated by a single mutation. Furthermore, three out of 11 haplotypes restricted to SP were found to cluster closer to WP haplotypes (Fig. 1).

Discussion

According to the existing literature (Castelló & Carballo 2001; Pereira *et al.* 2006), *Stenosoma nadejda* is a Mediterranean idoteid that has recently expanded to the Atlantic coasts of the Iberian Peninsula. However, the high genetic diversity and strong geographic structure in three levels of differentiation observed in the present

work contradict the hypothesis of a recent invasion and suggest instead that *S. nadejda* has been present in the Iberian Atlantic coasts for a long time.

At the highest level of differentiation, we found three haplogroups restricted to a specific region (Fig. 1), two of which are within the Atlantic area suggesting that isolating mechanisms to the west of Gibraltar are probably as old and efficient as those separating Mediterranean and Atlantic populations. No correlation between genetic and geographic distances was found as genetic distances among the three regional populations were very similar (Table 2). Such a pattern can be explained by a radiation event at the origin of the three regional populations that would have subsequently evolved without any further genetic exchange. Isolation between the two Spanish populations is probably related to the Strait of Gibraltar (Alberto *et al.* 1999) and the connectivity between Spanish and Portuguese populations in the Atlantic might be prevented by a stretch of 200 km of sandy coast (separating Chipiona and Olhos d'Água) and also by the complex current patterns observed in that area (García-Lafuente *et al.* 2006).

Intraregional structure was detected in Portugal where two subregions, SP and WP, could also be recognized by the analysis of pairwise F_{st}s (Table 2) and AMOVA. Within WP and SP, there seems to be no barriers to migration, given the low and nonsignificant F_{st} values. Individuals collected at Oliveirinha and Baleal, as well as those collected at Olhos d'Água and Porto de Mós, can be considered to belong to the same population respectively. Yet, an inversion of the pattern of connectivity was observed when comparisons were made between SP and WP, as the absence of shared variants among them suggests that, at present, connectivity between the southern and western coasts of Portugal is very low or absent. This genetic break may result from a combination of life-history traits and oceanographic features, such as local currents (García-Lafuente *et al.* 2006).

High connectivity along the western coast of Portugal had already been described for the amphipod, *Gammarus locusta* (Costa *et al.* 2004), as well as for the seaweed *Gelidium sesquipedale* (Alberto *et al.* 1999). For *G. locusta*, a unidirectional southeast migration

between the western coast and the western tip of the southern coast was also detected. In the case of *S. nadejda*, evidence for the present-day connectivity between SP and WP is not so obvious. However, haplotypes from SP and WP are not reciprocally monophyletic suggesting that these populations share a recent history. Either migration occurred in the past, or populations from both areas had a common origin that is not yet completely sorted.

Recent expansion or discrete persistence

So how can genetic and distributional evidence be reconciled? Either the species was misidentified or it passed unnoticed in the Atlantic Iberian coast. Because *S. nadejda* was only described in 1989 by Rezig, it would not be recognized as such in works preceding that date. Most likely, it would be identified as *Stenosoma capito* (Rathke 1837), a species with which it bears many morphological similarities. In fact, specimens of *S. capito* recorded for the Mediterranean coast of Spain before 1989 were found to be *S. nadejda* (Rezig 1989; Rodríguez-Sánchez *et al.* 2001). Even though there are no records of *S. capito* for the Atlantic coasts of Spain and Portugal, it was recorded further south, in the Atlantic coast of Morocco (Monod 1925; Daguerre de Hureaux 1968). It is reasonable to assume that these records may also correspond to *S. nadejda*, and although this still needs confirmation, it reinforces the idea that this species could have been occurring in southern Iberia, passing unnoticed given its scattered distribution. Interestingly, records of other *Stenosoma* species in the Portuguese coast are mostly occasional (Ozório 1892; Nobre 1903, 1938; Carvalho 1944). Even in more exhaustive works, which spanned several years, only a few individuals of the Atlantic species *Stenosoma lancifer* were sampled (Saldanha 1974; Monteiro Marques *et al.* 1982). This suggests that the presence of *Stenosoma* on the intertidal is essentially transient, and we found no apparent correlation between the sporadic high densities of these organisms and season, sites or even substrata (algae).

The significant rise of sea surface temperature along the western coast of Portugal and a series of unusual climatic events during 1997–1998 were correlated with changes in the distribution and densities of several intertidal species (Lima *et al.* 2006, 2007). These climatic changes could also have triggered changes in population dynamics of *S. nadejda* resulting in its presently greater abundances, but mtDNA sequence data cannot document such recent population expansions. The genetic variation observed in *S. nadejda* in the Atlantic shores of Iberia suggests a history of expanding populations and high effective population sizes, and even if abrupt changes in abundance characterized their history they were not sufficient to leave a trace in mtDNA.

Final remarks

Our study shows that what was thought to be a recent invasion is probably a native species that was overlooked and/or misidentified in the past. It is also further evidence that the study of poorly known taxa with complex/unresolved taxonomies (irrespective of the morphological or molecular nature of the data) often lead to erroneous inferences about their distributions and origins. We cannot completely rule out a recent invasion (either from the Atlantic coast of North Africa or from the Mediterranean), but our genetic data indicate that this is very unlikely. Still, pertinent questions remain unresolved: (i) what is the real distribution of *Stenosoma nadejda*? (ii) does it really have a Mediterranean origin? These questions may be answered by prospecting the Atlantic coast of North Africa and by extending the genetic analysis not only to this region, but also to the Mediterranean sea, using both mtDNA and nuclear DNA markers.

Finally, the present work is one more piece of evidence that our knowledge of the diversity of small intertidal invertebrates is still very incomplete and thus a continued effort to search, catalogue and identify species is essential. Many species still escape from our observation, possibly because they are not only rare but also due to the incomplete understanding of life histories and ecosystems. It is noteworthy that amongst the first exciting results from the 10-year Census of Marine Life, which have been recently reported (Gilbert 2008), is the discovery of more than 200 new species that highlights how fragmented is our knowledge of life in the oceans.

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