

Genetic Isolation by Distance among Populations of the Netted Dog Whelk *Nassarius reticulatus* (L.) along the European Atlantic Coastline

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Abstract

Estimates of the average distances by which marine larvae disperse are generally poorly described, despite the central role that larval dispersal plays in the demographic connectivity of populations across geographic space. Here, we describe the population genetic structure and average dispersal distance of the netted dog whelk *Nassarius reticulatus* (L.) (Mollusca, Gastropoda, Prosobranchia), a widespread member of European intertidal communities, using DNA sequence variation in a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (COI). An analysis of 156 individuals from 6 locations spread across ~1700 km of the European Atlantic coastline revealed weak and nonsignificant population structure (overall $\Phi_{ST} = 0.00013$). However, pairwise Φ_{ST} values revealed a slight but significant increase in genetic isolation with geographic distance (IBD), suggesting that populations are not panmictic across the sampled geographic range. If we assume that the isolation by distance is maintained by a stable, stepping stone model of gene flow, then the slope of the IBD is consistent with an average larval dispersal distance of ~70 km per generation. The spatial scale of larval dispersal in *N. reticulatus* is consistent with the life cycle of the species (planktotrophic veliger lasting 30–60 days before competent to settle). A mismatch analysis of the COI sequences revealed a signature of an ancient demographic expansion that began 61 500–160 000 years ago, well before the most recent Pleistocene glaciation event. The greatest levels of genetic diversity occur within the middle latitudes of the whelk's geographic range, consistent with the notion that historic populations of *N. reticulatus* might have expanded northward and southward from the centrally located Bay of Biscay.

The spatial scale of demographic connections between populations remains a central issue of marine biology because of its implications for the effective placement of management boundaries and the design of marine protected areas' networks (Botsford et al. 2003). Populations of many marine species often have enormous population sizes and a pelagic larval stage, and these traits have been assumed to connect populations demographically across a far broader spatial scale than most terrestrial species (Caley et al. 1996). However, a growing body of literature has demonstrated the existence of cryptic subdivision of populations over small distances in some organisms with a potentially high dispersal capacity (Grosberg and Cunningham 2001). Such barriers to dispersal have been found along hydrographical boundaries in the Atlantic, Pacific, and Indian oceans (e.g., Avise 1992; Star et al. 2003; Sotka et al. 2004) and across shifts in habitat

(e.g., Burton and Feldman 1981; Johnson and Black 1995; Riginos and Nachman 2001) though they are not ubiquitous. Overall, the evidence seems to indicate that the spatial scales of demographic connection between marine populations will depend on a complex mix of behavior, oceanography, and interactions with co-occurring species and most certainly varies with geographic context (Cowen et al. 2006).

Some of the empirical evidence for the spatial extent of larval dispersal comes from population genetics. For example, mitochondrial DNA have been successfully used to resolve genetic population structure in different marine taxa, including several gastropods (Kyle and Boulding 2000; Wilke and Davis 2000; Collin 2001; Marko 2004; Waters et al. 2005). Specifically, the gene cytochrome *c* oxidase subunit I (COI) has been the marker of choice in most studies; its popularity being largely based on the existence of

a robust, universal primer pair, which has proved successful for numerous invertebrate species (Folmer et al. 1994). In addition, mitochondrial loci also contain the signatures of historical demographic events that can play a major role in establishing contemporary biogeographic patterns and population structure. Fluctuating conditions of the Pleistocene must have had a profound influence on all temperate organisms. Yet, most of the research into the roles of the glacial–interglacial cycles for species has focused on terrestrial biota. In the marine and estuarine environments, concordant patterns of population structure have been found among several co-occurring species along the North American Atlantic coast (Awise 1992; Wares and Cunningham 2001), whereas little concordance has been reported along the European Atlantic. It has been argued that this apparent disparity between North American and European patterns could be a consequence of more complex responses after the retraction of ice sheets in Europe, or alternatively, a relative lack of European marine studies (Jolly et al. 2006).

Gathering data on the larval dispersal patterns and historical demography for all marine species is impossible. It has been proposed that model species (i.e., those organisms of limited economic and conservation importance) can serve as a proxy for species with comparable life-history characteristics (Palumbi et al. 2003) as their lack of commercial value lowers the risk that population structure is influenced by fishing depletion or aquaculture. In this vein, the netted dog whelk—*Nassarius reticulatus* (L.)—is a prosobranch neogastropod common along European shores from the Canaries and Azores to Norway; it has been also reported throughout the Mediterranean and Black Seas (Fretter and Graham 1994). This intertidal scavenger feeder is locally abundant in soft-bottom areas with high organic content and displays a broad tolerance to changes in salinity and temperature (Eriksson and Tallmark 1974). Its life cycle is characterized by the presence of planktotrophic veligers that emerge from benthic capsules; these pelagic larvae require 1–2 months before metamorphosis and settlement (Lebour 1931; Tallmark 1980). *Nassarius reticulatus* has been successfully utilized to biomonitor tributyltin pollution in Atlantic waters (Stroben et al. 1992; Barreiro et al. 2001). The only previous treatment of the population genetics of *N. reticulatus* utilized random amplified polymorphic DNA (RAPD) markers of Northwest Iberian populations to infer panmixia across 300-km spatial extent (Barreiro et al. 2006). Here, we expand our sampling to populations spread 1700 km along the European Atlantic coastline and analyze sequence variation at the mitochondrial locus cytochrome oxidase I in order to assess patterns of gene flow and historical demography of this whelk.

Materials and Methods

Sampling and DNA Extraction

Six populations of *N. reticulatus* were sampled along the South European Atlantic coast (>1700 km) in summer 2005 (Figure 1 and Table 1). Adult specimens (22–27 per sample)

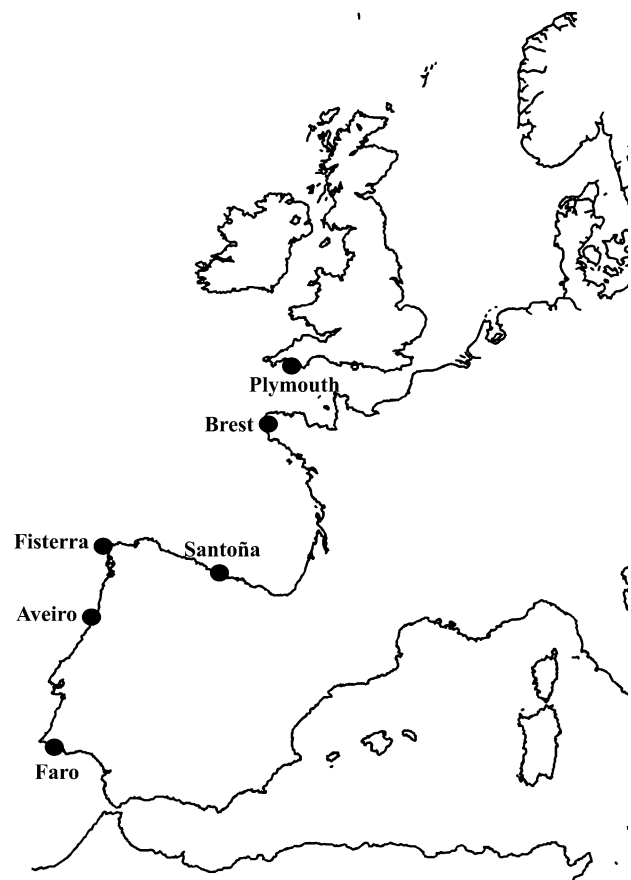


Figure 1. *Nassarius reticulatus*. Map of the South European Atlantic coast showing sampling sites.

were collected by hand or using fish traps, and after breaking the shell, the foot tissue of each animal was preserved in 96% ethanol. Total DNA was extracted from ethanol-preserved tissue using Chelex-100 resin (Estoup et al. 1996).

DNA Amplification and Sequencing

We amplified a 710-bp region of the mitochondrial COI gene using the universal primers described by Folmer et al. (1994). Amplifications were performed in 25 μ l of a solution containing 0.5 μ M of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1 \times Bio-Rad PCR buffer (containing 200 mM Tris–HCl [pH 8.4], 500 mM KCl), 0.4 U Bio-Rad *iTaq* DNA polymerase, and 1 μ l template DNA. Cycling conditions were 2 min denaturing at 95 $^{\circ}C$; (30 s at 95 $^{\circ}C$, 30 s at 45 $^{\circ}C$, and 1 min at 72 $^{\circ}C$) \times 39. In order to amplify several samples from the Plymouth population, we designed a new pair of internal primers: NasF, 5'-AAC AGC TCA CGC TTT CGT AAT-3' (forward), and NasR, 5'-TGC CAA TAC AGG CAA AGA TAA A-3' (reverse) and amplified using similar cycling conditions. After removing the excess of primers and nucleotides (shrimp alkaline phosphatase and exonuclease I enzymes), the samples were sequenced on

Table 1. Genetic diversity estimates for 6 populations of *Nassarius reticulatus* from South European Atlantic coasts

Population	Latitude	Longitude	<i>n</i>	H_t	H_u	<i>S</i>	$\pi \times 10^2$ (SD)	Hd (SD)
Plymouth	50°23'N	4°07'W	27	7	3	7	0.23 (0.05)	0.641 (0.082)
Brest	48°23'N	4°28'W	27	10	5	12	0.32 (0.06)	0.764 (0.063)
Santoña	43°26'N	3°27'W	26	14	8	16	0.41 (0.08)	0.849 (0.065)
Fisterra	42°53'N	9°15'W	27	9	4	13	0.34 (0.12)	0.684 (0.094)
Aveiro	40°37'N	8°45'W	27	11	5	16	0.38 (0.08)	0.781 (0.073)
Faro	37°1'N	7°55'W	22	7	1	6	0.25 (0.04)	0.753 (0.075)

n, sample size; H_t and H_u , number of total and unique (those only present in one individual) haplotypes, respectively; *S*, number of polymorphic positions; π (standard deviation), nucleotide diversity; Hd (standard deviation), haplotype diversity.

a Beckman Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequencing reactions were performed in one direction with the forward primer using CEQ™ DTCS Quick Start kit (Beckman Coulter) following suppliers' recommendations.

Data Analysis

Sequences were checked and edited using the program ChromasPro (Technelysium Pty. Ltd., Eden Praire, MN) and aligned using CLUSTAL X (Thompson et al. 1997) with default settings. After alignment and trimming, the final length of our sequences was 395 bp. MODELTEST 3.7 (Posada and Crandall 1998) identified Kimura's (1980) 2-parameter substitution model as the best-fit model of DNA evolution. This substitution model was utilized in subsequent analysis. DnaSP version 4 software (Rozas et al. 2003) calculated the number of polymorphic sites (*S*), number of total and unique haplotypes, nucleotide diversity (π), and haplotype diversity (Hd). The relationship between genetic diversity and geographic location was examined by performing regression analysis (second-order polynomial model) on the different diversity measures versus degree latitude. Pairwise Φ_{ST} statistics, the proportion of genetic variation within and among populations (i.e., analysis of molecular variance [AMOVA] procedure; Excoffier et al. 1992), the Tajima's *D*-test, and Fu's *F*-test were assessed using ARLEQUIN 2.0 (Schneider et al. 2000); the statistical significance of these indices was calculated by permuting the data for 10 000 replicates. A mismatch distribution was also constructed using this software. The evolutionary relationships between haplotypes were examined with the software Network (<http://fluxus-engineering.com/>) using the median-joining algorithm to build an unrooted cladogram (Bandelt et al. 1999). The correlation of genetic distances over geographical distances for all pairs of populations was tested with the Mantel permutation procedure (Mantel 1967) as implemented in IBD 1.52 (Bohonak 2002). Mean larval dispersal distance was estimated from genetic isolation-by-distance slope following Kinlan and Gaines (2003) and Palumbi (2003).

Results

We compared 156 sequences and found that 42 of 395 sites were polymorphic (10.6%), including 40 transitions and 3

transversions (Table 1). Thirty-six haplotypes were identified (GenBank accession numbers EF571446–80; EF599762): 2 were shared by all populations and represented 46.7% and 19.2% of the total number of individuals, 4 haplotypes were present in 3 populations, 4 haplotypes were present in 2 populations, and the rest (26) were sequenced within a single individual. On average, individuals differed by much less than 1% per base pair (Nei's nucleotide diversity [π] varied from 0.0023 [± 0.0005] to 0.0041 [± 0.0008], with an average of 0.0032). The number of haplotypes per site fluctuated between 14 (Santoña) and 7 (Plymouth and Faro), and the number of unique haplotypes between 8 (Santoña) and 1 (Faro), yielding a haplotype diversity (Hd) ranging from 0.641 (± 0.082) to 0.849 (± 0.065) with an overall average of 0.745.

There appears to be a unimodal relationship between genetic diversity and latitude; diversity was lower at both edges of our sampling range (Plymouth $\sim 50^\circ$ N and Faro $\sim 37^\circ$ N) while maximum values were registered at intermediate latitudes (Table 1). This correlation was statistically significant for the nucleotide diversity (π) and the number of polymorphic sites (*S*) (Figure 2).

The AMOVA revealed that 99.9% of the genetic variation was due to differences within populations; average

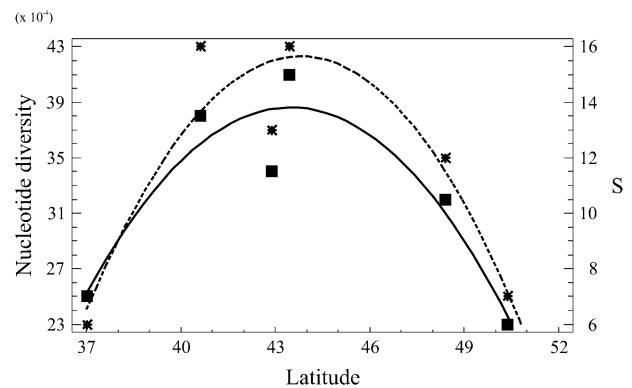


Figure 2. *Nassarius reticulatus*. Correlation between latitude and genetic diversity for 6 populations along South European Atlantic coasts. Squares, nucleotide diversity ($r = 0.94$, $P = 0.044$); asterisks, number of polymorphic sites ($r = 0.93$, $P = 0.048$).

Table 2. Pairwise Φ_{ST} and sequence divergence estimates for 6 populations of *Nassarius reticulatus* from South European Atlantic coasts

	Faro	Aveiro	Fisteria	Santoña	Brest	Plymouth
Faro	<i>0.991</i>	1.238	1.191	1.290	1.153	0.984
Aveiro	-0.008	<i>1.501</i>	1.413	1.542	1.394	1.208
Fisteria	0.010	-0.014	<i>1.364</i>	1.480	1.346	1.164
Santoña	-0.017	-0.015	-0.011	<i>1.628</i>	1.450	1.277
Brest	0.013	0.002	0.017	-0.004	<i>1.283</i>	1.074
Plymouth	0.035*	0.002	0.023*	0.007	-0.021	<i>0.910</i>

Average number of pairwise differences between populations above the diagonal, average number of pairwise differences within populations on the diagonal (italics), and Φ_{ST} values below diagonal.

* $P < 0.1$.

fixation index did not differ significantly from zero ($\Phi_{ST} = 0.00013$, $P = 0.4276$). Reflecting the lack of strong genetic structure, levels of pairwise Φ_{ST} values were low and statistically nonsignificant ($P > 0.05$), oscillating from -0.021 to 0.035. The highest level of Φ_{ST} corresponded to the pair composed of the 2 most distantly spaced populations, Plymouth and Faro, and was nearly statistically significant ($P < 0.1$; Table 2). Thus, despite the statistical determination of panmixia suggested by the AMOVA and Φ_{ST} analysis, the genetic differences between populations fitted to an isolation-by-distance model. The Mantel test showed a significant positive relationship between pairwise Φ_{ST} values and geographic distance ($r = 0.604$, $P = 0.022$; Figure 3). This significant pattern maintained itself when negative Φ_{ST} was converted to zero (Mantel's test $P = 0.024$) and with all possible log transformations of distance measures.

The haplotypic network of the COI sequences (Figure 4) indicates no significant geographic clustering of haplotypes: the 2 most common haplotypes differed by a single substitution and are centered within the network. The other haplotypes were generally separated from those most common haplotypes by 1 or 2 mutational steps, a star-like pattern consistent with a historical expansion of population size.

As suggested by the haplotype network, the mtDNA sequence data contain a signal of a relatively old population expansion. Both the Tajima's D (-2.48 , $P < 0.001$) and the Fu's F ($F_s = -28.79$, $P < 0.0001$) tests yielded significant negative results indicative of an excess of recent mutations. Further, the observed frequency distribution of pairwise differences between individual sequences (i.e., mismatch distribution) closely matches that expected given a population expansion (Rogers and Harpending 1992) (Figure 5).

If we assume the mismatch distribution is unimodal, then we can use the mismatch distribution analysis to approximate the timing of the beginning of the expansion. The software ARLEQUIN uses a nonlinear least-square analysis of the mismatch distribution to generate an empirical estimate of t (Schneider et al. 2000), which is equivalent to $2mT$, where m is the mutation rate of the studied fragment and T is the time since expansion started (see examples in Harpending 1994; Lessios et al. 2001; Sotka et al. 2005). We are unaware of a reliable molecular clock for

Nassarius or other closely related gastropods. We have instead used a sequence divergence rate of 2.4% per million years (mutation rate: 1.2×10^{-8} substitutions per base pair per year) that has been calculated from the COI loci of the marine gastropods *Tegula viridula* and *Tegula verrucosa* (Hellberg and Vacquier 1999). Because ARLEQUIN estimates $t = 1.26$, we infer that the expansion began along European shorelines 133 000 years ago. The 95% confidence interval (CI) for this estimate is extremely broad, suggesting that expansion could have begun 61 500–160 000 years ago, far longer than the last glacial maximum (~ 20 000 years BP).

Discussion

Population Connectivity

Our analysis of mitochondrial sequences suggests that *N. reticulatus* displays weak and nonsignificant population structure along 1700 km of the south European Atlantic coast. This was reflected by 1) a low and nonsignificant overall value of Φ_{ST} (0.00013), 2) low and nonsignificant values of pairwise Φ_{ST} statistics (-0.021 to 0.035; Table 2) and 3) a lack of obvious private haplotypes (i.e., clades of haplotypes that are geographically restricted). This result is consistent with a number of other nearshore gastropods with planktotrophic larvae that display weak population structure: similarly high levels of genetic variation within populations have been reported for *Littorina scutulata* along the west coast of North America (Kyle and Boulding 2000), *Nerita atramentosa* in southeastern Australia (Waters et al. 2005), and *Littorina brevicula* around Korean waters (Kim et al. 2003).

Weak genetic structure has also been documented for other invertebrates with high dispersal potential along European Atlantic coastlines (Sanjuán et al. 1996; Duran et al. 2004; Triantafyllidis et al. 2005). There are known phylogeographic breaks in this area at the English Channel (Jolly et al. 2005) and at the Strait of Gibraltar (Sanjuán et al. 1996; Pannacciulli et al. 1997; Roman and Palumbi 2004), the northern and southern boundaries, respectively, of this study. The *N. reticulatus* data set hints that the northernmost populations (Brest and Plymouth) are genetically distinct

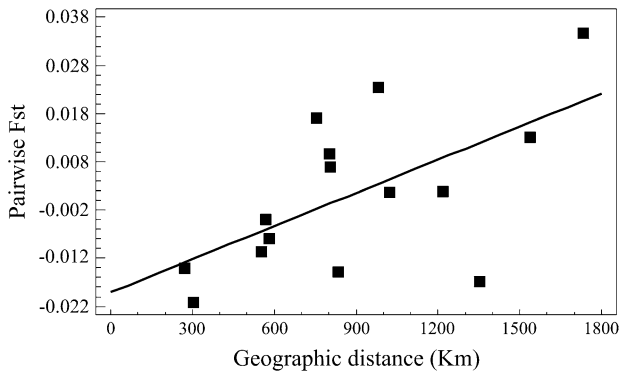


Figure 3. *Nassarius reticulatus*. Relationship between geographic (kilometers) and genetic (Φ_{ST} values) distances. The line shows the ordinary least-squares fit between both variables whose slope, indicative of Φ_{ST} accumulating at a rate of 0.023 per 1000 km, was used to estimate mean larval dispersal distance.

from the Iberian populations as the greatest levels of Φ_{ST} occurred between Iberian and northern populations (Table 2). However, none of these Φ_{ST} values were statistically significant (Table 2), and an analysis of molecular variance between Iberian and northern populations was similarly nonsignificant (results not shown). It is possible that expanding and more intensively sampling the geographic range of *N. reticulatus* would reveal whether these phylogeographic breaks among co-occurring marine animals also occur in *N. reticulatus*.

In spite of the nonsignificant levels of overall population structure in the 1700 km of sampled geographic range, an

analysis of the degree of genetic isolation with increasing geographic distance (IBD) was statistically significant (Figure 3), suggesting restricted gene flow among *N. reticulatus* populations. In theory, stable IBD slopes are generated and maintained via an equilibrational balance between genetic drift and migration. As a consequence, there is an inverse relationship between the IBD slope and the average dispersal distance of a species (Rousset 1997; Raybould et al. 2002). Following Kinlan and Gaines (2003), we used the modeling approach of Palumbi (2003) to calculate that the slope of the IBD (a Φ_{ST} of 0.023 per 1000 km) in *N. reticulatus* could have been generated by an average dispersal distance of approximately 70 km during its 1- to 2-month planktonic life. Even when the IBD slope is recalculated with negative pairwise Φ_{ST} values converted to zero, this estimate does not appear very broad (110 km). An average dispersal of ~ 70 km seems plausible for several reasons. First, the ancient age of the demographic expansion of this species (10s to 100s of thousands of years before present) suggests that the IBD slope is at equilibrium and not the consequence of a recent colonization, bottleneck, or other historical events (e.g., Hellberg et al. 2001). Second, the mean larval dispersal distance of *N. reticulatus* is in line with previous work along Northwest Spanish coasts using RAPD markers: no obvious geographical pattern was detected for the pairwise genetic distances across 300 km (Barreiro et al. 2006). Third, the dispersal distance value obtained in this work is consistent with direct and indirect estimates for other gastropods. A review of IBD slopes for several marine invertebrates inferred that planktonically developing gastropods dispersed from ~ 20 to 140 km on average (Kinlan and Gaines 2003). Likewise, direct estimates of dispersal (i.e., observations of the spatial distribution of larvae,

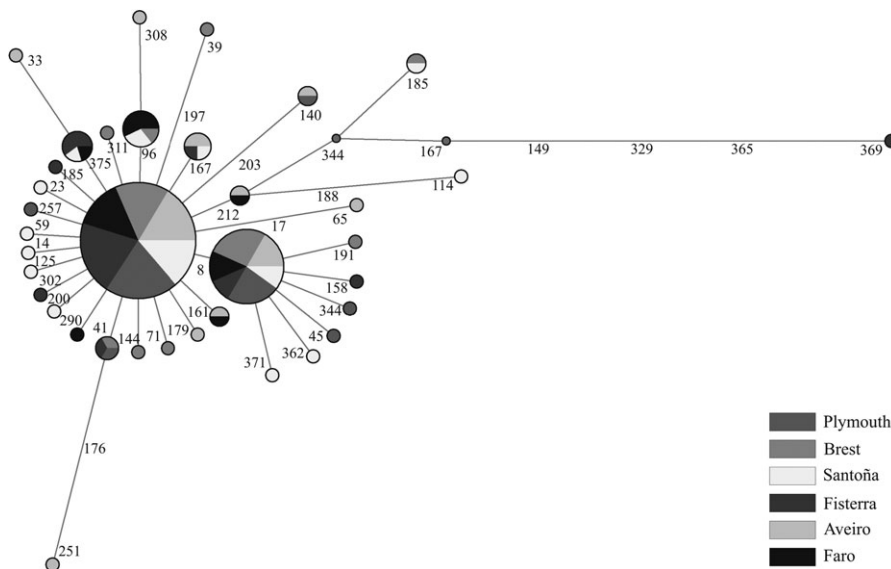


Figure 4. *Nassarius reticulatus*. Median-joining network of COI haplotypes from South European Atlantic coasts. Each haplotype is represented by a circle, and its area is proportional to its relative frequency; shared haplotypes are represented as frequency diagrams. Numbers correspond to mutational positions in the studied 395-bp fragment.

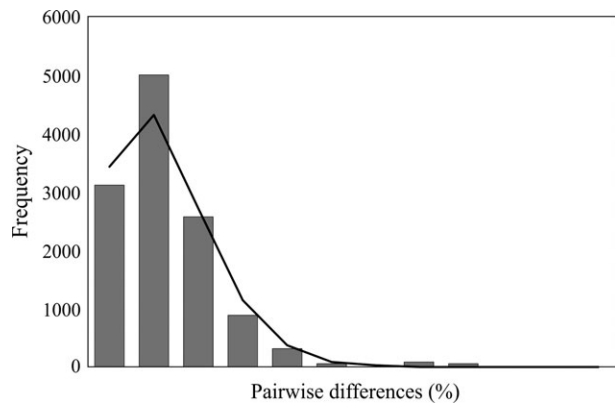


Figure 5. *Nassarius reticulatus*. Mismatch distribution for COI haplotypes from South European Atlantic coasts. Gray bars: observed distribution of pairwise differences among haplotypes; black line: expected distribution under a model of sudden expansion model.

experimental estimates, and observations of invasion fronts) compiled by Shanks et al. (2003) are also within the range of our estimate for *N. reticulatus* when species of similar larval duration are considered: 42 ± 40 km for the planktotrophic veliger larvae of the gastropod *Littorina littorea* which spend a month in the plankton; 33–130 km for the pelagic, feeding larvae of the marine fish *Lutjanus kasmira* that lasts 25–47 days; or 63 km for those of *Carcinus maenas* that spend 80 days dispersing.

Though our analyses may appear contradictory at first glance, the interpretation of pairwise F_{ST} and IBD-based analyses can differ for several reasons. For populations of marine species with high dispersal capability, F_{ST} values as low as 1% are probably common, but unfortunately, they are at the lower edge of the statistical power inherent to traditional pairwise F_{ST} measurements (Waples 1998). As such, these low pairwise F_{ST} values may indicate weak but real population differentiation or may be an artifact of sampling error in a panmictic population. In contrast, it has been argued that IBD analysis may allow researchers greater statistical power (Bohonak 1999) and can provide a more accurate analysis of dispersal distance (Palumbi 2003) than pairwise F_{ST} measurements, in part because multiple populations are simultaneously analyzed and because sampling error should be independent of geographic separation between populations. In any case, the results of this study are based on a moderately weak relationship from 6 populations and must be considered with caution; a further, wider study, including populations across the entire geographic range of the species, would be necessary to confirm the pattern described here.

Demographic History

The mitochondrial sequences also provided insight into the historical demography of *N. reticulatus*. Several lines of evidence in our results are consistent with a historical

population expansion: 1) significant Tajima's D and Fu's F tests, 2) a clearly unimodal mismatch distribution that fits the expected distribution under the sudden expansion model (Rogers and Harpending 1992), and 3) a star-shaped gene genealogy. Similar patterns in previous marine studies were also interpreted as the genetic signature of historical population expansions (e.g., Duran et al. 2004; Perrin et al. 2004; Provan et al. 2005). As is usually the case with mismatch distribution analyses, there is substantial variance in our estimate of the inception of the expansion. Yet, the 95% CI of our estimated values (61 500–160 000 BP) suggests that the expansion likely began during the late Pleistocene, far earlier than the most recent glacial maximum. Moreover, our estimate rests on a divergence rate based on a calibration across the Panamanian isthmus (Hellberg and Vacquier 1999) that a more recent recalibration from the fossil record suggests may be overestimated by at least 40% (Marko 2002). If true, then the time since the population expansion should be even more ancient than our estimate.

Such an old estimate of the time since expansion is consistent with those for 2 other species in the Northeast Atlantic, which suggest that penultimate (Saalian) glacial maximum was more important to their historical demography than the most recent glaciation: the sea urchin *Paracentrotus lividus* experienced a demographic expansion 129 000–282 000 BP (Duran et al. 2004), and the red seaweed *Palmaria palmata* expanded approximately 128 000 BP (Provan et al. 2005). More broadly, late Pleistocene has been reported as the epoch of population expansion for many marine organisms from disparate geographic regions (Benzie et al. 2002; Gopurenko and Hughes 2002; Uthicke and Benzie 2003).

Genetic diversity patterns are frequently employed to delineate potential routes for a species' range expansion. Recently colonized areas should harbor only a fraction of the genetic diversity originally found in territories where the organism has persisted longer. In our study, the highest diversity values were found for those samples collected at the North and Northwest Iberian Peninsula (40–43°N). This pattern contrasts with that of some co-occurring intertidal species where the highest diversity values were detected further north (Coyer et al. 2003; Olsen et al. 2004; Provan et al. 2005). In these species, the authors suggest that these northern latitudes held refugial populations during the glacial maxima. In contrast, our results for *N. reticulatus* would suggest that the hypothetical ancestral population might have been centered in the north of the Iberian Peninsula, a more southern region conceivably less influenced by glacial maxima. Interestingly, our results also hint at a southern attenuation of genetic diversity for our whelk. In some respects, it resembles what has been reported for other marine organisms where low diversity values were attributed to genetically impoverished populations at the edge of the species' distribution range (Coyer et al. 2003; Olsen et al. 2004). Whether this explanation is applicable to *N. reticulatus* is debatable as the currently accepted distribution range of this gastropod spans well into

the Mediterranean up to the Black Sea (Fretter and Graham 1994). Nonetheless, our study covered only a portion of the whelk's range, and the putative trend in diversity proposed here should be confirmed by a geographically more comprehensive study.

Conclusions

The goal of this study was to assess the degree of genetic structure displayed by *N. reticulatus* populations along South European Atlantic coastline. Our results revealed no overall genetic structure across more than 1700 km, but a significant trend of genetic isolation by distance reflects restricted dispersal and gene flow. Demographic processes appear to have left a signature of an ancient range expansion that began 61 500–160 000 years ago and may have been centered in the north of the Iberian Peninsula (Bay of Biscay).

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