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Molecular data delineate cryptic *Nassarius* species and characterize spatial genetic structure of *N. nitidus*

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Nassarius nitidus *is a marine snail species with a widespread distribution along European shorelines from the North Sea to the Mediterranean and Black Seas. Despite its widespread distribution,* N. nitidus *has been largely neglected due to taxonomic confusion with the congeneric* Nassarius reticulatus*. Discrimination between these two* Nassarius *is particularly challenging in areas where their ranges overlap: the European Atlantic and western Mediterranean. Here, we propose the use of the mtDNA gene cytochrome* c *oxidase subunit I (COI) to discriminate between these two morphologically similar congeneric species. A numerically comprehensive sampling of the areas of overlap reveals strong population-level differentiation in* N. nitidus*, particularly between the Mediterranean and the Atlantic. Despite this strong population structure, we found a wide (9.6 ± 1.6% mean K2P corrected sequence distance) barcoding gap with* N. reticulatus *that guarantees that COI barcodes may serve as a reliable diagnostic tool. A protocol using species-specific restriction patterns was developed to allow a quick and accurate discrimination between these two cryptic species.*

Keywords: *Nassarius reticulatus*, *Nassarius nitidus*, cryptic species, DNA barcoding, mtDNA, COI

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INTRODUCTION

Cryptic species are two or more distinct species classified as a single nominal one because they are superficially indistinguishable by morphological traits (Bickford *et al.*, 2007). Cryptic species are relatively common in the marine realm, in part because of inadequate information on the basic biology of many marine species but also because of the important role played by chemical recognition systems during speciation (Knowlton, 1993). Beyond their scientific relevance, these taxonomic uncertainties also have a negative impact on our ability to accurately estimate biodiversity (Hebert *et al.*, 2004), effectively manage fisheries (Borsa, 2002), biomonitor anthropogenic pollutants (Lobel *et al.*, 1990), or recognize invasive versus native members of local ecosystems (Geller *et al.*, 1997). Fortunately, molecular tools represent one of the most effective tools to detect and differentiate morphologically-similar species and their use on cryptic species has increased exponentially over the past two decades (Bickford *et al.*, 2007).

The netted dog whelk *Nassarius reticulatus* (Linnaeus, 1758) *sensu lato* is a marine snail common along Mediterranean and Black Seas coasts as well as in the Atlantic from Morocco to Norway. The morphological variation exhibited by this organism led to considerable debate over the taxonomic status of its various morphs (reviewed in Rolán & Luque, 1994). The consensus until the early 1990s defined only *N. reticulatus* as a 'valid' species, albeit with several recognized subspecies, varieties or morphs. However, morphological, behavioural, and ecological differences led Rolán & Luque (1994) to conclude that two morphs should be considered as separate species: *N. reticulatus* (Linnaeus, 1758) *sensu stricto* and *Nassarius nitidus* a name originally designated by Jeffreys (1867) for individuals specific to brackish, muddy environments. Earlier work had already shown the existence of chromatographic differences between both species (Collyer, 1961) that were later corroborated using allozymes (Sanjuán *et al.*, 1997) and, more recently, a geometric morphometric approach (Carvajal-Rodríguez *et al.*, 2006). Rolán & Luque (1994), after examining ample museum and private collection material, also concluded that the broad distribution range attributed to *N. reticulatus s.l.* was in fact that of *N. nitidus* while *N. reticulatus s.s.* (hereafter, *N. reticulatus* unless otherwise indicated) should be regarded as a mainly Atlantic organism. The latter species lives from the Atlantic margin of the British Isles and English Channel to Morocco and the Canary Islands, and enters into the western Mediterranean only up to the limits of the Gulf of Lyon. On the contrary, *N. nitidus* spans from the Black Sea to the Atlantic but it is confined to sheltered, muddy habitats (estuaries and saltmarshes) on Atlantic coasts where it may live in sympatry with *N. reticulatus*. Rolán & Luque (1994) noticed that three morphs seem to dominate different sections along this broad range (Eastern Mediterranean–Black Sea, Western Mediterranean–Iberian Peninsula and Bay of Biscay–English Channel), suggesting the existence of ecotypes, phenotypic plasticity, or a combination of both. Although their material did not include the North Sea, other studies suggest that *N. nitidus* may be the only congener in the region (Hansson, 1998; Dekker, 2004).

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Despite the morphological and biochemical evidence, distinguishing between these two congeners continues to be problematic. Neither the conventional morphological comparison nor the more recent geometric morphometric approach provides infallible discrimination (Rolán & Luque, 1994; Carvajal-Rodríguez *et al.*, 2006). Furthermore, neither of these techniques can be easily applied to early life-stages. Also, the aforementioned allozyme study (Sanjuán *et al.*, 1997) is of limited applicability because it was restricted to a single pair of sympatric populations and allozymes have been now largely outperformed by more convenient DNA-based diagnostic markers (Hebert *et al.*, 2003a).

The lack of appropriate resolving power is rather inconvenient. First, *N. reticulatus* is often used to biomonitor tributyltin pollution (Barreiro *et al.*, 2001) and it is officially recommended for biomonitoring the inshore maritime area covered by the Oslo and Paris (OSPAR) Convention (www.ospar.org). Biomonitoring studies rely on a species-specific response and often involve region-wide surveys. Therefore, an accurate determination of the specimens collected at different sites is essential. Second, a remarkable increase in the abundance of *N. reticulatus* in Dutch coastal waters (Netherlands Institute for Fisheries Research; RIVO) was recently reported, but the fact that the two *Nassarius* snails had been grouped into a single taxon in earlier surveys complicates the interpretation of the distributional changes (Craeymeersch & Rietveld, 2005).

Here, we used a fragment of the mtDNA gene cytochrome *c* oxidase subunit I (COI) to identify these two cryptic species (Hebert *et al.*, 2003a) and to explore the population structure of *N. nitidus*. To ensure the accuracy of our method, we obtained COI sequences for a large number of individuals from locations widely scattered along the regions where ranges of the two *Nassarius* overlap. We also describe a restriction-fragment approach for the fast, reliable discrimination of these snails in future surveys. To our knowledge, our results represent the first phylogeographical approach to the largely overlooked *N. nitidus*.

MATERIALS AND METHODS

COI gene sequences

We sequenced 90 *Nassarius nitidus* specimens from five locations across its distribution range from Venice, Italy to Lysekil, Sweden (Figure 1; Table 1). Protocols followed those reported in Couceiro *et al.* (2007). Total DNA was extracted from ethanol-preserved foot tissue using Chelex-100 (Bio-Rad Laboratories, Hercules, CA) (Estoup *et al.*, 1996). A 710-base pairs (bp) region of the mitochondrial COI gene was amplified using universal primers (Folmer *et al.*, 1994). Amplifications were performed in 25 μl of a solution containing 0.5 μM of each primer, 0.2 mM of each dNTP, 2 mM

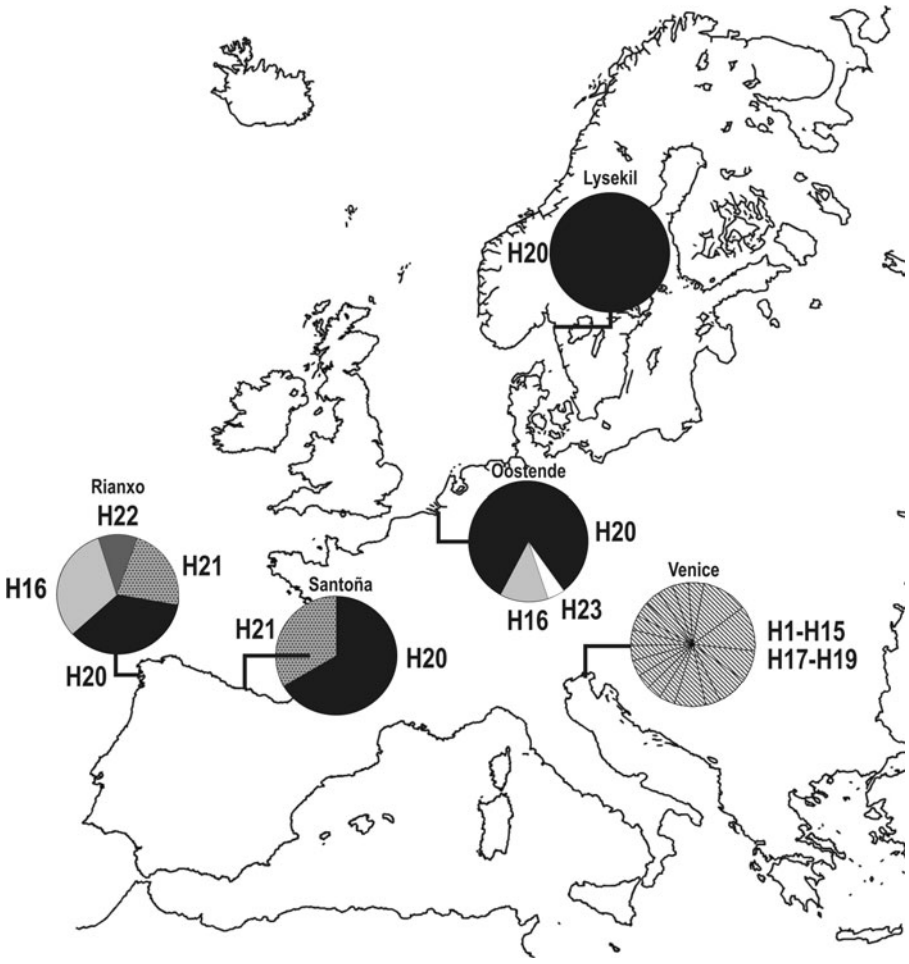

Fig. 1. Sampling sites and cytochrome *c* oxidase subunit I haplotype frequencies in *Nassarius nitidus*.

Table 1. Genetic diversity estimates for 5 populations of *Nassarius nitidus*. N, sample size; N_H, number of haplotypes, S, number of segregating sites; π (SD), Nei's nucleotide diversity (standard deviation); H_E (SD), haplotype diversity (standard deviation); H20, relative frequency of H20, the most common haplotype across the whole data set. Results for Santoña must be considered with caution given the small sample size. *, denotes sites where *N. reticulatus* was found in sympatry with *N. nitidus*.

Population	N	N_H	S	$\pi \times 10^2$ (SD)	H_E (SD)	H20 (%)
Lysekil, Sweden (58°16′16″N 11°27 ′17″E)	29	1	0	0.00 (0.00)	0.000 (0.00)	100
Ostend, Belgium (51°13′40″N 2°57 ′04″E)	22	3	3	0.15 (0.06)	0.325 (0.12)	82
Santoña*, Spain (43°26′14″N 3°27′14″W)	3	2	6	1.01 (0.48)	0.667 (0.31)	67
Rianxo*, Spain (42°38′40"N 8°49′08″W)	11	4	9	0.93 (0.19)	0.782 (0.08)	36
Venice, Italy (45°24′55"N 12°17′35″E)	25	18	23	0.91 (0.09)	0.970 (0.02)	0
N. nitidus	90	23	27	0.48 (0.07)	0.648 (0.058)	
N. reticulatus[a]	156	36	42	0.32 (0.03)	0.743 (0.033)	

[a], data from Couceiro *et al.* (2007).

$MgCl_2$, 1x polymerase chain reaction (PCR) buffer, 0.4 U AmpliTaq DNA polymerase (Applied Biosystems) and 1 μl template DNA. Cycling conditions were 2 minutes denaturing at 95°C; (30 seconds at 95°C, 30 seconds at 45°C and 1 minute at 72°C) × 40. After removing the excess of primers and nucleotides (shrimp alkaline phosphatase and exonuclease I enzymes), the samples were sequenced on an Applied Biosystems 3130*xl* Genetic Analyzer (Life Technologies Co., Carlsbad, CA). Sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following suppliers' recommendations.

Data analysis

Chromatograms were checked and edited with ChromasPro (www.technelysium.com). In a previous study with *Nassarius reticulatus* (Couceiro *et al.*, 2007) low DNA quality in specimens from one single site led us to restrict our analyses to a 395 bp fragment of the COI region. Here, and to facilitate comparison with *N. reticulatus* sequences, the same 395 bp fragment was identified in CLUSTAL X (Thompson *et al.*, 1997) and used for all analysis. Our decision to restrict the analysis to a 395 bp fragment could be considered as a safety measure for future studies in which it might be necessary to deal with partially degraded DNA. Intraspecific and intrapopulation variation was assessed using conventional DNA polymorphism estimates calculated by the software DnaSP v. 4.10.4 (Rozas *et al.*, 2003): haplotype diversity (H_E), Nei's (1987) nucleotide diversity (π), number of haplotypes (N_H) and number of segregating sites (S). The genetic differentiation between populations was assessed by an analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) and by estimating pairwise Φ_{ST} statistics; again, to facilitate comparison with our previous study of *N. reticulatus*, AMOVA and Φ_{ST} estimates were based on the Kimura 2-parameter (K2P) (Kimura, 1980) substitution model as implemented in Arlequin v3.11 (Excoffier, 2006) and their significance was tested by a permutational approach (10000 and 1000 permutations for AMOVA and pairwise Φ_{ST} estimates, respectively). The evolutionary relationships between haplotypes were inferred by the median-joining network approach implemented in Network version 4.2.0.1 (www.fluxus-engineering.com) using the maximum parsimony option to reduce the complexity of the resulting pattern (Bandelt *et al.*, 1999).

Following common practices in DNA barcoding (Hebert *et al.*, 2003b), the divergence between *N. nitidus* and *N. reticulatus* was calculated as pairwise distances between haplotypes using the K2P model and graphically displayed in a neighbour-joining (NJ) tree. COI sequences for *N. reticulatus* were obtained in a previous study by a comprehensive sampling across the species range (Couceiro *et al.*, 2007); sequences for representatives of other members of the genus were used as outgroups and obtained from GenBank. Intraspecific variation, as pairwise K2P distances between haplotypes within each species, was used to determine the magnitude of the barcoding 'gap' between intra- and interspecific variation as an indication of the effectiveness of barcoding. K2P distances were calculated with MEGA v. 4.0 (Tamura *et al.*, 2007); whenever average K2P values were estimated, standard errors were obtained by a bootstrap procedure (500 replicates) in MEGA v. 4.0.

Endonuclease restriction procedure

The presence of species-specific restriction endonuclease recognition sites in the consensus sequence of each species was investigated with the help of the software CLC Sequence Viewer (www.clcbio.com). To test the performance of the restriction enzyme identified as potentially informative, PCR products (10 μl) were digested without purification using 20 U of enzyme with the buffer provided by the manufacturer (Fermentas, Burlington, Ontario), incubated for 9 hours at 37°C. After thermal inactivation (65°C, 20 minutes), the products were visualized on 3% agarose gels stained with ethidium bromide.

RESULTS

Genetic variability and population structure in *N. nitidus*

Our 90 sequences for *Nassarius nitidus* comprised a total of 27 (6.8%) variable sites, an average of 0.48% (SD = 0.07%) substitutions per site, and 23 haplotypes (GenBank accession numbers EF571481-499, EU428828, EU623457 and FJ480182-3). Only one haplotype was found in Lysekil, our northernmost site, while both haplotype diversity (H_E) and number (N_H) increased with decreasing latitude and peaked in the Mediterranean location of Venice ($N_H = 18$, $H_E = 0.970$) (Table 1). The decline in haplotype diversity with decreasing latitude was largely explained by an increase in the relative frequency of haplotype H20 (EF571498), which was absent from Venice but it was the only haplotype sampled in Lysekil.

The AMOVA detected strong and significant spatial population structure (global $\Phi_{ST} = 0.135$, $P = 0.0003$). With the exception of comparisons with the poorly-sampled Santoña, pairwise Φ_{ST} statistics revealed that each population was significantly differentiated from the others (Φ_{ST} statistics from 0.104 to 0.356, P values from 0.0000 to 0.03). After Bonferroni correction, Venice was significantly ($P < 0.05$) differentiated from the two northernmost sites while Rianxo significantly differentiated from Lysekil.

None of the 18 haplotypes found in the Mediterranean was detected in the Atlantic and, likewise, none of the five haplotypes noticed in the Atlantic was observed in the Mediterranean (Figure 1). In general, there was a relatively shallow topology at COI across *N. nitidus* (Figure 2). More than half of the haplotypes found in the Atlantic (H16, H22, H23) or the Mediterranean (H1, H3–H7, H14, H15, H17) were phylogenetically close to the abundant H20. Atlantic haplotype H21 was phylogenetically distant (8 mutational steps) from H20 and was exclusively observed at the northern Iberian Peninsula. In the Mediterranean, both H12 and H13 were separated by four mutational steps from H20 while six haplotypes (H2, H8–H11, H19) formed a group of phylogenetically close sequences that may have radiated from H2.

Barcoding gap and species identification

To investigate the potential of the COI sequences to discriminate between *Nassarius nitidus* and *Nassarius reticulatus*, the 23 haplotypes of *N. nitidus* were compared with 36 COI haplotypes of *N. reticulatus* identified in a former study that covered most of the species range (Couceiro *et al.*, 2007). The NJ tree revealed that these two sets of sequences are reciprocally monophyletic and the distance between each cluster is comparable to the divergences estimated for other members of the genus *Nassarius* (Figure 3). K2P interspecific distances between *N. nitidus* and *N. reticulatus* were consistently larger than their intraspecific ones resulting in a clear-cut barcoding gap (Figure 4); the smallest interspecific distance (0.090) was more than triple the largest intraspecific one (0.026). The range of intraspecific distances was similar for the two *Nassarius* species (0.002–0.026), though the average distance was slightly larger within *N. nitidus* (0.011 ± 0.002; mean ± SE) than within *N. reticulatus* (0.008 ± 0.001). Average K2P corrected sequence divergence between species was 0.096 ± 0.016.

The analysis of the endonuclease recognition sites in our 395 bp fragment showed that enzyme *DdeI* (recognition site CTNAG) could provide a species-specific restriction fragment pattern. Folmer's primers originally employed in our study amplify a fragment considerably larger than the 395 bp portion included in our analysis. Hence, it is possible that the enzyme's recognition sites may be present in portions of the Folmer's fragment not included in our analysis. To avoid the interference of any unnoticed recognition site, we designed a new set of primers that more exclusively amplified the analysed portion. The resulting set (NasF 5′–AAC AGC TCA CGC TTT CGT AAT–3′; NasR 5′–AGG AAG ACG CTC AAA T–3′) produced a 373 bp fragment that largely overlapped (332 bp) with the 395 bp originally used in our recognition site analysis. Along this 373 bp fragment, there are three recognition sites for *DdeI* in *N. reticulatus* (producing 4 fragments of lengths 31, 138, 90 and 114 bp) and only 1 *N. nitidus* (2 fragments 31 and 342 bp long) that render a clearly distinctive pattern for each species (Figure 5). The presence of at least one recognition site for *DdeI* in each species was considered a convenient protection against false identifications (i.e. cases where no restriction is produced due to protocol failures rather than to the absence of recognition sites) because fragments must always be shorter after a successful restriction.

DISCUSSION

Strong population structure in *N. nitidus*

At the onset of this study we had anticipated that *Nassarius nitidus* would possibly display a shallow phylogeographical pattern comparable to that of *Nassarius reticulatus*

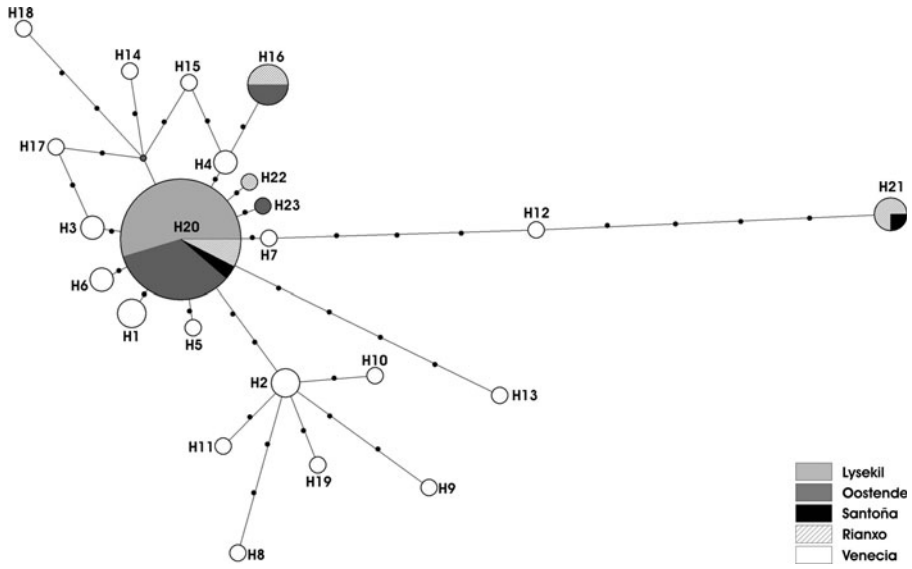

Fig. 2. Median-joining network of *Nassarius nitidus* cytochrome *c* oxidase subunit I haplotypes. Each haplotype depicted as a circle of area proportional to its relative frequency; shared haplotypes shown as pie-charts. Open symbols (Venice) denote Mediterranean haplotypes; shaded (shading varies with locality) symbols represent Atlantic ones. Dots indicate missing haplotypes. Each line between haplotypes represents a single mutation step between haplotypes.

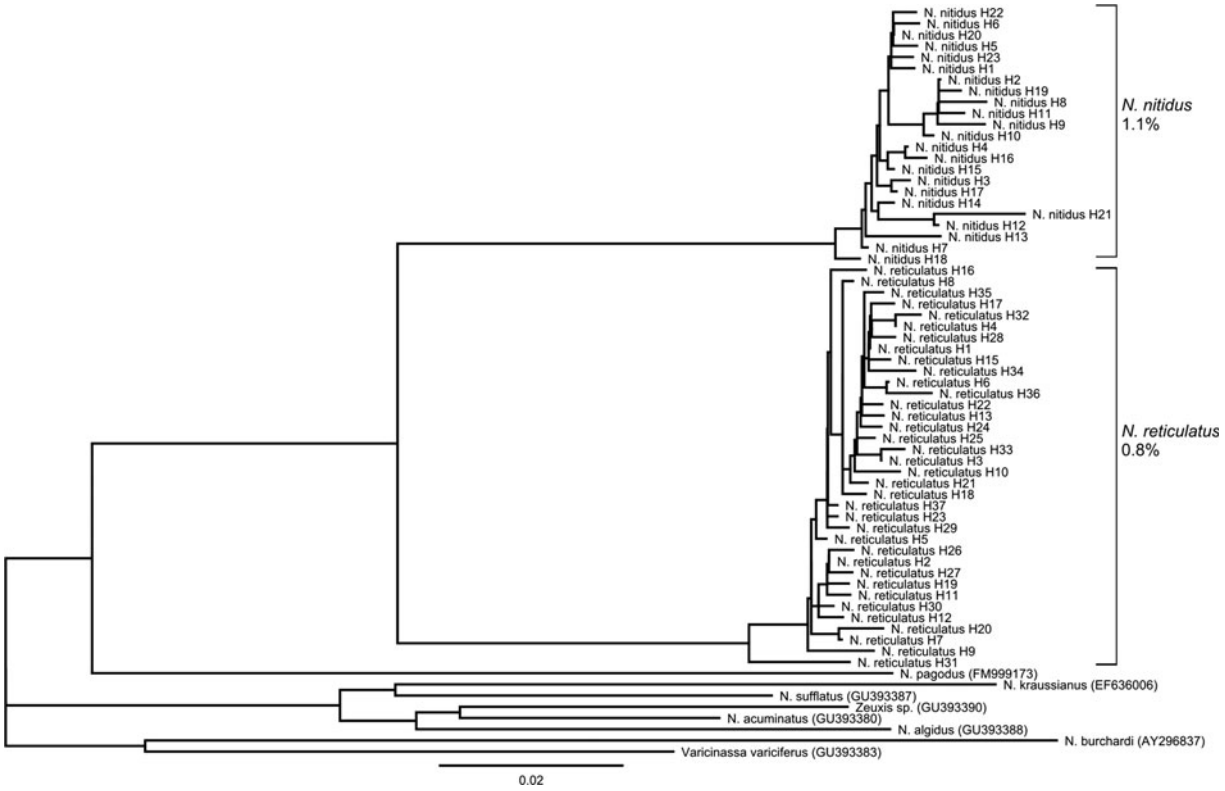

Fig. 3. Neighbour-joining tree based on Kimura-2-parameter (K2P) distances for cytochrome *c* oxidase subunit I DNA haplotypes from *Nassarius nitidus* and *Nassarius reticulatus* (visual representation of species diversity and relative divergence, not to be interpreted as a robust phylogenetic analysis). Average K2P divergence within each clade is listed. Sequences for other species of *Nassarius* were obtained from GenBank; species names retain those originally reported in GenBank (accession numbers indicated in parentheses). Scale bar units are substitutions per site.

(Couceiro *et al.*, 2007) because both species have a relatively long pelagic larval stage (1–2 months at typical ambient temperatures; Lebour, 1931; Tallmark, 1980). For most benthic marine species, dispersal occurs primarily during this pelagic larval stage (Cowen & Sponaugle, 2009) and longer pelagic stages typically provide greater potential for dispersal and gene flow. In this regard, several genetic studies with marine invertebrates reported a correspondence between increased potential for larval dispersal and diminished genetic differentiation among geographical locations (Palumbi, 2003; Avise, 2004; Bradbury *et al.*, 2008). This seemed to be the case of *N. reticulatus* where mtDNA data for locations spread across 1700 km of its European Atlantic range found only a weak, non-significant population structure (Couceiro *et al.*, 2007).

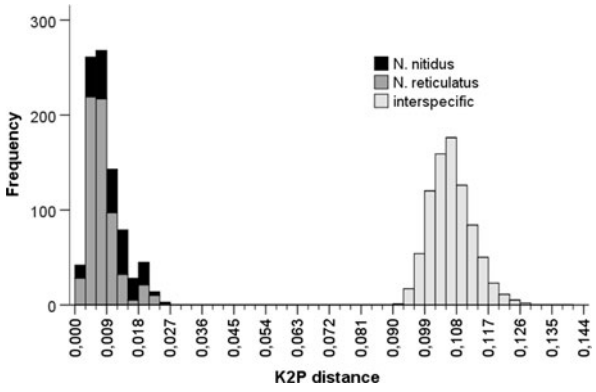

Fig. 4. Mismatch distribution of intraspecific variability and interspecific divergence between *Nassarius nitidus* and *Nassarius reticulatus*. Estimates are based on pairwise Kimura-2-parameter (K2P) distances between cytochrome *c* oxidase subunit I haplotypes.

In contrast with our expectations, and despite their biological similarity, our results reveal a pronounced population structure in *N. nitidus*. It could be argued that this difference might be an artefact from the fact that the current study covered a considerably wider range (more than 6500 km from Venice to Lysekil) that even crosses known phylogeographical breaks (English Channel and Strait of Gibraltar) not contained in the *N. reticulatus* study. However, even when locations separated by a comparable geographical distance are considered for *N. nitidus* (Ostend versus Rianxo, about 1500 km, pairwise $\Phi_{ST} = 0.195$, $P = 0.013$), we still find high, statistically significant differentiation estimates well above the maximum estimates recorded for *N. reticulatus* (Faro versus Plymouth, about 1700 km, pairwise $\Phi_{ST} = 0.035$, $P < 0.1$; Couceiro *et al.*, 2007).

Other authors have found that pelagic larval duration is not an infallible predictor of population genetic structure (see also Bird *et al.*, 2007). Larval duration is just one component of the dispersal process and larval behaviour, local hydrographic conditions, or both can also determine actual dispersal (Johnson & Black, 1991; Parsons, 1996). Along north-eastern Atlantic coastlines, *N. reticulatus* is commonly found at both sheltered and open-coast sites while *N. nitidus* mostly occurs in sheltered habitats (estuaries and saltmarshes) (Jeffreys, 1867; Rolán & Luque, 1994). It could be speculated that its dependence on habitats that commonly appear as isolated enclaves along the shoreline may have promoted the genetic differentiation among the various local populations. Evidence suggests that estuaries and other semi-enclosed

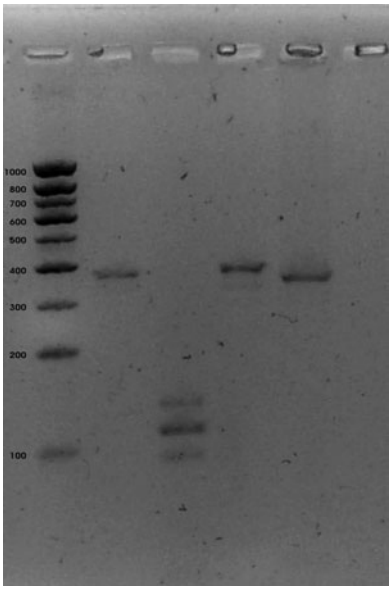

Fig. 5. Example of the distinctive restriction fragment pattern obtained in *Nassarius reticulatus* (lanes 2–3) and *Nassarius nitidus* (lanes 4–5). Agarose-gel electrophoresis of the 373 base pairs (bp) cytochrome *c* oxidase subunit I fragment before (lanes 2 and 4) and after (lanes 3 and 5) restriction with endonuclease *DdeI*. Fragment concentrations normally obtained after polymerase chain reaction do not allow the detection of the smallest (31 bp) restriction fragment on an agarose-gel. Lane 1 shows a 100 bp DNA ruler for reference.

water bodies may restrict gene flow and this effect may be more pronounced for species with a low ability to disperse, occur higher up in the estuary or both (Bilton *et al.*, 2002; Watts & Johnson, 2004). In a previous regional-scale study, we found no evidence of enhanced genetic differentiation between samples of *N. reticulatus* collected within and outside an estuary (Barreiro *et al.*, 2006). However, at least in the northern Iberian Peninsula, *N. reticulatus* abounds at mid–low reaches and adjacent open-coast sites while *N. nitidus* is mostly confined to the upper parts of the rias (drowned river valleys) suggesting that the isolating influence of the estuarine environment may be stronger in the latter.

Our results also show some evidence of a strong latitudinal decrease in genetic diversity, driven in large part by an increase in the relative abundance of haplotype H20. This pattern evokes the signature conventionally attributed to a postglacial recolonization from glacial refugia (Maggs *et al.*, 2008). Likewise, the large split in haplotype frequencies among Mediterranean and Atlantic populations combined with the occurrence of unique haplotypes within each region suggests a history of low genetic exchange between Atlantic and Mediterranean populations as noted in other marine organisms (Patarnello *et al.*, 2007). Although more work is clearly needed to corroborate this preliminary picture, it still suggests that *N. nitidus* might be an interesting candidate in the cumbersome search of phylogeographical patterns among coastal organisms in Europe (for a review see Maggs *et al.*, 2008).

Reliable species discrimination in the area of range overlap

Although molecular markers have yielded success in delineating species boundaries, their effectiveness can be limited in situations in which there is substantial overlap between intra- and interspecific variation (i.e. there is no barcoding 'gap'; Moritz & Cicero, 2004). Thus, a detailed analysis of barcoding performance within the species-rich cypraeid marine gastropods estimated a near 20% failure rate that was consistent with the observation that 23% of animal species may be not monophyletic (Funk & Omland, 2003; Meyer & Paulay, 2005). Cases such as that of *N. nitidus*, where a single species displays several morphs along its range, can be particularly worrisome as it might be suspected that this morphological variation may hide comparable genetic differences. Despite these expectations, the detection of reciprocal mitochondrial monophyly between our two species of *Nassarius* confirms the applicability of the barcoding approach to their discrimination. In fact, the magnitude of the sequence divergence detected between *N. nitidus* and *N. reticulatus* is similar to the divergence observed between other valid species within the genus (average K2P divergence = 16.2%, range 7.0–22.1%).

We did find a strong population structure in the area where *N. nitidus* overlaps with *N. reticulatus*. The separation is particularly pronounced between the Mediterranean and the Atlantic to the point that each region showed reciprocally unique haplotypes. However, the level of intraspecific variation is similar to what was recorded for *N. reticulatus* even though the latter only showed minimal signs of population structure across its European range (Couceiro *et al.*, 2007). More importantly, the magnitude of the intraspecific variation is considerably smaller than the differences detected between the two species. As a result, there is a substantial barcoding gap that allows the reliable discrimination between these two snails all along the area of range overlap. In fact, our between-species estimate (9.6 $\pm$ 1.6%) is very close to the average 11.1 $\pm$ 5.1% ($\pm$ standard deviation) sequence divergence obtained for 1155 congeneric molluscan species pairs (Hebert *et al.*, 2003b).

The restriction fragment protocol presented here allows for a quick species-specific identification. Restriction fragment patterns are a faster, albeit not necessarily cheaper, alternative to fragment sequencing, and may be useful in broad field surveys or serve as a diagnostic tool in the sorting of specimens for laboratory assays (together with non-destructive sampling; Palmer *et al.*, 2008). Thus, our method represents an alternative/complement to current diagnostic tools based on conventional taxonomy (Rolán & Luque, 1994) and on geometric morphometric approaches (Carvajal-Rodríguez *et al.*, 2006). Unlike those tools, barcoding and restriction analyses should offer unambiguous discrimination and the ability to deal with samples unmanageable by other techniques (tissue fragments, incomplete or damaged specimens, young stages).

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