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Behind the mask: cryptic genetic diversity of *Mytilus galloprovincialis* along southern European and northern African shores

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ABSTRACT

Morphological uniformity in geographically widespread species may cause genetically distinct entities to pass unnoticed if they can only be detected by molecular approaches. The importance of uncovering such cryptic diversity is prompted by the need to understand the putative adaptive potential of populations along species ranges and to manage biodiversity conservation efforts. In this study, we aim to assess cryptic intraspecific genetic diversity and taxonomic status of the widely distributed intertidal mussel *Mytilus galloprovincialis*, along Atlantic southwestern (SW) Iberian, Atlantic northwestern (NW) Moroccan and Mediterranean Tunisian shores. By using mitochondrial (16S restriction-fragment length polymorphism) and nuclear (polyphenolic adhesive protein gene, Glu-5′) markers, we discovered a more complex taxonomic diversity of *M. galloprovincialis* than previously known. Both Atlantic and Mediterranean haplogroups of *M. galloprovincialis* were detected along Atlantic SW Iberian shores along with *M. galloprovincialis*/*edulis* hybrids (92.2% Atlantic, 3.9% Mediterranean and 3.9% hybrids). In contrast, NW Moroccan populations consisted solely of Atlantic *M. galloprovincialis*. The Mediterranean populations did not include *M. galloprovincialis*/*edulis* hybrids, but both Atlantic (58%) and Mediterranean (42%) lineages were detected. Divergent selection between coastlines and/or indirect larval dispersal by human activities may be the drivers of this geographically structured genetic diversity.

INTRODUCTION

Phenotypic uniformity can conceal intraspecific genetic diversity or even cryptic species (Bickford *et al.*, 2007; Geller, Darling & Carlton, 2010). Surprisingly, cryptic genetic diversity occurs not only in poorly studied taxa, but also in widely studied species that may include several distinct genetic entities (e.g. Lee & Foighil, 2004; Kadarusman *et al.*, 2012) or even novel species (e.g. Zardi *et al.*, 2011a,b). This undetected diversity leads to widespread underestimation of biodiversity. The assessment of the genetic dimension of biodiversity allows the identification of evolutionarily significant units and an understanding of species' adaptive potential (e.g. Gibson & Dworkin, 2004; Paaby & Rockman, 2014). Elucidation of cryptic complexes is particularly relevant to a functional definition of management and conservation efforts (Bickford *et al.*, 2007). When cryptic genetic diversity is revealed, complex distributional patterns of species and/or cryptic clades often emerge (e.g. Bierne *et al.*, 2002; Lee & Foighil, 2004; Johnson, Warén & Vrijenhoek, 2008; Kadarusman *et al.*, 2012). Moreover, in contact zones, hybrid populations may develop, characterized by offspring of mixed ancestry (Väinölä, 1991; Bierne *et al.*, 2003; Hilbish *et al.*, 2012).

Blue mussels of the genus *Mytilus* are dominant components of temperate marine rocky shores throughout the northern and southern hemispheres (Hilbish *et al.*, 2000). Genetic analyses have revealed three closely related species: *M. edulis* (Linnaeus, 1758), *M. trossulus* (Gould, 1850) and *M. galloprovincialis* (Lamarck, 1819) (Koehn, 1991; McDonald, Seed & Koehn, 1991; Plazzi & Passamonti, 2010; Plazzi *et al.*, 2011). *Mytilus trossulus* is a boreal species distributed in the northeastern and northwestern (NW) Pacific, NW Atlantic, North Sea, Baltic Sea, White Sea and northern Norway and Barents Sea (e.g. McDonald *et al.*, 1991; Väinölä & Strelkov, 2011; Śmietanka *et al.*, 2013). *Mytilus edulis* is a cold-temperate species found from the White Sea through Iceland and the British Isles to southern France (Edwards & Skibinski, 1987; Sanjuan, Zapata & Alvarez, 1994 and references therein), reaching its southernmost European limit in the Bay of Biscay (Hilbish *et al.*, 2012). The warm-temperate *M. galloprovincialis* evolved in the Mediterranean and later expanded along Atlantic shores as far as the British Isles and North Africa (Gosling, 1992). This widespread geographical distribution comprises distinct genetic entities (Hilbish *et al.*, 2000): a North Atlantic haplogroup that includes *M. edulis* and a Mediterranean haplogroup.

Hybridization occurs between sympatric *Mytilus* species or genetic entities (e.g. Hilbish *et al.*, 2012); several hybrid zones have been reported between *M. edulis* and *M. trossulus* in the Baltic Sea (Stuckas *et al.*, 2009) and North America (Toro, Thompson & Innes, 2002). Furthermore, *M. galloprovincialis* and *M. edulis* have partially overlapping distributions along the Bay

of Biscay (e.g. Hilbish *et al*., 2012; Gosset & Bierne, 2013), southwestern (SW) England (Edwards & Skibinski, 1987) and Ireland (Gosling, Doherty & Howley, 2008), creating a mosaic structure where pure genotypes of both species alternate with hybrids.

Within *M. galloprovincialis*, an additional hybrid contact zone has been reported along the European side of the Almeria-Oran front (AOF; stretching from Almeria on the Spanish coast to Oran on the Algerian coast; Quesada, Zapata & Alvarez, 1995c). This oceanographic front separates Atlantic from Mediterranean waters, driven by major current eddies (Tintore *et al*., 1988). This hybrid zone separates two distinct lineages: an Atlantic lineage, occurring from northern Spain around Portugal to southeastern Spain (Almeria), and a Mediterranean lineage, from Alicante into the Mediterranean. The stretch of coast between Almeria and Alicante corresponds to a discontinuity in mussel distribution, possibly being unsuitable for mussels due to unfavourable environmental conditions (Quesada *et al*., 1995c).

Through interoceanic dispersion, *M. galloprovincialis* naturally invaded southern hemisphere shorelines between 0.84 Ma (Gérard *et al*., 2008) and 1.2 Ma (Hilbish *et al*., 2000), evolving into a genetically distinguishable southern lineage (Westfall, Wimberger & Gardner, 2010). In the southern hemisphere, the native range of *M. galloprovincialis* includes Australia, New Zealand and Chile (Hilbish *et al*., 2000; Gérard *et al*., 2008; Westfall & Gardner, 2010, 2013) and it has been accidentally introduced to South Africa (Grant & Cherry, 1985).

Notably, recent studies have shown extensive invasion of northern hemisphere *M. galloprovincialis* in Australia, Chile and New Zealand; in fact 10–80% of *M. galloprovincialis* sampled in the southern hemisphere were identified as of northern hemisphere origin (Westfall & Gardner, 2010). Additionally, ongoing hybridization has been reported from the southern hemisphere where northern and southern lineages of *M. galloprovincialis* coexist (Westfall & Gardner, 2013).

In contrast to southern hemisphere and northern European shores, the taxonomic status of *Mytilus* along Atlantic SW Iberian and NW Moroccan coastlines remains relatively unexplored (but see Quesada *et al*., 1995c; Comesaña, Posada & Sanjuan, 1998; Daguin & Borsa, 1999; Daguin & Borsa, 2000; Fraïsse *et al*., 2014; Ouagajjou & Presa, 2015). The present study addresses genetic diversity of native *Mytilus* and assesses possible inter-hemispheric invasion along southern European and northern African shores, using a combination of diagnostic species and lineage-specific nuclear and mitochondrial markers for the genus *Mytilus*. Specifically, a mitochondrial DNA 16S restriction-fragment length polymorphism (RFLP) assay is here employed to distinguish: (1) *M. trossulus*; (2) invasive southern hemisphere *M. galloprovincialis* haplogroup; (3) Mediterranean, northern hemisphere *M. galloprovincialis* haplogroup and (4) North Atlantic northern hemisphere *M. edulis*/*M. galloprovincialis* haplogroup (Rawson & Hilbish, 1995; Hilbish *et al*., 2000; Westfall *et al*., 2010). The 16S data cannot distinguish between *M. edulis* and *M. galloprovincialis*, so we also used the size of polymerase chain reaction (PCR) amplified fragments of the nuclear-DNA Glu-5′gene to characterize these species (Inoue *et al*., 1995). Used in conjunction, these markers allow ready taxonomic identification among the three species within the *M. edulis* complex.

MATERIAL AND METHODS

Sampling and DNA extraction

A total of 249 samples of *Mytilus galloprovincialis* were collected from 11 locations along the SW Iberian, NW Moroccan and Mediterranean Tunisian intertidal shores between November 2010 and April 2012 (Table 1; Fig. 1). Mantle tissue (20–30 mg) was dissected from each individual, preserved in 96% ethanol and stored at −20 °C. To avoid the complication of doubly-uniparental inheritance (Skibinski, Gallagher & Beyonon, 1994; Zouros *et al*., 1994) in gonadal tissue, only female mtDNA was targeted in our study by selecting muscle tissue. Total genomic DNA extraction was performed using a standard Proteinase K protocol (adapted from Sambrook, Fritseh & Maniatis, 1989).

DNA markers: 16S RFLP

Universal primers amplifying portions of the 16S rRNA gene (16SAR 5′-CGCCTGTTTAACAAAAACAT-3′ and 16SBR 5′-CCGGTTTGAACTCAGATCACGT-3′, Palumbi, 1996) were used for PCR. PCR was performed in 25 μl containing 1–10 ng of total DNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 1 mM of MgCl2, 1× GoTaq Flexi Buffer (Promega, USA) and 1 U GoTaq DNA Polymerase (Promega, USA). Amplification used an initial denaturation during 5 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 46 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. PCR products were sequenced directly in an automated DNA sequencer (ABI PRISM 3130, Applied Biosystems) using PCR primers and the BigDye Terminators v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster-City, CA, USA).

The 16S rRNA sequences were virtually digested using the web-based tool Restriction Digest available at http://www.bioinformatics.org/sms2/rest_digest.html (accessed 20 November 2014). The virtual digestion using *Eco*RV, *Nhe*I and *Spe*I enzymes allows us to distinguish between *M. trossulus*, North Atlantic *M. edulis*/*M. galloprovincialis* haplogroup and northern Mediterranean and southern hemisphere *M. galloprovincialis* (Hilbish *et al*., 2000; Westfall *et al*., 2010). Published partial

Table 1. List of sampling locations in southwestern Iberia, Tunisia and northwestern Morocco with coordinates and sample size for each.

Country	Location	Code	Coordinates	Sample size
Portugal	Sagres	SG	37°00′23.69″N; 8°56′55.80″W	18
Portugal	Vilamoura	VL	37°04′05.50″N; 8°06′42.64″W	24
Portugal	Tavira	TV	37°06′41.06″N; 7°36′57.99″W	25
Spain	Punta del Moral	PM	37°10′57.76″N; 7°19′46.83″W	25
Spain	Atlanterra	AT	36°05′24.78″N; 5°48′43.02″W	23
Tunisia	Bizerte	BZ	37°15′10.41″N; 9°56′41.15″E	22
Tunisia	Kourbous	KR	36°49′29.04″N; 10°33′59.97″E	21
Morocco	Rabat	RB	34°1′57.26″N; 6°50′27.96″W	23
Morocco	Casablanca	CB	33°39′7.22″N; 7°29′3.11″W	22
Morocco	Sidi Bouzid	SB	33°13′6.11″N; 8°34′23.19″W	25
Morocco	El Beddouza	EB	32°32′42.33″N; 9°16′55.34″W	21

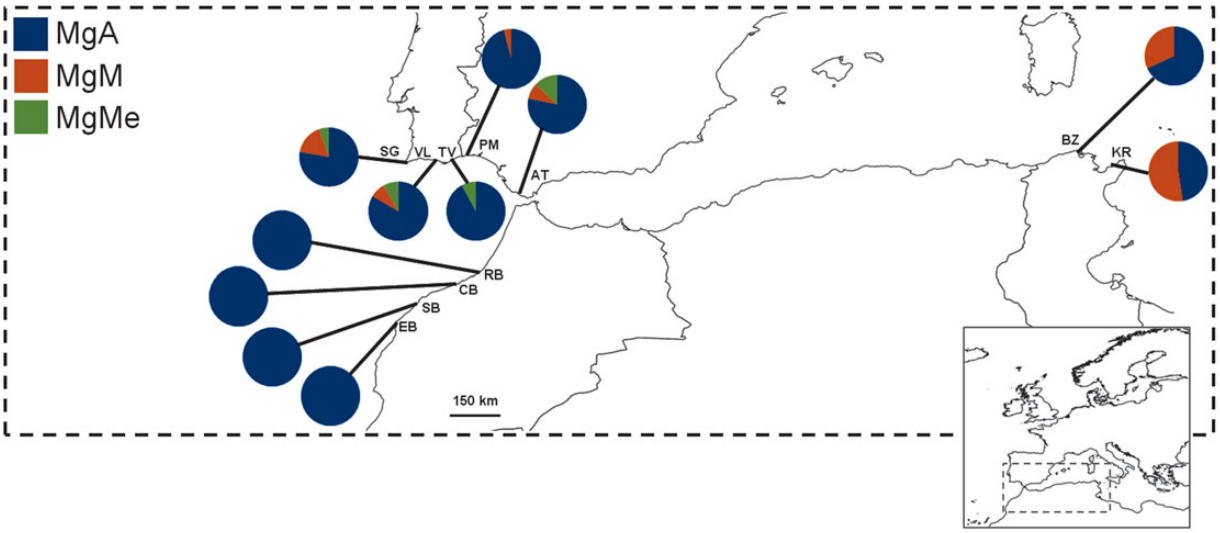

Figure 1. Geographic location of southwestern Iberian, northwestern Moroccan and Mediterranean Tunisian sampling sites and results of combined16S and Me 15/16 identification. Abbreviations: MgA, Atlantic *Mytilus galloprovincialis*; MgM, Mediterranean *M. galloprovincialis*; MgMe, hybrids of *M. galloprovincialis* and *M. edulis*. Location codes are according to Table 1.

sequences representing the different haplogroups were retrieved from GenBank (accession numbers: GQ455380–GQ455405). Following an alignment and trimming of GenBank sequences and the ones from this study with subsequent triple (virtual) digestion, *M. trossulus* is expected to display three bands (band sizes: 370, 85 and 82 bp), North Atlantic *M. edulis*/*M. galloprovincialis* haplogroup four bands (band sizes: 342, 85, 82 and 28 bp), northern Mediterranean *M. galloprovincialis* two bands (band sizes: 342 and 195 bp) and southern *M. galloprovincialis* three bands (band sizes: 342, 167 and 28 bp) (from Westfall *et al.*, 2010; Table 2). Individuals were classified according to band sizes and the proportion of allocation to each haplogroup was quantified for each sampling location.

To confirm the identification of each specimen as determined by band sizes, a phylogenetic tree was built with sequences retrieved from GenBank (for southern *M. galloprovincialis* sequences with GQ455381–GQ455383, GQ455385–GQ455389, GQ455391, GQ455394–GQ455395; for northern Mediterranean *M. galloprovincialis* sequences with GQ455380, GQ455384, GQ455398; for North Atlantic *M. edulis*/*M. galloprovincialis* sequences with GQ455392–GQ455393, GQ455396–GQ455397, GQ455399, GQ455405 and for *M. trossulus* sequences with GQ455400, GQ455402, GQ455404). These and our sequences were combined in one dataset and haplotypes were estimated through COLLAPSE v. 1.2 (Posada, 2004). The Akaike Information Criterion implemented in jModelTest v. 0.1.1 (Posada, 2008) selected TnR + G as the best-fit model to be used in MrBayes v. 3.2 (Ronquist *et al.*, 2012). The phylogenetic tree was built using Bayesian inference (BI) performed with MrBayes v. 3.2, running for 500,000 generations (three simultaneous Monte Carlo Markov chains; sample frequency 500). Two independent runs were performed. Burn-in was set to the first 25% of generations. The robustness of the inferred Bayesian trees was determined using Bayesian posterior probabilities (as obtained from majority-rule consensus trees of the post burn-in trees).

Partial sequences representing the 249 individuals used in this study were deposited in GenBank (accession numbers: KM056770–KM056975 and KP202882–KP202924).

DNA markers: Glu-5′

Primers Me 15/16 (Me15, 5′-CCAGTATACAAACCTGTGAAGA-3′ and Me16, 5′-TGTTGTCTTAATAGGTTTGTAAGA) targeted the nuclear polyphenolic adhesive protein gene (Glu-5′, Inoue *et al.*, 1995). Amplification was performed in a 10 μl reaction volume containing 1–10 ng of total DNA, 0.25 μM of each primer, 0.05 mM of each dNTP, 1× GoTaq Flexi Buffer (Promega, USA), 3 mM of MgCl2 and 1 U GoTaq DNA Polymerase (Promega, USA). Amplified fragments were obtained under the following PCR conditions: initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 90 s and a final extension at 72 °C for 4 min. PCR products stained with GelRed were run and scored on 2% agarose gel.

The size of the Me 15/16 PCR fragments is species-specific and used to distinguish between *M. trossulus*, *M. edulis* and *M. galloprovincialis* (Inoue *et al.*, 1995). The expected fragment size for *M. trossulus* is 168 bp, for *M. edulis* is 180 bp and for *M. galloprovincialis* is 126 bp (Inoue *et al.*, 1995; Table 2).

Table 2. Identification of *Mytilus* species and genetic haplogroups based on number and size of bands for both 16S RFLP and Glu-5′.

16S RFLP				Glu-5′		
Mt (bp)	Me/MgA (bp)	MgM (bp)	MgS (bp)	Mg (bp)	Me (bp)	Mt (bp)
370	342	342	342	126	180	168
85	85	195	167			
82	82		28			
	28					

Abbreviations: Mt, *M. trossulus*; Me/MgA, North Atlantic *M. edulis/M. galloprovincialis* haplogroup; MgM, Mediterranean *M.* galloprovincialis; MgS, southern hemisphere *M. galloprovincialis*; Mg, *M. galloprovincialis*; Me, *M. edulis*; Mt, *M. trossulus*.

RESULTS

16S RFLP

Virtual digestion revealed two distinct haplogroups, Atlantic *Mytilus edulis*/*M. galloprovincialis* (northern hemisphere; North Atlantic haplogroup) and northern Mediterranean *M. galloprovincialis* (northern

hemisphere, hereafter referred to as Mediterranean haplogroup according to Westfall & Gardner, 2013), while no *M. trossulus* individuals were recorded. BI analyses confirmed virtual digestion results (Supplementary material, Fig. S1). However, one individual showed the typical number and size of southern *M. galloprovincialis* fragments, while it clustered together with *M. edulis*/*M. galloprovincialis* haplogroup in the phylogenetic tree (individual SB02 in Supplementary material, Fig. S1). Haplogroup frequency revealed that the Atlantic *M. edulis*/*M. galloprovincialis* haplogroup represented 89.6% of all individuals (223 out of 249) while the Mediterranean *M. galloprovincialis* haplogroup represented 10.4% (26 out of 249). The Mediterranean *M. galloprovincialis* haplogroup was found only at four Iberian and two Tunisian sites and not at Moroccan locations, whereas the Atlantic *M. edulis*/*M. galloprovincialis* haplogroup was widespread across Iberian, Moroccan and Tunisian locations (Fig. 1). The Mediterranean *M. galloprovincialis* haplogroup occurred at much higher frequency inside the Mediterranean (41.9%) than in Atlantic locations (3.9%). The SW Iberian coast was comprised of 93% Atlantic *M. edulis*/*M. galloprovincialis* and 7% of Mediterranean *M. galloprovincialis*. The NW Moroccan coast was 100% Atlantic *M. galloprovincialis*, while Tunisia was 58% Atlantic and 42% Mediterranean *M. galloprovincialis*.

Glu-5′

The Me 15/16 PCR assay targeting the Glu-5′ gene did not detect any *M. edulis* or *M. trossulus* individuals (i.e. no band sizes typical of these two species appeared). Because band sizes were 126 bp, 241 individuals previously classified (by DNA 16S RFLP assay) as Atlantic *M. edulis*/*M. galloprovincialis* or Mediterranean *M. galloprovincialis* were assigned to *M. galloprovincialis*. Additionally, 8 individuals out of 249 (3.2%) dispersed among the SW Iberian locations (Fig. 1) and classified as *M. edulis*/*M. galloprovincialis* haplogroup displayed two fragments (126 and 180 bp), typically found in hybrid forms of *M. galloprovincialis* and *M. edulis* (Inoue *et al.*, 1995; hereafter referred to as *M. galloprovincialis*/*M. edulis* hybrids).

DISCUSSION

Our results show extensive cryptic taxonomic diversity in the genus *Mytilus* along Atlantic SW Iberian, NW Moroccan and Mediterranean Tunisian shores. Recent studies have described a widespread invasion of northern hemisphere *M. galloprovincialis* and interspecific hybrids (of *M. edulis* and *M. galloprovincialis*) into the southern hemisphere (Westfall & Gardner, 2010; Westfall & Gardner, 2013). Although our study did not reveal invasive southern hemisphere *Mytilus* in the northern hemisphere, the combined information provided by the mitochondrial DNA 16S RFLP assay and nuclear-DNA Glu-5′ highlights a more complex distributional pattern of lineages and hybrids than previously shown.

Hybrids of *M. galloprovincialis*/*M. edulis* are well described from northern Spain, France (Coustau, Renaud & Delay, 1991; Bierne *et al.*, 2002; Hilbish *et al.*, 2012) SW England (Edwards & Skibinski, 1987) and Ireland (Gosling *et al.*, 2008), but have rarely been reported from Atlantic southern European shores (Comesaña *et al.*, 1998; Kijewski *et al.*, 2011). Here, we show that these hybrids are broadly distributed at relatively low frequency in SW Iberia. Interestingly, hybrids were not detected at any of the NW Moroccan or Mediterranean sites. Divergent environmental conditions may be the cause of such sharp geographical structure.

Sea surface temperature (SST) is recognized as a major determinant of geographic distributions of many marine species over large (latitudinal) geographic scales (Parmesan, 2006; Jones, Lima & Wethey, 2010). SST clines can define species' distributional limits and shifts, either directly acting on organisms' thermal tolerance or indirectly by changing competition or predation dynamics (Southward, Hawkins & Burrows, 1995; Parmesan, 2006; Jones *et al.*, 2010). Atlantic shores between the Iberian Peninsula and North Africa are characterized by a strong, persistent latitudinal gradient in SST (Lima *et al.*, 2007; Nicastro *et al.*, 2013). This environmental cline marks distributional boundaries of many cold- and warm-water species (e.g. Lima *et al.*, 2007) and could explain the lack of cold-temperate *M. edulis* hybrids along Moroccan shores. The role played by SST in latitudinally segregating distinct *Mytilus* entities is further supported by the absence of the pure, cold-water species *M. edulis* along our study area. The absence of *M. edulis* from southern Iberia was also reported by Fraïsse *et al.* (2014). Similarly, relatively warmer SST along Tunisian shores (Goddard Earth Sciences, Data and Information Services Center; http://disc.sci.gsfc.nasa.gov, accessed 22 November 2014) could exclude *M. edulis* and its hybrids from Mediterranean waters.

Wave action is one of the major stresses affecting intertidal communities (e.g. Zardi *et al.*, 2008). The capability of a mussel to resist waves and avoid being dislodged depends primarily on its byssal attachment strength. While *M. galloprovincialis* is more strongly attached to the substratum than *M. edulis* and thus less easily dislodged by waves (Schneider *et al.*, 2005), intermediate attachment strength values have been found in hybrids of the two species (Willis & Skibinski, 1992). These differences in attachment strength indicate that wave action is a potential determinant of competitive interactions and ultimately habitat segregation (Schneider *et al.*, 2005). However, it is unlikely that hydrodynamic stress is responsible for the exclusion of species or lineages at latitudinal scales; waves are more likely to affect species zonation at small (Zardi *et al.*, 2006) and/or meso-scales (Nicastro *et al.*, 2008), rather than at large geographic scales as in the patterns described in the present study.

In addition to the limited geographical distribution of *M. galloprovincialis*/*M. edulis* hybrids along our study area, individuals belonging to the Mediterranean *M. galloprovincialis* haplogroup were restricted to four SW Iberian and the two Mediterranean sites, and absent from Moroccan shores. The study area is characterized by several oceanographic currents with contrasting directionalities and intensities. The SW coast of the Iberian Peninsula, as well as the northwest Moroccan coastline, is mainly affected by the eastward-flowing Azores Current (Martins, 2002). The eastward branch of this current enters the Gulf of Cadiz and flows south, pushed by eddies and gyres occurring offshore in front of the southern coast of Portugal (Martins, 2002). The Canary Current transports the North Atlantic Central Water southwards, along the Atlantic western coast of Morocco (Knoll *et al.*, 2002). All of these Atlantic oceanographic features are likely to promote larval dispersal, thus reducing between-region genetic structuring of broadcast-spawning invertebrates inhabiting these shores (e.g. Quinteiro, Rodríguez-Castro & Rey-Méndez, 2007; Lourenço, 2012).

The Strait of Gibraltar is the meeting point between Atlantic and Mediterranean waters. A flux of surface Atlantic waters enters the Mediterranean through the Strait of Gibraltar and circulates in two anticyclonic gyres: the west Alboran gyre and the east Alboran gyre (EAG) (Helguen *et al.*, 2002). The eastern edge of the EAG flowing along the Algerian coast corresponds to the AOF. The AOF is a sharp hydrogeographical transition located at the easternmost boundary of the Alboran Sea; it is characterized by strong salinity and SST gradients coupled with strong currents, eddies and gyres separating Western Mediterranean and Atlantic waters (Tintore *et al.*, 1988). The AOF is recognized as barrier to larval dispersal, causing several species to show clear genetic structuring (Borsa, Blanquer & Berrebi, 1997; Patarnello, Volckaert & Castilho, 2007). In

M. galloprovincialis a distinct genetic break at the AOF has been shown with a wide range of markers: mitochondrial DNA (Quesada, Beynon & Skibinski, 1995a; Sanjuan, Comesaña & De Carlos, 1996), nuclear DNA (mac-1: Daguin & Borsa, 1999; Daguin, Bonhomme & Borsa, 2001; microsatellites: Ouagajjou & Presa, 2015) and a combination of mitochondrial and nuclear markers (Śmietanka, Burzyński & Wenne, 2010; Kijewski *et al.*, 2011). Much weaker (but still visible) divergence was detected with allozymes (locus ODH; Sanjuan *et al.*, 1994; Quesada *et al.*, 1995c) and microsatellites (Diz & Presa, 2008). It has been suggested that the AOF genetic structure is the result of secondary contact between an Atlantic and a Mediterranean lineage that occurred before the formation of the AOF (Quesada, Wenne & Skibinski, 1995b). In addition to present day hydrography and environmental features, the complex geological and climatic history of the Mediterranean might have contributed to the biogeographical separation between Atlantic and Mediterranean biota (Patarnello *et al.*, 2007 and references therein). Over the last few million years, the Mediterranean Sea has been affected by several geological events; recurrent glaciations during the Pleistocene and dramatic drop of the sea level during the 'Messinian salinity crisis' influenced contemporary intra- and interspecific diversity within the Mediterranean and between the Mediterranean and the Atlantic Ocean. However, marker-specific genetic patterns, together with the widespread occurrence of Mediterranean and Atlantic *M. galloprovincialis* in Atlantic and Mediterranean waters reported in our study, suggest that, at the AOF, a semipermeable genetic barrier is acting together with oceanographic (e.g. currents) and environmental (e.g. SST) barriers (Galarza *et al.*, 2009; Bierne *et al.*, 2011 and references therein; Sá-Pinto *et al.*, 2012). Although the divergence time of the Atlantic and Mediterranean lineages remains unknown, the complex geological history of the Mediterranean (e.g. Messinian salinity crisis, when the connection between Atlantic and Mediterranean waters was lost) might have shaped and separated these two lineages which then evolved in allopatry.

In the Anthropocene era (Frawley & McCalman, 2014) the effect of human activities on the redistribution of marine biota must be considered in addition to environmental divergent selection and changing climate. Ballast water has been recognized as a primary source of invasive species in coastal freshwater and marine ecosystems (Carlton, 1999). Ballast water can transport thousands of species per day throughout the world (exceeding 10,000 species; Carlton, 1999), which can be released in a completely different region, erasing the effects of any natural geographical barriers (Flagella, Soria & Buia, 2006; Gollasch *et al.*, 2002). It has also been shown that hull fouling and seawater ballast tanks are major vectors for the spread of invasive organisms (Coutts, Moore & Hewitt, 2003; Hopkins & Forrest, 2008). There is evidence that the distribution of Mytilidae has been enhanced by both ballast-water transport and ship fouling (Wonham, 2004). Moreover, unmanaged vessel fouling and incorrect vessel cleaning procedures can contribute to the propagation of invasive species, by acting as suitable substratum or releasing individuals to new areas (Hopkins & Forrest, 2008; Cunha *et al.*, 2014), thus affecting the ecological equilibrium of recipient communities. The southwest Iberian coast is an area known for intense shipping and it lies on the main route to northern regions from the Mediterranean (Flagella *et al.*, 2006; Janeiro, Martins & Relvas, 2012). Maritime traffic has been increasing around the world as a response to human demands and the traffic inside Mediterranean is likely to continue increasing as well (Flagella *et al.*, 2006). The predicted increase in shipping activities will presumably enhance invasive events as it has in the past decades (Ruiz *et al.*, 1997; Flagella *et al.*, 2006; Gollasch, 2006). In 2001, around 39,000 ship arrivals have been recorded for the international Italian harbours of Naples and Salermo (Flagella *et al.*, 2006), revealing an intense maritime traffic for just two single harbours. In particular, the connectivity of Mediterranean harbours, such as of these two Italian harbours, is more frequent with Iberia than with North Africa, thus increasing the opportunity for transportation of Mediterranean *M. galloprovincialis* specimens to southern Portugal and Spain rather than to Morocco (Flagella *et al.*, 2006).

In conclusion, the combination of the species-specific nuclear Glu-5′ and mitochondrial DNA 16S RFLP assay has identified novel and geographically structured distribution of *M. galloprovincialis* lineages and hybrids. The Atlantic lineage is widely spread over the study area and the Mediterranean lineage is dispersed along SW Iberian and Mediterranean shores, while hybrids of *M. galloprovincialis*/*edulis* are restricted to SW Iberian shores. We anticipate that the use of these markers to characterise Atlantic North European and Mediterranean mussel populations will be crucial to unveil further the complexity and geographic distribution of *M. galloprovincialis*.

The significance of our findings extends beyond theoretical interest. Because different genetic entities may display diverse ecophysiological attributes (e.g. Pfennig, Rice & Martin, 2007; Zardi *et al.*, 2011a,b), the detection of intraspecific genetic variation and hybrid zones is of critical importance for a complete understanding of ongoing and future population dynamics.

In addition to individual biological attributes, habitat complexity and community composition of different *Mytilus* (species or hybrid) bed assemblages deserve to be investigated. Different mussel taxa can display distinct bioengineering properties (e.g. Nicastro *et al.*, 2012) that can affect diversity and abundance associated with mussel aggregations (e.g. Cole, 2010).

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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