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Re-assessing the origins of the invasive mussel *Mytilus galloprovincialis* in southern Africa

G. I. Zardi[A], *C. D. McQuaid*[A], *R. Jacinto*[B], *C. R. Lourenço*[A], *E. A. Serrão*[B] *and K. R. Nicastro*[B,C]

[A]Department of Zoology and Entomology, Rhodes University, Grahamstown, 6140, South Africa.
[B]CCMAR (Centro de Ciencias do Mar), CIMAR Laboratório Associado, Universidade do Algarve, Campus de Gambelas, PT-8005-139 Faro, Portugal.
[C]Corresponding author. Email: katynicastro@gmail.com

Abstract. Retracing the origins of invasive species is a first critical step in identifying potential mechanisms of introduction, implementation of management strategies and forecasting the spread of the invader. *Mytilus galloprovincialis* is an intertidal mussel that is widely distributed in many temperate and subtropical regions. It is invasive worldwide and the most successful invasive marine species in southern Africa. Previous studies have examined genetic relationships between a few South African populations from the south-western coast and other worldwide populations, presenting evidence of a north-eastern Atlantic origin of the invasion. Here, a combination of nuclear (Me15/16 PCR-based) and mitochondrial (16S restriction fragment-length polymorphism; 16S RFLP) DNA assays was applied to infer the origin of this strong invader across its entire southern African distribution (South Africa and Namibia). The 16S RFLP confirmed the northern hemisphere as being the likely sole source of invasion. Additionally, the frequencies of haplotypes at the 16S marker and alleles at the Me15/16 locus point to north-eastern Atlantic shores as the most likely origin throughout the Namibian and South African distribution of the species.

Additional keywords: biogeography, intraspecific biodiversity, invasive species, mitochondrial DNA markers, nuclear DNA markers.

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Introduction

Recognising the full identity of an invasive species, at both the inter-specific and intra-specific levels, is critical to the understanding of the origins of introduction and of the potential ecological impacts of the invasion. Additionally, it is a prerequisite to highlight global hotspots vulnerable to invasion that can inform management and conservation efforts that aim at the prevention or early detection of invasive species (e.g. Hulme 2009; Geller *et al.* 2010).

The blue mussel, *Mytilus galloprovincialis*, is one of three commercially and ecologically important sibling species in the *M. edulis* species complex: *M. edulis* (Linne. 1758), *M. galloprovincialis* (Lam. 1819) and *M. trossulus* (Gould, 1850). *Mytilus* spp. are antitropically distributed throughout cold, temperate and subtropical shores of both hemispheres (Hilbish *et al.* 2000). *Mytilus trossulus* is restricted to the Baltic Sea, north-eastern Pacific and north-western Atlantic (e.g. McDonald *et al.* 1991). *Mytilus edulis* is present in the White Sea through Iceland and the British Isles, down to the southern coast of France (Sanjuan *et al.* 1994, and references therein). *Mytilus galloprovincialis* has the most extensive distribution of the three; it successfully occupies shores on every continent except Antarctica (McQuaid *et al.* 2015).

This smooth-shelled blue mussel has a complex evolutionary history; it is native to the Mediterranean and the Atlantic coasts of Europe and north Africa (Gosling 1992), and dispersed to the southern hemisphere during the Pleistocene between 0.5 million and 1.3 million years ago (Gérard *et al.* 2008) where it now represents a genetically distinct southern subclade (Hilbish *et al.* 2000) with a range that includes Australia, New Zealand and Chile (Westfall and Gardner 2010). Along North Atlantic shores, its distribution partially overlaps with that of *M. edulis*, and extensive *M. galloprovincialis*–*M. edulis* hybrid zones have been identified along Atlantic French shores (Hilbish *et al.* 2012; Gosset and Bierne 2013).

Northern hemisphere *M. galloprovincialis*, including *M. edulis*–*M. galloprovincialis* hybrids, have been widely introduced in the southern hemisphere, including Australia, Chile and New Zealand (Westfall and Gardner 2010).

The most rapid invasion by *M. galloprovincialis* took place in southern Africa (Grant and Cherry 1985; Branch and Steffani 2004), where it has spread along more than 2800 km of coast (Grant and Cherry 1985; Assis *et al.* 2015). Of all the different mechanisms responsible for aquatic invasions, ballast water is the primary vector for the transport and discharge of planktonic organisms (Carlton and Geller 1993). It is thus commonly

assumed that *M. galloprovincialis* larvae arrived in South Africa in the late 1970s traveling in ballast water (e.g. Branch and Steffani 2004). In general, ports are the most common sources and recipients of introduced species and (e.g. Drake and Lodge 2004; Voisin *et al.* 2005); southern Africa has several major international commercial ports including important centres of worldwide shipping activities. The recent arrival of *M. galloprovincialis* in southern Africa is confirmed by low genetic diversity over its entire geographic range there (Zardi *et al.* 2007) and the absence of blue mussels from prehistoric shell middens and museum shell collections (Grant and Cherry 1985). *Mytilus galloprovincialis* has been particularly successful on the western coast of southern Africa, where, as a result of its extremely high growth and recruitment rates, it has partially replaced the indigenous mussels *Aulacomya ater* and *Choromytilus meridionalis* and has become the dominant intertidal mussel (Robinson *et al.* 2007). On sheltered shores, limpets (*Scutellastra granularis* and S. *argenvillei*) have also been negatively affected by the colonising abilities of *M. galloprovincialis* (Steffani and Branch 2003, 2005). On the southern coast of South Africa, *M. galloprovincialis* has not completely displaced the indigenous mussel *Perna perna* and the two exhibit spatial segregation along the intertidal (Bownes and McQuaid 2009; Nicastro *et al.* 2010).

Despite its various negative ecological impacts, the introduction of *M. galloprovincialis* has positive aspects. The endangered African black oystercatcher, *Haematopus moquini*, has changed its diet from indigenous limpets and mussels to the invasive mussel and there is evidence that population sizes and breeding productivity have increased as a result of the increase of prey biomass and availability during low tide because of the arrival of *M. galloprovincialis* on the upper shore (Vernon 2004; Brown and Hockey 2007; Coleman and Hockey 2008). The positive economic impact of *M. galloprovincialis* has also been significant as the South African mussel-culture industry is mostly based on this invasive species (Department of Agriculture, Forestry and Fisheries 2012).

There are several requirements for genetically identifying the source of an invasion, with a significant geographic genetic structure in the native range being the most important (Geller *et al.* 2010). When native populations are genetically homogeneous because of either high gene flow or lack of variability in the chosen markers, locating the origin of an invasion is impossible at any meaningful geographic scale. In contrast, when genetic structure exists across the native range, the source of an invasion may be determined.

Genetic relationships between *M. galloprovincialis* from South Africa and other worldwide populations have been previously investigated; evidence from nuclear DNA markers indicate the north-eastern Atlantic as the origin of *M. galloprovincialis* invasion (Daguin and Borsa 2000). However, only a few South African populations in the Cape Town area were considered in these studies and sampling was undertaken reasonably early (1990s) in the history of this invasion when the distribution of *M. galloprovincialis* was restricted to the south-western coast of South Africa.

In the present study, we re-examine the genetic identity of this southern African invader. *Mytilus galloprovincialis* populations along the Namibian and South African shores were assessed with nuclear (Me15/16 PCR-based) and mitochondrial (16S restriction fragment-length polymorphism; 16S RFLP) DNA assays to infer the most likely origin of invasion. Specifically, we aim at understanding whether populations along the entire invaded range share the same Atlantic origin as populations assessed in earlier studies (Daguin and Borsa 2000) or if introductions from other regions (Mediterranean and southern hemisphere) have also occurred.

Table 1. List of sampling locations and codes, with coordinates and sample size for each

Location	Code	Coordinates	*N*
Swakopmund	SW	22°40′21.13″S, 14°31′24.93″E	30
Port Nolloth	PN	29°15′1.04″S, 16°52′2.22″E	30
Saldanha Bay	SD	33°0′24.72″S, 17°56′47.84″E	30
Cape Town	CT	33°56′27.75″S, 18°22′18.23″E	29
Mossel Bay	MB	34°11′0.87″S, 22°9′30.10″E	30
Port Alfred	PA	33°36′13.00″S, 26°54′4.08″E	30

Materials and methods

In total, 179 specimens of *M. galloprovincialis* were collected from six locations along the mid-intertidal shores of southern Africa between 2012 and 2014 (Table 1, Fig. 1). Mantle tissue (20–30 mg) was dissected from each individual and total genomic DNA extraction was performed using a standard proteinase-K protocol (adapted from Sambrook *et al.* 1989).

Me15/16

The nuclear DNA marker Me15/16 is located within the nuclear gene encoding a polyphenolic adhesive protein, which is important in the attachment of mussels to the substratum (Waite 1992). This locus contains an insertion–deletion (indel) zone, whose amplification reveals three alleles (Inoue *et al.* 1995). Several studies consider the distinct alleles as diagnostic within the *Mytilus edulis* complex (e.g. Inoue *et al.* 1995; Lourenço *et al.* 2015). In particular, the expected fragment size for *M. edulis* is 180 bp (E-allele), for *M. galloprovincialis* it is 126 bp (G-allele), whereas for *M. galloprovincialis*–*M. edulis* hybrids, the following two fragments are detected: 126 bp and 180 bp (GE-alleles; Inoue *et al.* 1995). Critically, other works have highlighted the fact that, although strongly differentiated between the two species, the Me15/16 marker is not fully diagnostic between *M. edulis* and *M. galloprovincialis* and that GE-type heterozygotes can simply be explained by introgression (e.g. Hamer *et al.* 2012).

Despite not being fixed, Me15/16 allele frequencies show clear variations over large geographic scales (e.g. Mediterranean *v.* Atlantic or Atlantic Iberia *v.* Atlantic Morocco; Hamer *et al.* 2012; Lourenço *et al.* 2015). Comparison of allele frequencies between native and invaded locations can, thus, provide a useful molecular tool to infer origin of invasion.

We performed polymerase chain reaction (PCR) using primers (Me15 5′-CCAGTATACAAACCTGTG AAGA-3′ and Me16 5′-TGTTGTCTTAATAGGTTTGTAAGA-3′; Inoue *et al.* 1995). Amplification was performed in a 10-μL reaction

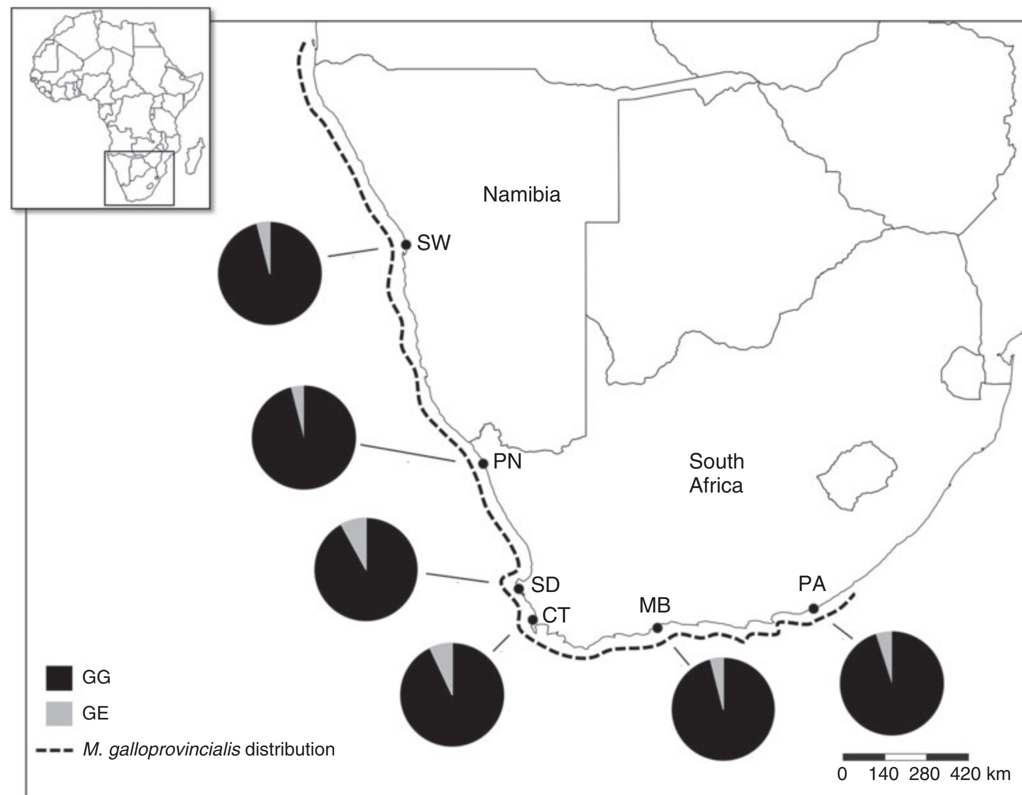

Fig. 1. Allele frequencies at the Me15/16 locus at each sampling site (for full name, see Table 1).

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, Madison, WI, USA), 3 mM of $MgCl_2$ and 1 U GoTaq DNA polymerase (Promega). Amplified fragments were obtained under the following PCR conditions: initial denaturation at 94°C during 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.

Proportion of homozygote (GG- and EE-type alleles) and heterozygote (GE-type alleles) individuals was quantified for each sampling location.

16S RFLP

Westfall *et al.* (2010) developed a RFLP based on 16s rRNA gene sequences. This assay distinguishes between northern and southern hemisphere *M. galloprovincialis*. Specifically, southern hemisphere *M. galloprovincialis* (MgS) haplogroup is expected to display three bands (342, 167 and 28 bp), northern hemisphere *M. galloprovincialis* (MgN) two bands (342 and 195 bp) and northern hemisphere shared *M. edulis–M. galloprovincialis* haplogroup (MgN–Me) four bands (342, 85, 82 and 28 bp; Westfall *et al.* 2010).

The PCR reaction with the 16S RFLP primers (16sAR 5′-CGCCTGTTTAACAAAAACAT-3′ and 16sBR 5′-CCGGTTTGAACTCAGATCACGT-3′; Palumbi 1996) was performed in 25-µL reaction volume, containing 1–10 ng of total DNA, 0.2 µM of each primer, 0.2 mM of each dNTP, 1 mM of $MgCl_2$, 1× GoTaq Flexi Buffer (Promega) and 1 U GoTaq DNA polymerase (Promega). 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, Foster City, CA, USA) using PCR primers and the BigDye Terminators version 3.1 Cycle Sequencing Kit (Applied Biosystems). The 16S rRNA sequences were virtually digested with EcoRV, *Nhe*I and *Spe*I restriction enzymes, using the web-based tool restriction digest available at http://www.bioinformatics.org/sms2/rest_digest.html (accessed 20 November 2017). Individuals were classified according to the resulting size and number of bands, and the proportion of allocation to each haplogroup was quantified for each sampling location.

To confirm the identification of each sample as determined by band sizes and to allow comparison with previous studies (Westfall and Gardner 2010; Lourenço *et al.* 2015), sequences in the present study were aligned with partial sequences of the different haplogroups (for MgS sequences with GQ455381–GQ455383, GQ455385–GQ455389, GQ455391, GQ455394–GQ455395; for MgN sequences with GQ455380, GQ455384, GQ455398; for MgN–Me sequences with GQ455392–GQ455393, GQ455396–GQ455397, GQ455399, GQ455405 and for *M. trossulus* sequences with GQ455400, GQ455402,

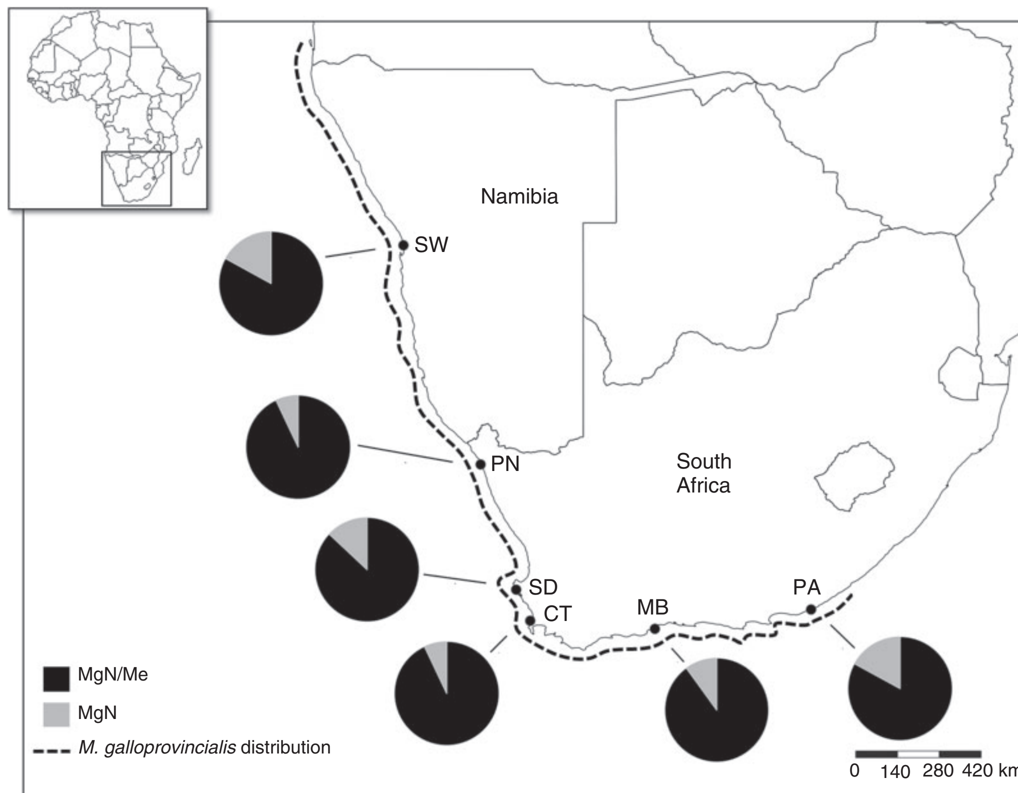

Fig. 2. Haplotype frequencies at the 16S marker at each sampling site (for full name see Table 1).

GQ455404) retrieved from GenBank and the same virtual digestion procedure was applied. A phylogenetic tree was inferred using Bayesian criteria. Haplotypes from a combined dataset with sequences retrieved from the GenBank and sequences from the present study were estimated using COLLAPSE, ver. 1.2 (D. Posada, see http://darwin.uvigo.es, accessed 17 November 2017). The Akaike information criterion implemented in jModelTest, ver. 0.1.1 (Posada 2008), selected GRT+gamma as the best-fit model to be used in MrBayes, ver. 3.2 (Ronquist *et al.* 2012). Two independent analyses were each run for 520 000 generations, with chains sampled every 100 generations. Burn-in was set to the first 25% of generations. The robustness of the inferred trees was determined using Bayesian posterior probabilities estimated from majority-rule consensus trees of the post burn-in trees. Partial sequences of the present study were deposited in GenBank (Accession numbers: KP223497–KP223675).

Results

Me15/16

The amplification of all samples at the Me15/16 locus was successful. The *M. galloprovincialis* (G) allele was the most frequent (92–96%) and was identified in all specimens at all investigated sites (Fig. 1). Most mussels displayed a homozygote genotype (GG-type alleles). Lower frequencies (4–8%) of the heterozygote genotype (GE-type alleles) were also detected at all locations. Allele frequencies and distribution did not show any obvious geographic distributional pattern.

16S RFLP

The assay confirmed the northern hemisphere origin of the *M. gallorpovincialis* invasion in southern Africa. No southern hemisphere *M. galloprovincialis* individuals (MgS) were recorded (Fig. 2).

Restriction digestion showed two distinct haplogroups, namely, the northern hemisphere *M. galloprovincialis* (MgN) and northern hemisphere shared *M. edulis*–*M. galloprovincialis* haplogroup (MgN–Me).

There was no clear geographical structuring of either genetic entity. Haplogroup frequencies showed that MgN–Me was present at all sampling sites and was the most frequent (84.9%). Haplogroup MgN was also detected at all locations at relatively low frequencies (7–20%) compared to MgN–Me.

Sequence-based Bayesian phylogeny confirmed virtual digestion results (Fig. S1, available as Supplementary material to this paper).

Discussion

In our study, frequencies of haplotypes at the 16S marker and alleles at the Me15/16 locus support a European north-eastern Atlantic origin throughout the species' Namibian and South African distribution, confirming results of earlier studies based on samples from a limited area on the south-western coast of South Africa (Grant and Cherry 1985; Sanjuan *et al.* 1997; Daguin and Borsa 2000). The 16S RFLP results did not show southern hemisphere haplotypes, indicating that the northern hemisphere is the sole origin of invasion.

The absence of southern hemisphere *M. galloprovincialis*, above all from major shipping centres (Sites SD, CT, SW) is, thus, rather unexpected, particularly considering that other marine invaders from the southern hemisphere have been reported along southern African shores (Mead *et al.* 2011).

Substantial, widespread invasion of northern hemisphere *M. galloprovincialis* to the southern hemisphere has been reported in other studies at locations ranging from international harbours to isolated areas far from the influence of shipping (Westfall and Gardner 2010, 2013; Gardner and Westfall 2012). Multiple, widespread introductions emphasise the ecological and genetic threat that these invasions may pose in terms of the displacement of native species. Introgressive swamping of native genotypes has been suggested (Westfall and Gardner 2013), although it is not supported by current theory (Currat *et al.* 2008). Recent evidence indicates that, genetically, native species are largely unaffected by invasions and that the ecological risk of being displaced poses a more serious threat (e.g. Saarman and Pogson 2015). The extent to which the geographic distribution of this aggressive invader complex has been reshaped and enhanced by human activities is a cause for concern. In Australia and Chile, northern invaders pose a minimal threat to the native populations. However, in New Zealand, recurrent immigration, introgression and minor hybridisation biased towards non-native maternal parents have the potential to lead to the displacement of native lineages (Westfall and Gardner 2013).

The *M. galloprovincialis* invasion in southern Africa represents a scenario different from that of other southern hemisphere locations because the establishment of non-indigenous populations occurred where native southern hemisphere *M. galloprovincialis* never existed (Grant and Cherry 1985; Zardi *et al.* 2007). Although this invader has not affected native mussel species genetically, its arrival has restructured the entire southern African rocky intertidal by altering the arrangement of resident communities and by dramatically increasing the overall biomass of mussels on these shores with positive second-order effects reaching higher trophic levels (Vernon 2004; Brown and Hockey 2007; Coleman and Hockey 2008; Nicastro *et al.* 2008; Kohler *et al.* 2011).

The *M. galloprovincialis* invasion of southern Africa is also exceptional in that all of its introductions in the northern hemisphere (California, Japan) have a Mediterranean origin (e.g. Daguin and Borsa 2000; Westfall and Gardner 2010).

In the native range of *M. galloprovincialis*, Me15/16 allele frequencies show clear variations over large geographic scales (e.g. Mediterranean *v.* Atlantic or Iberian *v.* Morocco). Me15/16 analyses of *M. galloprovincialis* populations at 22 sites along the eastern Adriatic coast (Croatia) showed that most specimens are fixed for the G allele (GG-type alleles; Hamer *et al.* 2012). Only one specimen of the 110 analysed showed a heterozygote genotype with the GE-type allele. This corresponds to an E allele frequency of 0.45%. The presence of the E allele in French Mediterranean *M. galloprovincialis* samples has also been reported at similarly very low frequencies (<1%; Borsa *et al.* 1999; Śmietanka *et al.* 2004), whereas no E alleles have been found in Tunisian populations (Lourenço *et al.* 2015).

In the Atlantic, E allele frequencies are comparatively higher and more variable than those found in the Mediterranean (e.g. Luttikhuizen *et al.* 2002; Bierne *et al.* 2003). In *M. galloprovincialis* from Brittany, E allele frequency is 4% (Bierne *et al.* 2003), whereas it reaches 41% in samples from the Irish Sea (Gosling *et al.* 2008). In five *M. galloprovincialis* populations sampled along Atlantic southern Iberian shores (Portugal and Spain), E allele frequency is ~4% (Lourenço *et al.* 2015). In addition, primers Me15 and Me17 (that amplify a larger segment of the Glu gene) have also been used to analyse Iberian populations, showing similar frequencies of GE heterozygotes (Bierne *et al.* 2003). Interestingly, only homozygote mussels (GG-type alleles) have been found in four Atlantic Moroccan populations (Lourenço *et al.* 2015). Our assessment of E and G allele frequencies showed overall frequencies matching those found along Atlantic European shores (4 and 96% respectively).

As for Me15/16, our 16S RFLP results indicated that 16S haplotype frequencies match those from Atlantic European sites, with frequencies varying between 7 and 17% and between 83 and 93% for MgN and MgN–Me respectively. The 16S RFLP assay not only distinguishes between northern and southern hemisphere individuals, but, as with Me15/16 alleles, haplotype frequencies at this marker also show distinct large-scale regional variations. Only the MgN–Me haplogroup has been reported along north African Atlantic shores, whereas in the Mediterranean Sea, the MgN and MgN–Me haplogroups have similar frequencies (42 and 58% respectively; Lourenço *et al.* 2015); along the southern European Atlantic coast, haplogroup frequencies are 7 and 93% for MgN and MgN–Me respectively (Lourenço *et al.* 2015).

It is important to stress that estimation of the putative origin of invasion based on allele frequencies should be interpreted with caution. In fact, there is abundant evidence that population bottlenecks and genetic drift during founder events can strongly affect patterns of diversity and population structure (e.g. Roman and Darling 2007; Vera *et al.* 2016). It is also interesting to note the genetic homogeneity of all of the populations sampled in our study. Generally, homogenous genetic structure covering large spatial distances is the result of fairly rapid population expansion after introduction (Drygala *et al.* 2016, and references therein). This is in line with previous studies reporting very rapid colonisation of the western and southern coastlines of southern Africa (McQuaid and Phillips 2000), triggered by high fecundity and recruitment rate, long pelagic larval duration, high dispersal potential and the lack of any substantial barrier to dispersal (e.g. Assis *et al.* 2015).

In addition to supporting a north-eastern Atlantic origin of *M. galloprovincialis* invasion, our work has established a baseline for tracking future genetic variation of this aggressive invader. Spatial changes in a populations' genetic composition can pre-date demographic changes at the species and ecosystem levels. It is, thus, important to keep track of any changes in the intra- or inter-specific diversity of invasions because these can presage further changes in indigenous biodiversity or community structure. Detailed knowledge about invasion routes and genetic composition of the invading populations is critical to evaluating the environmental and evolutionary factors that favour a successful invader (e.g. Darling *et al.* 2008) and, in practical terms, it is of great importance for the design of

strategies aimed at controlling or preventing future invasions (Estoup and Guillemaud 2010).

Conflicts of interest

The authors declare that they have no conflict of interest.

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