

mtDNA analysis of *Mytilopsis* (Bivalvia, Dreissenidae) invasion in Brazil reveals the existence of two species

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Abstract The genus *Mytilopsis* includes some of the most invasive estuarine bivalves in the world, and *M. leucophaeata* (native to the Gulf of Mexico) and *M. sallei* (indigenous in the Caribbean Sea) are the most often reported species. Molecular investigations of the COI gene in invasive populations of *M. leucophaeata* in Europe found only one haplotype, whereas the invasive *M. sallei* in Asia had high haplotype diversity. The present study investigated COI variability of invasive populations previously

identified as *M. leucophaeata* in Brazil, located at the cities of Recife and Rio de Janeiro. Our genetic analysis revealed that the record of *M. leucophaeata* from Recife appears erroneous; this population forms a clade adjacent to but genetically separated from the Asian *M. cf. sallei*. Similarly, samples from Rio de Janeiro clustered close to but not within European/North American sequences of *M. leucophaeata*. The Automatic Barcode Gap Discovery test suggested the existence of three species: *M. leucophaeata*, *M. cf. sallei* (Asia), and *M. cf. sallei* (Recife), which merit confirmation with nuclear DNA sequences. The discovery of two lineages of *Mytilopsis* in Brazil augments concerns about their expansion.

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Introduction

Globalization has led to intense shipping traffic between ports around the world, eliminating ecological and geographical barriers for aquatic species (Seebens et al., 2013). Aquatic invasions are increasing dramatically, mostly via hull fouling and ballast water (Fofonoff et al., 2003; Molnar et al., 2008), and estuarine habitats are usually prone to harbor more invasive species than marine or freshwater sites (Nehring, 2006). Invasive bivalves require targeted management efforts, as they often reach high densities and act as ecosystem engineers that heavily impact the diversity of local biota (Ricciardi et al., 1998; Sousa et al., 2009; Boltovskoy & Correa, 2015).

The bivalve family Dreissenidae, known for the widespread aquatic invasions of some of its species, is composed of three genera (Bouchet, 2011): *Congeria* Partsch, 1835, with three valid extant species, all restricted to caves (Bilandzija et al., 2013); *Dreissena* Van Beneden, 1835, with 10 valid extant species (Rosenberg, 2013); and *Mytilopsis* Conrad, 1857, with eight valid extant species (Bouchet, 2012). *Mytilopsis* usually inhabits brackish or fresh waters, with a few marine records of fossil species; all extinct and extant species are believed to be epifaunal and byssally attached (Nuttall, 1990a, b). Species of *Mytilopsis* often have shown explosive population growth and where introduced have become pests, particularly *Mytilopsis leucophaeata* (Conrad, 1831) and *Mytilopsis sallei* (Récluz, 1849).

Mytilopsis leucophaeata (popular names: dark false mussel or Conrad's false mussel) tolerates wide temperature variations and lives in temperate to tropical waters; adults occur in waters with salinities ranging from 0.1 to 18–21 ppt (Verween et al., 2010; Kennedy, 2011; van der Gaag et al., 2016). *Mytilopsis leucophaeata* occurs in low densities in its native range in the Gulf of Mexico (and possibly part of the eastern coast of the U.S.A.), and has spread widely through European estuaries since the nineteenth century (Verween et al., 2010; Kennedy, 2011; Oliver, 2015). Recent expansions in the Northern Hemisphere

include the northern Baltic Sea, the Black Sea, the Sea of Azov, and the Caspian Sea (Heiler et al., 2010; Zhulidov et al., 2015; Forsström et al., 2016). This species was first reported in the Southern Hemisphere in two widely separated localities in Brazil (~ 1,875 km apart), based on morphological identification: one in the estuary of the Capibaribe and Tejipió rivers, Recife city, northeastern Brazil (Souza et al., 2005), the other in the coastal lagoon Lagoa Rodrigo de Freitas, Rio de Janeiro city, southeastern Brazil (Rizzo et al., 2014).

Mytilopsis sallei (popular names: black-striped mussel or Caribbean false mussel) tolerates a wide range of oxygen conditions, surviving in highly polluted waters, and those with large sediment and contaminant loads (Puyana, 1995; Devi, 1996; Cai et al., 2014). Although it is less cold tolerant than *M. leucophaeata*, *M. sallei* presumably is more tolerant to high, even nearly marine salinities (Morton, 1981; Raju et al., 1988; Wangkulangkul, 2009), although larval development appears optimal in salinities lower than 25 ppt (Raju et al., 1975; Wangkulangkul, 2017). This species may produce extremely dense macrofouling on intertidal and subtidal hard substrata, with a preference for rough surfaces such as asbestos and wood (Raju et al., 1988; but see Wangkulangkul, 2009). Its native range includes the entire Caribbean and part of the Gulf of Mexico, where it may overlap distribution with *M. leucophaeata* (Marelli & Gray, 1983; Nuttall, 1990a). *Mytilopsis sallei* has invaded sites in Asia, from India to Japan (e.g., Ganapati et al., 1971; Habe, 1980; Tan & Morton, 2006), and has expanded to Australia (Willan et al., 2000) and Israel (Galil & Bogi, 2009).

As aggressive foulers and ecosystem engineers, these two species fundamentally change the structure of the benthic community by furnishing shelter and food for other animals, in addition to altering the nutrient dynamics due to their high filtration rates, and possibly affecting macroalgae growth and the plankton community (Lin & Yang, 2006; Cai et al., 2014). In some locations, they have displaced economically important native bivalves (Freitas, 2009; Wangkulangkul, 2009). Severe economic losses due to their biofouling in electricity-generating stations and discharge pipes of power plants were observed (Rajagopal et al., 1997; Laine et al., 2006; Florin et al., 2013), in addition to damages to fisheries and

aquaculture operations (Wangkulangkul, 2009; Liao et al., 2010).

Genetic investigations of *Mytilopsis* species are scarce, despite their worldwide impact. Heiler et al. (2010) studied the genetics of *M. leucophaeata*, generating 14 mitochondrial DNA COI (cytochrome c oxidase subunit I) sequences from five invaded sites (eastern U.S.A., Belgium, Germany, Ukraine, Iran). They found just a single haplotype, and noted that more variable markers other than COI should be investigated, just like the genetic structure in the species' native range. *Mytilopsis sallei* was investigated in invaded sites in Asia (Taiwan, Hong Kong, Singapore, and India), but populations in the native range have not been sampled (Wong et al., 2011). The latter study found a higher haplotype diversity for COI (254 sequences, 15 haplotypes), indicating its suitability to reconstruct the invasion history of *M. sallei*.

The present study evaluated the genetic diversity of invasive species of *Mytilopsis* in Brazil, based on COI sequences, comparing their haplotype diversity to populations previously studied in other invaded areas. The new data inform a discussion about the processes of invasion of *Mytilopsis* in Brazil, its ecological aspects, and concerns about the correct identification of species in this genus.

Materials and methods

Following the two records of *M. leucophaeata* in Brazil (Souza et al., 2005; Rizzo et al., 2014), samples were taken in the cities of Recife, in November 2016, and Rio de Janeiro, in February of 2016 and 2017 (Table 1). At Recife, the locality sampled in Bacia do Pina was near the mouth of the Capibaribe River, where substantial loads of organic pollution are discharged, leading to eutrophic and hypoxic conditions in the estuary, which is separated from the sea by a natural dike of sandstone and calcareous reefs (Freitas, 2009). Its mean salinity is 27 ppt, varying from marine levels to less than 15 ppt; the water temperature ranges from 27 to 31°C (Freitas, 2009). At Rio de Janeiro, Lagoa Rodrigo de Freitas is a eutrophic coastal lagoon surrounded by a dense urban area and connected to the sea by a small channel; the lagoon receives fresh water from heavily polluted rivers. A system of floodgates usually maintains local salinity between 13 and 18 ppt (Altir Neto, pers. comm.), with

the water temperature typically varying between 22 and 28°C (Soares et al., 2012).

A total of 28 specimens were collected in Recife, and 31 in Rio de Janeiro. They were fixed and stored in 95% ethanol, with a small piece of mantle tissue removed for DNA extraction. Although dreissenids have not been reported to possess heteroplasmic mitochondrial DNA (Plazzi, 2015; Soroka et al., 2017), as is common in many bivalves (Zouros, 2013; Plazzi, 2015), extraction of gonad tissues was avoided. Shells were photographed with an Olympus DP70 camera coupled to an Olympus SZX7 stereoscope.

Initial DNA extraction was based on a standard protocol of salting out, with minor modifications (Howe et al., 1997). A NucleoSpin Tissue kit (Macherey–Nagel GmbH & Co. KG) was used for specimens from Recife, following the manufacturer's protocol. In both cases, the quality of DNA extraction was verified with a Nanodrop 2000 spectrophotometer. The following reagents were used for the amplification of the gene COI: 1x Taq buffer, 2.5 mM MgCl₂, 0.8 mg/mL BSA, 0.16 mM dNTP, 0.32 μM for each of the primers HCO-2198 and LCO-1490 (Folmer et al., 1994), 1 U Taq DNA polymerase, in addition to 1.0 μL of DNA (50–200 ng), totaling 25 μL per reaction. The thermocycler settings were initial denaturation at 95°C (5'); 35 cycles of denaturation at 95°C (1'), annealing at 50°C (1'), extension at 72°C (1'); followed by a final extension at 72°C (5'). PCR quality was evaluated with TAE 1.5% agarose gel electrophoresis. PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea) for both directions, with the same universal primers used for PCR.

In complement to the samples obtained from Brazil, we used sequences of *M. leucophaeata* and *M. sallei* available in GenBank and BOLD-Barcode of Life Database (Table 1). Sequences were aligned with the algorithm ClustalW available in MEGA 7 (Kumar et al., 2016), and the quality of the alignment was checked by eye. Owing to the disparity of the length of sequences available in GenBank, various *indel*-free alignments were carried out: a phylogenetic tree with *Mytilopsis* sequences and the measurement of genetic distances aligned for 570 nt, whereas the alignment for phylogeographic analyses and haplotype/nucleotide diversities for each species comprised 488 nt for *M. leucophaeata* and 372 nt for *M. sallei*.

Table 1 Sequences used in the present study

Voucher	Individuals	Coordinates	Locality	GenBank sequences
Originally identified as <i>Mytilopsis leucophaeata</i>				
MNRJ 28892	28	08°04'44"S, 34°53'25"W	Recife city (Brazil)	<u>MF139867</u> – <u>MF139894</u>
MNRJ 36090	5	22°58'45"S, 43°12'55"W	Rio de Janeiro city (Brazil)	<u>MF139862</u> – <u>MF139866</u>
MNRJ 36091	9	22°58'31"S, 43°12'05"W	Rio de Janeiro city (Brazil)	<u>MF139853</u> – <u>MF139861</u>
MNRJ 36092	10	22°58'20"S, 43°12'57"W	Rio de Janeiro city (Brazil)	<u>MF139843</u> – <u>MF139852</u>
MNRJ 36093	7	22°57'48"S, 43°12'35"W	Rio de Janeiro city (Brazil)	<u>MF139836</u> – <u>MF139842</u>
–	1	–	New York (U.S.A.)	U47649.1
–	5	–	Maryland (U.S.A.)	KU905843.1; KU905894.1; KU905904.1; KU905989.1; KU906056.1
–	1	–	Florida (U.S.A.)	KX713485.1
–	2	–	Belgium	HM100251.1; EF414477.1
–	2	–	Sweden	OZIMP032-15 ; OZIMP033-15
–	2	–	Germany	HM100253.1; HM100254.1
–	1	–	Ukraine	HM100255.1
–	3	–	Caspian Sea (Iran)	HM100257.1; HM100258.1; HM100260.1
Originally identified as <i>Mytilopsis sallei</i>				
–	25	–	Eastern India	DQ078480.1; DQ078485.1; DQ078487.1; DQ078489.1; DQ078490.1; DQ078491.1
–	181	–	Singapore	DQ078480.1–DQ078490.1; DQ078492.1–DQ078494.1
–	93	–	Southern China	FJ696654.1–FJ696701.1; EU780672.1; JX099435.1; DQ078480.1–DQ078481.1; DQ078485.1; DQ078487.1; DQ078489.1–DQ078490.1; DQ078494.1
–	1	–	Northern Australia	OZIMP031-15
Outgroup				
<i>Dreissena polymorpha</i>				AF474404.1

Coordinates and voucher material are provided only to Brazilian localities, which represent the single sources of new sequences (underlined). Sequences in bold are exclusively found at BOLD (Barcode of Life Database)

MNRJ Museu Nacional do Rio de Janeiro, Rio de Janeiro, Brazil

A maximum-likelihood tree was computed in PhyML 3.0 (Guindon et al., 2010), following AIC (Akaike Information Criterion) automatic selection, with 1000 bootstrap (BS) replications, subtree-pruning-regrafting extensive heuristic search, and an initial tree automatically calculated with Neighbor-Joining. Bayesian inference was conducted with MrBayes 3.2.1 (Ronquist et al., 2012) with no a priori model, and 1,000,000 generations applied to two independent runs with four chains each, trees sampled after every 100th cycle; the burn-in was defined as 25%, and posterior probabilities (PP) were estimated from the 50% majority-rule consensus tree.

Genetic distances (p-distances) between main clades were calculated with MEGA 7. To evaluate whether the genetic distances were characteristic of separate species, the program ABGD-Automatic Barcode Gap Discovery (Puillandre et al., 2011) was executed following the default settings (Pmin 0.001, Pmax 0.1, relative gap width 1.5, algorithm Kimura-2-Parameter), both for the total alignment of 570 nt and the smaller alignment of 372 nt (comprising more sequences of *M. sallei*). Haplotype and nucleotide diversities were estimated with Arlequin 3.5 (Excoffier & Lischer, 2010) following the default settings, but not considering gaps. DnaSP 5.0 (Librado & Rozas, 2009) was used to define the haplotypes. Haplotype networks were processed with Network 5.0 under a median-joining network (Bandelt et al., 1999).

Along this work, the group defined as ‘Asia’ for *M. cf. sallei* includes the single sequence available from Australia and the sequence available from Florida (U.S.A.), in addition to those from Asia, all retrieved from GenBank/BOLD (Table 1). The group defined as ‘North’ for *M. leucophaeata* includes sequences from GenBank/BOLD from eastern U.S.A., Europe, and the Caspian Sea. The groups ‘Recife’ and ‘Rio de Janeiro’ correspond to samples herein obtained from the cities of Recife and Rio de Janeiro (Brazil), respectively.

Results

Prior to the genetic analysis, some differences in shell morphology were observed between specimens from the two localities in Brazil. Although shells from Rio de Janeiro (Fig. 1a–b) and Recife (Fig. 1c–d) shared some similar features, e.g., elongated outline, convex

dorsal margin, and rounded posterior margin, they were distinguished by: outline of ventral margin (straight or slightly concave in shells from Rio de Janeiro, concave in those from Recife); morphology of the apophysis (rounded and not prominent in the former, pointed and prominent in the latter); and outline of the umbo (not projected in the former, slightly projected in the latter). In addition, shells from Rio de Janeiro had smaller dimensions than those from Recife, with the largest specimen recorded from Rio de Janeiro measuring 31 mm in length (Altir Neto, pers. comm.), and that from Recife measuring 38 mm (Freitas, 2009).

The consensus tree from maximum-likelihood and Bayesian inference placed the populations from Rio de Janeiro and Recife in two distinct lineages (Fig. 2). Sequences from Rio de Janeiro formed a well-supported clade (BS: 1.00; PP: 1.00) together with *M. leucophaeata* from the Northern Hemisphere (eastern U.S.A., Europe and Caspian Sea). The population from Rio de Janeiro formed a clade with internal low resolution in the consensus tree, in a basal position of the clade herein termed ‘*M. leucophaeata*.’ The other main clade grouped sequences from Recife and those of *M. sallei* from GenBank (BS: 0.64; PP: 1.00), in addition to the sequence previously named as *M. leucophaeata* by Combosch et al. (2016) from southern Florida, southeastern U.S.A. (GenBank accession number: KX713485.1); we termed this entire group ‘*Mytilopsis cf. sallei*.’ Particularly for Recife, this is a well-supported clade (BS: 0.99; PP: 1.00) with two clearly defined subgroups (Fig. 2).

Mytilopsis leucophaeata and *M. cf. sallei* are highly divergent in the COI gene, with a pairwise genetic distance of 13.5–15.6% (Table 2). The two groups of *M. leucophaeata*, herein termed ‘North’ and ‘Rio de Janeiro,’ have 2.4–3.1% genetic divergence between them, close to the hypothetical 3% cut-off between species (Hebert et al., 2003). In contrast, the greater divergence of 8.0–8.8% between the groups of *M. cf. sallei*, i.e., ‘Recife’ and ‘Asia’, suggests the existence of two species in this clade (Table 2). The two distinct subgroups within the Recife population had a 2.4% genetic distance. ABGD suggested the existence of two clear barcode gap zones, separating *M. leucophaeata*, *M. cf. sallei* (Asia), and *M. cf. sallei* (Recife), although differences in the size of the alignments employed led to inconsistencies in the integrity of *M. leucophaeata* as a single species

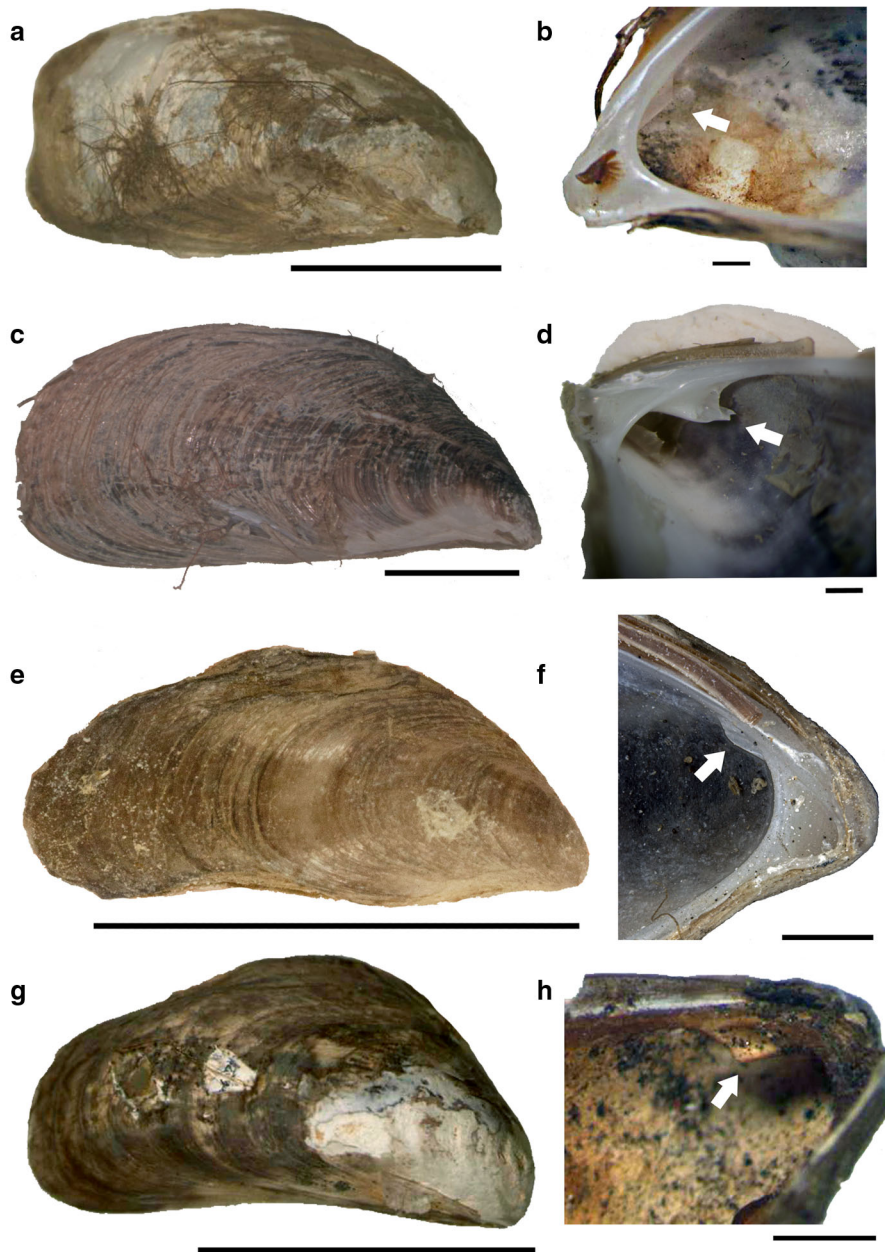


Fig. 1 a, b *Mytilopsis leucophaeata* (Conrad, 1831), MNRJ 36092, from Rio de Janeiro. c, d *Mytilopsis* cf. *sallei* (Récluz, 1849), MNRJ 28892, from Recife. e, f. Lectotype of *Mytilopsis rossmaessleri* (Dunker, 1853), ZMB 108737 [Museum für Naturkunde, Berlin], credits to Christine Zorn. g, h. *Mytilopsis*

sp., MCP 8504 [Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre], 23°52'S, 46°22'W, Cubatão, São Paulo state, Osmar Domaneschi coll. White arrows indicate the apophysis. Scale bars: a, c, e, g, 1 cm; b, d, f, h, 1 mm

(Fig. 3). The problematic taxonomic status of the genus leads us to adopt a conservative approach of terming the groups 'Asia' and 'Recife' as a single entity, even though it probably contains more than one species (see "Discussion").

The alignment of 47 sequences of *M. leucophaeata* yielded 488 nt, with 17 polymorphic sites and 10 haplotypes (Online Resource 1). The alignment of 333 sequences of *M. cf. sallei* included 372 nt, with 50 polymorphic sites and 24 haplotypes (Online Resource

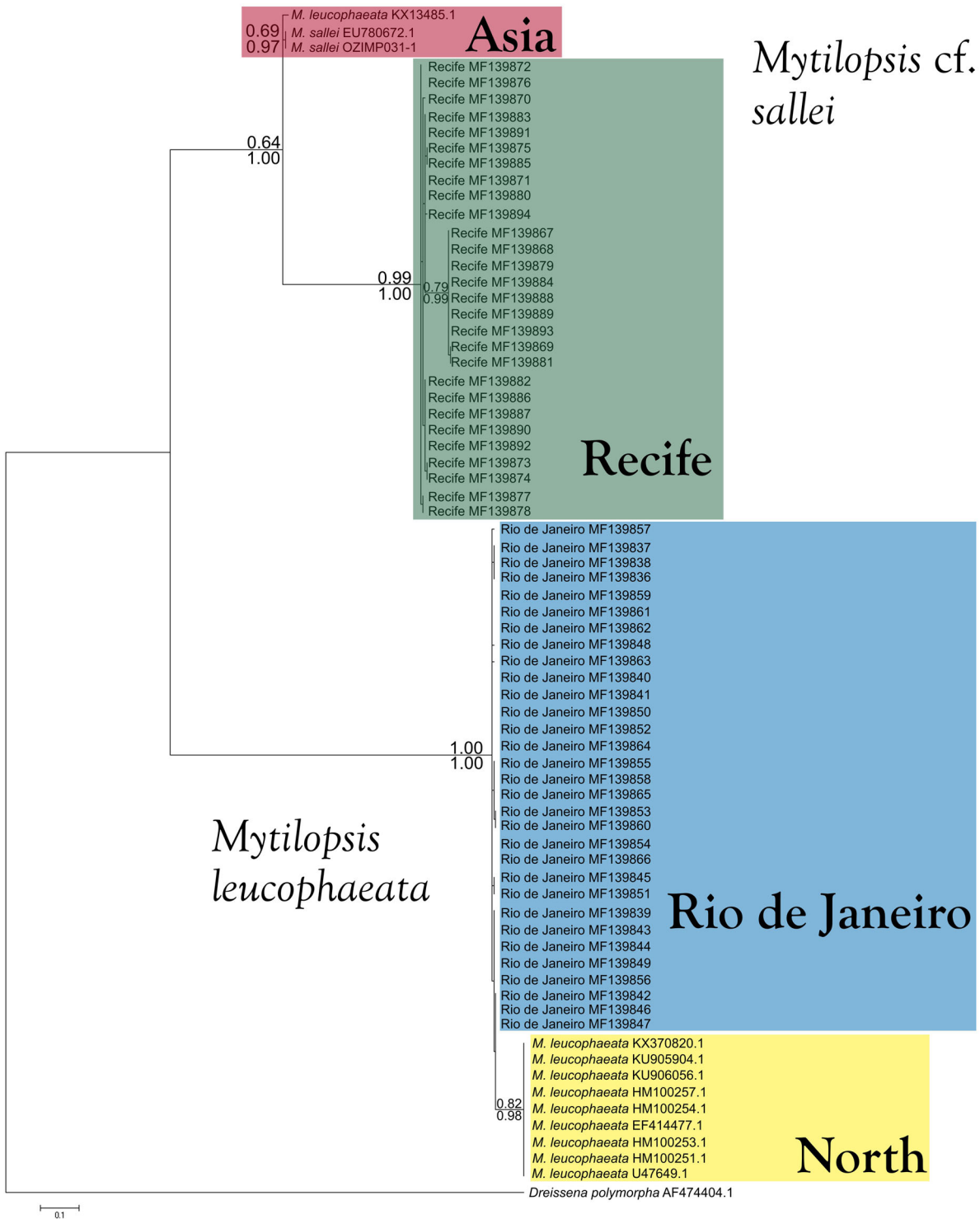


Fig. 2 Consensus tree of maximum-likelihood (ML) and Bayesian inference, showing topology recovered by ML; color background represent different populations within the two main clades. Indices of bootstrap and posterior probability are

respectively displayed above and under branches only for clades recovered with more than 60% of support for both analyses

1). Populations from Rio de Janeiro and Recife had haplotype diversities of 0.837 and 0.786, respectively (Table 3). The former contrasts with the single known haplotype of *M. leucophaeata* from the Northern Hemisphere (Heiler et al., 2010), and the latter is similar to the haplotype diversity of the ‘Asian’ *M. cf. sallei* (Wong et al., 2011).

The invasive populations of *M. leucophaeata* in the North and in Rio de Janeiro are separated by 10 mutational steps (Fig. 4). The nine haplotypes from Rio de Janeiro differed by single mutational steps, with two singletons, no medium vectors, and a central

Fig. 4 Haplotypes of introduced *Mytilopsis leucophaeata* (a–b) and *Mytilopsis cf. sallei* (c–d). Mutational steps indicated by rectangular black marks, inferred haplotypes by empty circles

haplotype (H3) present in 35% of the individuals (Fig. 4).

Although the phylogeography of invasive *M. cf. sallei* in Asia has been discussed previously by Wong et al. (2011), we note that one Asian haplotype is shared with Australia (H11 = H6 of Wong et al., 2011) and another one with southern Florida

Table 2 Genetic distances (p-distances) between invasive populations of *Mytilopsis*

Species (locality)	<i>M. leucophaeata</i> (North)	<i>M. leucophaeata</i> (Rio de Janeiro)	<i>M. cf. sallei</i> (Asia)	<i>M. cf. sallei</i> (Recife)
<i>M. leucophaeata</i> (North)	–			
<i>M. leucophaeata</i> (Rio de Janeiro)	2.4–3.1%	–		
<i>M. cf. sallei</i> (Asia)	14.2–14.5%	13.5–14.0%	–	–
<i>M. cf. sallei</i> (Recife)	15.1–15.6%	15.3–15.6%	8.0–8.8%	

Refer to “Materials and methods” in order to verify the meaning of localities

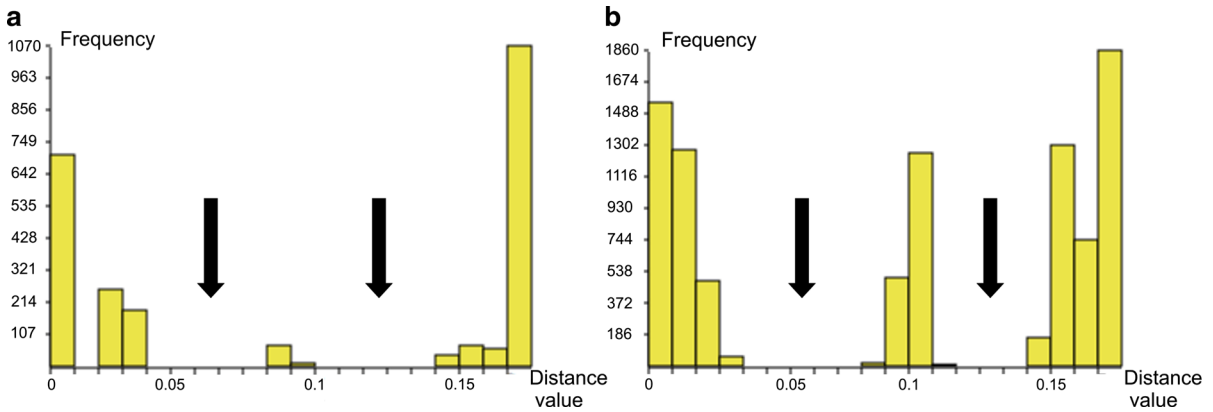
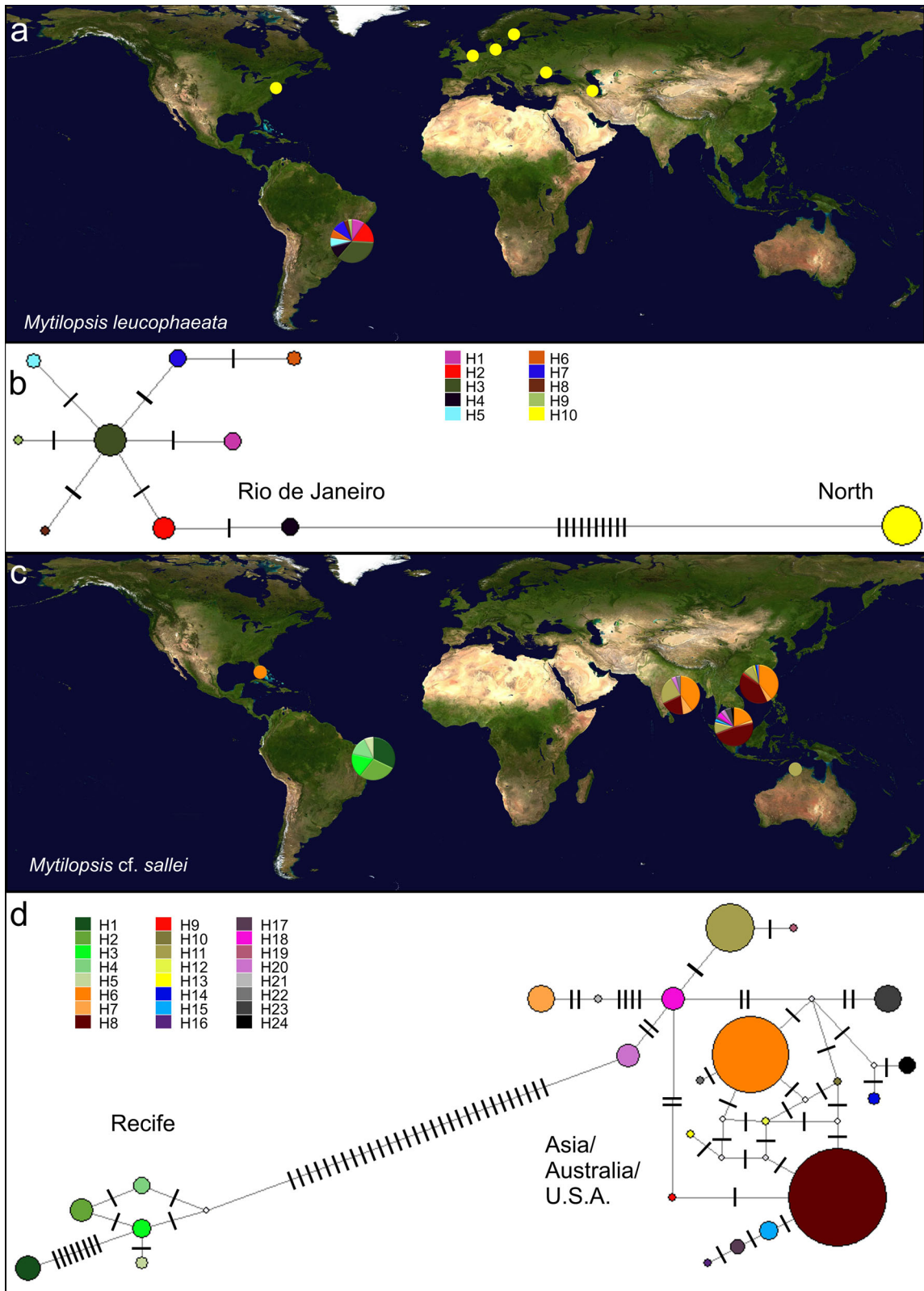


Fig. 3 Automatic Barcode Gap Discovery (ABGD) results. **a** Distribution of pairwise differences for the alignment of 570 nt. **b** *Idem*, for the alignment of 372 nt. Black arrows indicate clear barcode gap zones

Table 3 Haplotype (h) and nucleotide (π) diversity indices of invasive populations of *Mytilopsis*

	<i>M. cf. sallei</i>	(Asia)	(Recife)	<i>M. leucophaeata</i>	(North)	(Rio de Janeiro)
<i>h</i>	0.770 ± 0.017	0.728 ± 0.018	0.786 ± 0.038	0.825 ± 0.036	0	0.837 ± 0.048
π	0.022 ± 0.011	0.009 ± 0.005	0.012 ± 0.007	0.012 ± 0.007	0	0.003 ± 0.002

Refer to “Materials and methods” in order to verify the meaning of localities



(H6 = H11 of Wong et al., 2011), from the indigenous range of *M. sallei*. Separated by 30 mutational steps from ‘Asian’ *M. cf. sallei*, eight mutational steps separate the two subgroups from Recife; one has just a single haplotype (H1), and the other four haplotypes (H2–H5), each separated by a single mutational step (Fig. 4).

Discussion

The present study revealed unexpected results, considering the prior assumption that *M. leucophaeata* was the sole dreissenid believed to have invaded Brazilian estuaries at Recife and Rio de Janeiro. This investigation found a divergent lineage in Recife, which appears to be more closely related to the invasive *M. cf. sallei* in Asia, and discerned a genetic variability of the population in Rio de Janeiro. The two invasive populations of *Mytilopsis* in Brazil are likely a consequence of high propagule pressures, and their genetic variability reinforces concerns about their potential to expand to nearby estuaries (Rizzo et al., 2014).

Even after sampling several localities (but with few individuals per locality), Heiler et al. (2010) found a single haplotype in Europe and stated that the COI gene is not appropriate to reveal the genetic structure of *M. leucophaeata*. The higher COI haplotype diversity herein found in the invasive population in Rio de Janeiro shows that this marker is variable in this species, although another marker would be indeed necessary to reveal the genetic structure of *M. leucophaeata* in the invasion of European estuaries. The presumed single COI haplotype widespread in Europe and the Caspian Sea was probably originated by a founder effect from the eastern U.S.A., which shares the same haplotype. Another founder effect originating the eastern U.S.A. population from the native range may have occurred through a recent invasion (Kennedy, 2011) or through a natural genetic bottleneck derived from deglaciation periods, as observed to *Dreissena* (Stepien et al., 2014). In contrast, a high propagule pressure likely drove the invasion of *M. leucophaeata* in Rio de Janeiro, probably from native source(s); future sampling within the natural range of *M. leucophaeata* in the Gulf of Mexico may reveal intermediate haplotypes linking the populations

from Rio de Janeiro and ‘North’ (separated by 2.4–3.1% in COI).

Contrary to the traditional view of using the name *M. sallei* for populations from Asia and Australia (Morton, 1981), some authors questioned the use of this name (Willan et al., 2000) and others recommended adoption of the eastern Pacific species *Mytilopsis adamsi* Morrison, 1946 for all Asian occurrences (Marelli & Gray, 1985; Salgado-Barragán & Toledano-Granados, 2006; Wangkulangkul, 2009). However, the single available sequence from a common native site of both *M. sallei* and *M. leucophaeata* (KX713485.1, from southern Florida, U.S.A.), although originally identified as the latter (Combosch et al., 2016), grouped with Asian sequences; it constitutes haplotype H6 in the present study, the second most common haplotype in Asia (Fig. 4). The possibility of additional species in the native range of *M. sallei* should be investigated with further sampling and more genes, regarding whether larval retention in estuaries or their dispersal into marine waters occur (Page et al., 2013). In that case, the lineages from Asia and Recife could be related to different species currently allocated under a broad (but perhaps inadequate) application of the name “*M. sallei*.”

The species *Mytilopsis rosmaessleri* (Dunker, 1853) was possibly originated from Brazil, perhaps from Pernambuco state in northeastern Brazil (Nuttall, 1990b); Dunker (1853) admitted uncertainty regarding the type locality because the specimen was obtained from a shell dealer, and its original label does not mention Recife or Pernambuco (Dr. Christine Zorn, pers. comm.). *Mytilopsis rosmaessleri* is currently considered a synonym of *M. sallei* (Marelli & Gray, 1983; Nuttall, 1990a, b), although the type material of the former has never been examined. Nuttall (1990a) discarded that *M. sallei* naturally occurs south of Venezuela, and no authors after Dunker (1853) reported new shells of *M. sallei* or *M. rosmaessleri* from northeastern Brazil. Although the shell outline resembles *M. sallei*, the type of *M. rosmaessleri* (Fig. 1e–f) shows an apophysis somewhat similar to *M. leucophaeata*. Because of its unclear origin and identity, *M. rosmaessleri* must be regarded as a *species inquirenda*. Even though, it is possible that *Mytilopsis* may have invaded Recife via shipping in the 1850s, considering the historical importance of the local port. Similarly, *Mytilopsis africana* (van

Beneden, 1835) is a supposed lineage of *M. sallei* that invaded the western coast of Africa during the period of slavery commerce (Nuttal, 1990a, b). An alternative scenario is that *Mytilopsis* from Recife represents a naturally occurring species that was not detected in early surveys owing to low densities, but which increased in population density after some environmental change.

Comparing our data to those of Marelli & Gray (1983, 1985), we observed that the shell outline of specimens from Brazil is more similar to *M. leucophaeata* than to native *M. sallei*. However, shells from Recife usually have the umbo and apophysis (Fig. 1c–d) resembling more native *M. sallei* than *M. leucophaeata*, whereas shells from Rio de Janeiro have the apophysis (Fig. 1b) expected for *M. leucophaeata*. The maximum shell length attained is slightly discrepant between populations, possibly because of their genetic differentiation or of plasticity due to variable growth conditions: *M. leucophaeata* (Europe: 27 mm; Rio de Janeiro: 31 mm) vs. *M. cf. sallei* (Asia: 31 mm; Recife: 38 mm) (Morton, 1981; Freitas, 2009; Kennedy, 2011).

The clade herein termed *M. cf. sallei* may share a higher tolerance to elevated salinities than *M. leucophaeata*, considering that the population established in Recife inhabits a wide salinity range of up to 31 ppt (Freitas, 2009), similarly to some nearby marine sites recorded for Asian specimens (Raju et al., 1975; Wangkulangkul, 2017). The modes of introduction of *Mytilopsis* species over long marine distances by ballast water (larvae) or ship fouling (adults) have been extensively debated (e.g., Verween et al., 2010; Kennedy, 2011; Zhulidov et al., 2015). Illustrating it, larvae of *M. cf. sallei* were observed in ships mooring in Asian ports (Chu et al., 1997) and larvae of *M. leucophaeata* may develop in nearby marine salinities (Siddall, 1980), whereas adult specimens of *M. cf. sallei* from Recife were observed fouling the hulls of ships after traveling over moderate distances (Farrapeira et al., 2007). Although dispersal via ballast water is the most probable cause for the invasion of both *Mytilopsis* species after long-distance travels, ship/boat fouling may propagate them over shorter distances.

The sampling localities at Recife (Bacia do Pina) and Rio de Janeiro (Lagoa Rodrigo de Freitas) are close to important ports, with intense traffic of Brazilian and foreign vessels. These urban areas are

highly polluted, with fewer natural predators or competitors with the exotic *Mytilopsis*, serving as a warning for future invasions at similar Brazilian ports. Preventing these mussels from spreading to other areas is a major and central challenge, requiring more control on ships arriving (biofouling and ballast water) at large scales, and controls on dispersal vectors and routes (e.g., small boats and waterways that can facilitate the dispersal of larvae) on small scales (Wasson et al., 2001), although both may be limited by high costs (Marbua et al., 2014). Routine examination of the hull is especially important for boats moored for long periods, but also necessary for overland transport of boats (Farrapeira et al., 2007; De Ventura et al., 2016). Particularly for Rio de Janeiro, some boats in Lagoa Rodrigo de Freitas were observed to be covered by *Mytilopsis* (pers. obs.), constituting possible vectors to other coastal lagoons such as Marapendi and Jacarepaguá (Rizzo et al., 2014). At Recife, small range expansions of *Mytilopsis* were observed in the neighboring municipality of Olinda soon after the first record of this bivalve (Farrapeira et al., 2009). Monitoring of adjacent areas may help to prevent the future spread of invasions, and modern techniques such as metabarcoding are capable of detecting the presence of *Mytilopsis* larvae at new introduction sites (Brown et al., 2016).

A previous invasion of *Mytilopsis* occurred in Brazil: Domaneschi & Moretzsohn (1989) recorded *Mytilopsis* sp. in the municipality of Cubatão (São Paulo state, southeastern Brazil), in an estuary close to the international port of Santos. Specimens collected by Osmar Domaneschi were originally labeled as *M. sallei* and indeed show a curved shell outline typical of individuals from the native range of this species (Fig. 1g–h). A recent field survey (M. R. Fernandes and I. C. Miyahira, November 2016) at Cubatão and nearby municipalities did not succeed in finding more specimens of *Mytilopsis*. Plausible explanations for this apparently failed invasion may be the intense water pollution at that locality, a result of the historical presence of the main Brazilian port (at Santos) and a large industrial park, or even a chemical biocide release in the water just after the invasion of *Mytilopsis* (Dr. Flávio Passos, pers. comm.). A large number of dead *Mytilopsis* specimens was also observed in a highly polluted area at Recife (M. R. Fernandes, March 2016), agreeing with the recurrent death of individuals there in the beginning of the rainy season,

which might be related to an increase in water pollution (Freitas & Souza, 2015). Similarly, high mortalities of *Mytilopsis* in Taiwan may have resulted from excessive sedimentation and algal blooms (Minchin et al., 2016).

Research on the biology of any *Mytilopsis* species requires correct taxonomic identifications and delimitations, which help in the development of conservation strategies to mitigate their impacts. For a comprehensive knowledge of the source of invasive populations, further studies with increased number of samples (especially from native ranges) and using other molecular markers (e.g., from nuclear DNA) should be carried out to complement data based on shell morphology. By comparison, the genetic investigation of another invasive dreissenid, *Dreissena polymorpha*, is far more advanced, with the use of several microsatellite markers (e.g., Stepien et al., 2014; Peñarrubia et al., 2016) and the description of its complete mitochondrial genome (Soroka et al., 2017). Public authorities should also give more attention to preventing the spread of these invasive taxa, especially by adopting rigorous procedures for surveillance of ballast water and inspection of the hulls of ships and boats.

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