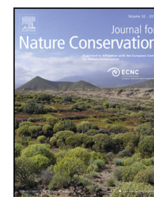
Journal for Nature Conservation 39 (2017) 58–67

Contents lists available at ScienceDirect

Journal for Nature Conservation

journal homepage: www.elsevier.de/jnc



Detection of invasive and cryptic species in marine mussels (Bivalvia, Mytilidae): A chromosomal perspective

Daniel García-Souto [a], Auriel Sumner-Hempel [a], Susana Fervenza [a], Concepción Pérez-García [a], Angel Torreiro [a], Rodrigo González-Romero [b], José M. Eirín-López [b], Paloma Morán [a], Juan J. Pasantes [a,*]

[a] Departamento de Bioquímica, Xenética e Inmunoloxía, Universidade de Vigo, Spain
[b] Department of Biological Sciences, Florida International University, USA

ARTICLE INFO

Article history:
Received 16 March 2017
Received in revised form 10 July 2017
Accepted 14 July 2017

Keywords:
Marine mussels
Invasive species
Cryptic species
Karyotype
Fluorescence *in situ* hybridization
Barcoding

ABSTRACT

Marine mussels illustrate a stunning variability in shape and color. Such variability, added to the scarcity of reliable morphological characters for their identification, can mislead recognition prompting the assignation of specimens of a single species to different ones or incorporate specimens belonging to different taxa into a single one. DNA barcoding is widely used for species identification; however, as this method relies on the previous morphological identification of the specimens, some of the DNA sequences stored in DNA databases are incorrectly assigned to a given species. In view of this uncertainty, further criteria beyond morphological characters and DNA sequences in databases are required to more reliably and accurately identify marine mussels. In this work we mapped ribosomal RNA and histone gene clusters to chromosomes of four species of marine mussels and compared them with those from another eight marine mussel taxa. Specimens of these twelve taxa were also DNA barcoded. Our results clearly demonstrated that the chromosomal analysis of marine mussels could shed light on their identification and, therefore, solve contradictions posed by morphological and molecular data.

© 2017 Elsevier GmbH. All rights reserved.

1. Introduction

An ever growing amount of marine biological invasions have been recorded throughout the last few decades (Rilov & Crooks, 2009). These invasions not only have posed a tremendous threat to marine ecosystems but have also denoted that our knowledge of marine life is rather scarce and that the identification of marine species is often very difficult even for expert taxonomists (Geller, Darling, & Carlton, 2010). In order to overcome these problems, more and more researchers have applied genetic methods to the study of marine organisms. Among these methods, the use of the 5′ end of the mitochondrial DNA cytochrome c oxidase subunit I (COI) gene to identify known taxa and to aid in the discovery of new animal species (DNA barcoding, Hebert, Cywinska, Ball, & deWaard, 2003) is paramount. Although the use of DNA barcoding has revealed an extraordinary amount of hidden marine diversity in the form of unrecognized cryptic species, the broad application of this method is not without controversy; mostly due to suspicions about the correct identification of the species in some of these studies (Huber, 2015).

The marine mussels belonging to the family Mytilidae (50 genera, 400 species) are characterized by presenting stunning variability in shape and color (Huber, 2010). Such variability, added to the scarcity of reliable morphological characters for their identification, can mislead recognition. The erroneous identification can, on the one hand, prompt the assignation of specimens of a single species to different ones and, on the other, incorporate specimens belonging to different taxa in a single one. Furthermore, as DNA data obtained relying on incorrect morphological identification is then directly stored in DNA databases, mistakes can be unintentionally perpetuated. In view of these uncertainties, further criteria beyond morphological characters are needed to reliable identify marine mussels.

The broad increase in the number of Mytilidae taxa either described as invasive or cryptic species further enhances this requirement. The World Register of Introduced Marine Species (WRIMS) database (http://www.marinespecies.org/introduced/) describes 22 species of mussels as introduced. Many of these species are well known invaders, i.e. the Asian date mussel

* Corresponding author at: Departamento de Bioquímica, Xenética e Inmunoloxía, Universidade de Vigo, E-36310 Vigo, Spain.
E-mail address: pasantes@uvigo.es (J.J. Pasantes).

http://dx.doi.org/10.1016/j.jnc.2017.07.005
1617-1381/© 2017 Elsevier GmbH. All rights reserved.

Arcuatula senhousia (Crooks & Khim, 1999; Katsanevakis et al., 2012), the Atlantic ribbed mussel *Geukensia demissa* (Sousa, Gutiérrez, & Aldridge, 2009), the golden mussel *Limnoperna fortunei* (Boltovskoy, Correa, Cataldo, & Sylvester, 2006), the Mediterranean mussel *Mytilus galloprovincialis* (Branch & Steffani, 2004), the brown mussel *Perna perna* (Holland, 2001; Rajagopal, Venugopalan, van der Velde, & Jenner, 2006), the Asian green mussel *Perna viridis* (Baker et al., 2007) and the pigmy mussel *Xenostrobus securis* (Pascual et al., 2010). Regarding marine mussel cryptic speciation, besides the classical case of the *Mytilus edulis* complex (e.g. Knowlton, 1993; Lourenço, Nicastro, Serrão, Castilho, & Zardi, 2015), cryptic species have been described mainly for the genera *Brachidontes* (*B. exustus*, Lee & Ó Foighil, 2004; *B. variabilis*, Terranova, Lo Brutto, Arculeo, & Mitton, 2007) and *Bathymodiolus* (Smith, McVeagh, Won, & Vrijenhoek, 2004) and postulated for *Xenostrobus* (Colgan & da Costa, 2013).

As displayed in Table 1, marine mussels present a wide variety of diploid chromosome numbers (2n = 22 to 32; reviewed by Nakamura, 1985 and Dixon, Jolly, Vevers, & Dixon, 2010; Pérez-García, Cambeiro, Morán, & Pasantes, 2010; Pérez-García, Guerra-Varela, Morán, Pasantes, 2010; Pérez-García, Morán, & Pasantes, 2011; Thiriot-Quiévreux, 1994, 2002) even among congeneric species (i.e. *Brachidontes* 2n = 28–32, *Mytilaster* 2n = 26–28, *Perna* 2n = 28–30). In contrast, the species belonging to the genus *Mytilus* are usually portrayed as presenting conserved chromosome numbers (2n = 28) and quite similar karyotypes. The application of molecular cytogenetic techniques demonstrated that this was not exactly the case and that species with apparently similar karyotypes showed remarkable differences in number and chromosomal location of repetitive DNA clusters (Pérez-García, Morán, Pasantes, 2014), therefore allowing identification of mussel taxa.

The objective of this work is to provide solid criteria to aid in the identification of marine mussel species. To this aim, we applied fluorescent *in situ* hybridization (FISH) techniques to map ribosomal RNA (rRNA) and histone gene clusters to chromosomes of four species of marine mussels of different genera (*Brachidontes*, *Gibbomodiola*, *Mytilus* and *Perna*) and compared them with those from another eight marine mussel taxa previously FISH karyotyped in our lab (Pérez-García, Cambeiro et al., 2010; Pérez-García, Guerra-Varela, et al., 2010; Pérez-García et al., 2011, 2014). In addition to this, we amplified the 5′ end of the mitochondrial DNA cytochrome oxidase I gene from karyotyped specimens of these twelve taxa in order to compare the accuracy of the chromosome identification with DNA barcoding data.

2. Materials and methods

2.1. Biological material

Specimens of the scorched mussel *Brachidontes exustus* (Linnaeus, 1758), the tulip mussel *Gibbomodiola adriatica* (Lamarck, 1819), the Chilean blue mussel *Mytilus platensis* d'Orbigny, 1842, and the brown mussel *Perna perna* (Linnaeus, 1758) were transported alive to the lab, maintained in tanks of 5 L filtered seawater at 18 ± 1 °C and fed on microalgae (*Isochrysis galbana*) to promote somatic growth (Martínez-Expósito et al., 1994a, 1994b).

In order to broaden the scope of the present research, we also included eight mussel species, previously analyzed by FISH mapping (Pérez-García, Cambeiro et al., 2010; Pérez-García, Guerra-Varela, et al., 2010; Pérez-García et al., 2011, 2014), for COI analysis.

All specimens were carefully identified according to external and internal shell characteristics (Fig. 1). The taxa analyzed and the sampling localities appear in Table 2. The nomenclature utilized follows the World Register of Marine Species (WoRMS) database (http://www.marinespecies.org/).

2.2. Chromosome spreading

Chromosome spreads were obtained from mussels treated overnight with colchicine (Pasantes, Martínez-Expósito, Martínez-Lage, & Méndez, 1990; Méndez, Pasantes, & Martínez-Expósito, 1990). Gills and gonads were hypotonized in diluted seawater and fixed in ethanol/acetic acid before disaggregating in 60% acetic acid. After spreading cell suspensions onto warm microscope slides, chromosome preparations were checked by phase-contrast microscopy (García-Souto, Pérez-García, Morán, & Pasantes, 2015; García-Souto, Kendall, Pérez-García, & Pasantes, 2016).

2.3. DNA extraction and PCR amplification

DNA was extracted from adductor muscles with the EZNA Mollusc DNA Kit (OMEGA). For DNA barcoding purposes, a fragment of the COI gene was amplified by PCR using either the universal primers *LCO1490* and *HCO2198* (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994) or the *Brachidontes* specific *COI F* and *COI R* (Cunha, Lopes, Reis, & Castilho, 2011) and *CO1aF* and *CO1aR* (Trovant, Ruzzante, Basso, & Orensanz, 2013). In order to generate species specific FISH probes, a portion of the 28S rDNA was amplified with primers *LR10R* (Hopple & Vilgalys, 1994) and *LR12* (Vilgalys & Hester, 1990). The amplifications of the entire 5S rDNA repeat, the H3 and the H1 histone genes used primers described by Pérez-García, Cambeiro et al. (2010), Giribet and Distel (2003) and Pérez-García et al. (2011), respectively. Amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems) as previously described (Pérez-García et al., 2011, 2014; García-Souto et al., 2015, 2016). PCR products were examined by electrophoresis on a 2% agarose gels.

2.4. DNA sequencing and phylogenetic analysis

Purified mitochondrial COI gene sequences (ExoSAP-IT® PCR Product Cleanup, Affymetrix) were sequenced (CACTI, University of Vigo) in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The edited sequences (BioEdit v. 7.1.11, Hall, 1999) were aligned using MEGA7 (Kumar, Stecher, & Tamura, 2016). Sequence similarity searches in the GenBank were performed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). To check the quality of the marine mussel sequences stored in GenBank, we also searched for marine mussel COI sequences using both the currently accepted scientific names and the former synonymies listed in WoRMS. Relationships among sequences were inferred from a maximum likelihood (ML) tree using a JC + G substitution model. Branch support was assessed with 500 bootstrap replicates. Analyses were performed on MEGA7 (Kumar et al., 2016).

2.5. Fluorescent *in situ* hybridization (FISH)

Single, double and sequential FISH experiments were performed as described by Pérez-García, Cambeiro et al. (2010), Pérez-García, Guerra-Varela, et al. (2010), Pérez-García et al. (2011, 2014). Chromosome preparations were digested with RNase and pepsine, denatured in 70% formamide and hybridized overnight. Labeled probes were detected with avidin/anti-avidin and anti-digoxigenin antibodies. Slides were counterstained with 4′-6-diamidin-2-fenilindol (DAPI: 0.14 μg mL^{-1} in 2xSSC) and mounted with antifade (Vectashield, Vector). Chromosome preparations were analyzed with a Nikon Eclipse-800 microscope. A DS-Qi1Mc CCD camera (Nikon) controlled by the NIS-Elements software (Nikon)

Table 1
Diploid chromosome numbers (2n) in Mytilidae

Genus	Species	2n	Reference
Arcuatula Jousseaume in Lamy (1919)			
	Arcuatula arcuatula (Hanley, 1843)	24	Ghobashy, Mona, Yasseen, and Desouky (1995)
	Arcuatula senhousia (Benson, 1842)	30	Ieyama (1977)
Aulacomya Mörch (1853)			
	Aulacomya atra (Molina, 1782)	26	Thiriot-Quiévreux (1984a)
Bathymodiolus Kenk and Wilson (1985)			
	Bathymodiolus azoricus Cosel and Comtet (1999)	32	Dixon et al. (2010)
	Bathymodiolus thermophilus Kenk and Wilson (1985)	32	Dixon et al. (2010)
Brachidontes Swainson (1840)			
	Brachidontes darwinianus (d'Orbigny, 1842)	30	Introini, De Magalhaes, and Recco-Pimentel (2010)
	Brachidontes mutabilis (Gould, 1861)	30	Ieyama et al. (1994)
	Brachidontes pharaonis (P. Fischer, 1870)	28	Vitturi et al. (2000)
	Brachidontes puniceus (Gmelin, 1791)	32	Pérez-García, Guerra-Varela, et al. (2010)
	Brachidontes rodriguezii (d'Orbigny, 1842)	32	Torreiro, Martínez-Expósito, Trucco, and Pasantes (1999)
Choromytilus Soot-Ryen (1952)			
	Choromytilus chorus (Molina, 1782)	30	Palma-Rojas, Guerra, Brown, and Von Brand (1997)
Crenomytilus Soot-Ryen (1955)			
	Crenomytilus grayanus (Dunker, 1853)	28	Ieyama (1984)
Gregariella Monterosato (1884)			
	Gregariella difficilis (Deshayes, 1863)	32	Ieyama (1984)
Ischadium Jukes-Browne (1905)			
	Ischadium recurvum (Rafinesque, 1820)	30	Diuportex-Chong, Rodríguez-Romero, Uribe-Alcocer, and Laguarda-Figueras (1978)
Leiosolenus Carpenter (1857)			
	Leiosolenus lischkei M. Huber (2010)	32	Ieyama (1984)
Limnoperna Rochebrune (1882)			
	Limnoperna fortunei (Dunker, 1857)	30	Ieyama et al. (1994)
Modiolus Lamarck (1799)			
	Modiolus barbatus (Linnaeus, 1758)	32	Libertini, Boato, Panozzo, and Fogato (1996)
	Modiolus auriculatus (Krauss, 1848)	32	Ieyama (1984)
Musculus Röding (1798)			
	Musculus cupreus (A. A. Gould, 1861)	30	Ieyama (1984)
	Musculus discors (Linnaeus, 1767)	28	Ieyama (1984)
Mytilaster Monterosato (1884)			
	Mytilaster lineatus (Gmelin, 1791)	26	Libertini et al. (1996)
	Mytilaster minimus (Poli, 1795)	28	Thiriot-Quiévreux (2002)
	Mytilaster solidus Monterosato (1883)	28	Libertini et al. (1996)
Mytilisepta Habe (1951)			
	Mytilisepta keenae (Nomura, 1936)	28	Ieyama (1983)
	Mytilisepta virgata (Wiegmann, 1837)	28	Ieyama (1983)
Mytilus Linnaeus (1758)			
	Mytilus californianus Conrad (1837)	28	Ahmed and Sparks (1970)
	Mytilus edulis Linnaeus (1758)	28	Ahmed and Sparks (1970)
	Mytilus galloprovincialis Lamarck (1819)	28	Thiriot-Quiévreux and Ayraud (1982)
	Mytilus platensis d'Orbigny (1842)	28	Thiriot-Quiévreux (1984b)
	Mytilus trossulus Gould (1850)	28	Insua, Labat, and Thiriot-Quiévreux (1994)
	Mytilus unguiculatus Valenciennes (1858)	28	Ieyama (1984)
Perna Philipsson (1788)			
	Perna canaliculus (Gmelin, 1791)	30	Libertini et al. (1996)
	Perna perna (Linnaeus, 1758)	28	Ahmed (1974)
	Perna viridis (Linnaeus, 1758)	30	Ahmed (1974)
Perumytilus Olsson, 1961			
	Perumytilus purpuratus (Lamarck, 1819)	32	Pérez-García, Cambeiro et al. (2010)
Septifer Récluz, 1848			
	Septifer bilocularis (Linnaeus, 1758)	26	Ieyama (1983)
	Septifer excisus (Wiegmann, 1837)	26	Ieyama (1983)
Xenostrobus Wilson, 1967			
	Xenostrobus atratus (Lischke, 1871)	22	Ieyama (1977)
	Xenostrobus securis (Lamarck, 1819)	30	Pérez-García et al. (2011)

Nomenclature: World Register of Marine Species database.

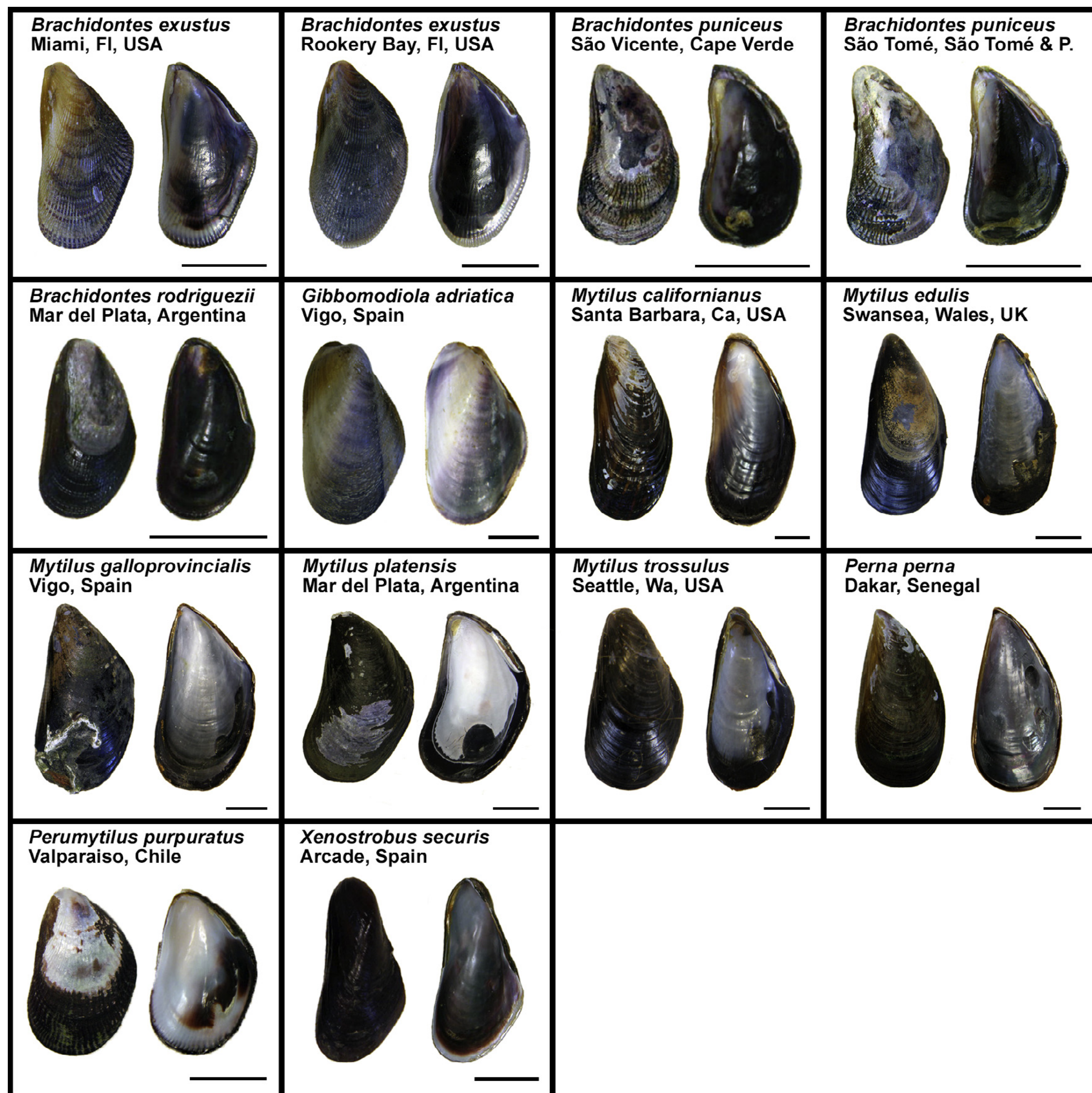


Fig. 1. External and internal shell morphologies of representative specimens of the 12 marine mussel taxa analyzed. Scale bars, 1 cm.

enabled the acquisition of separated images for each fluorochrome that were then merged using Adobe Photoshop. Chromosomes were measured (Micromeasure 3.3, Reeves & Tear, 2000) and classified according to centromeric indices and relative lengths (Levan, Fredga, & Sandberg, 1964).

3. Results

3.1. Chromosome analysis

Representative marine mussel metaphase plates and karyotypes are presented in Fig. 2. In agreement with previous reports (Ahmed, 1974; Thiriot-Quiévreux, 1984b), both *M. platensis* and *P. perna* showed diploid chromosome numbers of 2n = 28. The chromosome complements of 2n = 30 for *B. exustus* and 2n = 32 for *G. adriatica* are described here for the first time. Karyotypes also showed clear differences in chromosome composition (Fig. 2). Whereas metacentric and submetacentric chromosome pairs were relatively abundant in *G. adriatica* (7 m, 2 sm, 5 st, 2 t/st), *M. platensis* (6 m, 8 sm/st) and *P. perna* (4 m, 7 sm, 3 st), in *B. exustus* (2 m, 13 st) they were much scarcer.

45S rDNA clusters, subterminal in the four taxa (Fig. 2, Table 2), mapped to a single locus in *G. adriatica* and *P. perna* and to two loci in *B. exustus* and *M. platensis*. In *B. exustus* one of the major rDNA clusters occupies the entirety of the short arms of a subtelocentric chromosome pair (11p). Regarding minor rDNA, *B. exustus* and *P. perna* showed 5S rDNA clusters intercalary to the long arms of two chromosome pairs (Fig. 2, Table 2). In contrast, *G. adriatica* presented five clusters on five chromosome pairs and *M. platensis* four clusters on three chromosome pairs. In both cases, in addition to intercalary signals, subterminal and subcentromeric ones were also present.

Even though H3 histone genes mapped to two loci in all four marine mussels, their chromosomal locations diverged (Fig. 2, Table 2), intercalary in *B. exustus*, intercalary and subterminal in *G. adriatica*, intercalary and subcentromeric in *P. perna* and subcentromeric and subterminal in *M. platensis*. The FISH mapping of the H1 histone genes only gave good results in two of the four species analyzed; the signals appeared at a single locus in *G. adriatica* and at two loci in *M. platensis*.

Double color FISH and rehybridization experiments, besides confirming the location of the signals, allowed detection of the presence of overlapping 45S rDNA and H3 histone gene signals in *G.*

Table 2
Collection localities, diploid chromosome numbers and chromosomal location of rDNA and histone gen clusters in the marine mussels studied

Species	Locality	2n	45S rDNA	5S rDNA	H3 histone genes	H1 histone genes	Reference
Brachidontes exustus (Linnaeus, 1758)	Miami, FL, USA	30	11p (st)	4q ic (st)	1p ic (m)		This work
	Rookery Bay, FL, USA		14q ter (st)	5q ic (st)	5q ic (st)		
Brachidontes puniceus (Gmelin, 1791)	São Vicente, Cape Verde	32	15q ter (st)	4q ic (st)	6q ic (sm/st)		Pérez-García, Guerra-Varela, et al. (2010)
	São Tomé, São Tomé & Principe			6q ic (sm/st)	10q ic (sm)		
Brachidontes rodriguezii (d'Orbigny, 1842)	Mar del Plata, Argentina	32	4p (t/st)	3q ic (st)	3q ic (st)		Pérez-García, Guerra-Varela, et al. (2010)
			6p (t/st)	4q ic (t/st)	9q ic (st)		
Gibbomodiola adriatica (Lamarck, 1819)	Vigo, Galicia, Spain	32	2q ter (m)	4q ic (m)	2q ter (m)	10q cen (st)	This work
				12q ic (t) 13q ic (st) 15q ic (m) 16p cen (st)	14p ic (st)		
Mytilus edulis Linnaeus (1758)	Swansea, Wales, UK	28	4q ter (sm/st)	1p ter, 1p ic (m)	6p cen (m)	1p ter (m)	Pérez-García et al. (2014)
			10q ter (st/sm)	5q ic (m) 8p cen (m)	7q ter (st/sm)	12p ter (m)	
Mytilus galloprovincialis Lamarck (1819)	Baiona, Galicia, Spain	28	4q ter (sm/st)	1p ter, 1p ic (m)	6p cen (m)	1p ter (m)	Pérez-García et al. (2014)
			10q ter (sm)	5q ic (m) 8p cen (m)	7q ter (st)	12p ter (m)	
Mytilus platensis d'Orbigny (1842)	Mar del Plata, Argentina	28	4q ter (sm/st)	1p ter, 1p ic (m)	6p cen (m)	1p ter (m)	This work
			10q ter (sm)	5q ic (m) 8p cen (m)	7q ter (st/sm)	12p ter (m)	
Mytilus trossulus Gould (1850)	Seattle, WA, USA	28	4p ter (m)	1p ter, 1p ic (m)	5p cen (m)	1p ter (m)	Pérez-García et al. (2014)
			8p ter (sm) 11p ter (sm)	4q ic (m) 6p cen (m)	7q ic (sm)	12p ter (m)	
Mytilus califonianus Conrad (1837)	Santa Barbara, CA, USA	28	2p ter (sm)	1p ter, 1q cen (sm)	4p cen (m)	1p ter (sm)	Pérez-García et al. (2014)
			13p ter (sm)		6q ter (st/sm)	12p ter (m)	
Perna perna (Linnaeus, 1758)	Dakar, Senegal	28	4p ter (sm)	5q ic (sm)	11p ic (m)		This work
				10q ic (st)	12q cen (st)		
Perumytilus purpuratus (Lamarck, 1819)	Valparaiso, Chile	32	4q ter (st)	4p ter, 4q cen (st)	8q cen (st/sm)		Pérez-García, Cambeiro et al. (2010)
			10q ter (st)	9q cen (st)			
Xenostrobus securis (Lamarck, 1819)	Vigo, Galicia, Spain	30	1p ic (m)	2p ic, 2q ic (sm)	7p cen (sm)	12q cen (sm)	Pérez-García et al. (2011)
			3p cen (m)	5q cen (sm) 6q cen (sm) 8p cen (m)	8q ter (m) 10p cen (m) 13q cen (sm)	14q cen (m/sm)	

p: short arm; q: long arm; cen: subcentromeric; ic: intercalary; ter: subterminal; (m): metacentric; (sm): submetacentric; (st): subtelocentric; (t): telocentric.

adriatica (2q) and 5S rDNA and H1 histone gene signals in *M. platensis* (1p); separated intercalary signals for 5S rDNA and H3 histone genes were present in the long arms of *B. exustus* chromosome pair 5. Fig. 3 summarizes chromosomal data for the species analyzed in this work together with those for the other eight marine mussel taxa previously studied in our lab (Pérez-García, Cambeiro et al., 2010; Pérez-García, Guerra-Varela, et al., 2010; Pérez-García et al., 2011, 2014).

3.2. COI gene sequences

All partial COI gene sequences obtained (Table 3) were independently compared using BLAST with those stored in GenBank. The nucleotide sequences for 11 of the marine mussel taxa studied displayed a high degree of similarity (97–100%) with sequences retrieved from GenBank, mostly from mussels identified with the same specific names, therefore confirming their correct morphological and chromosomal identification. The only exception to this behavior were the sequences obtained from *Gibbomodiola adriatica*, for which the closest sequences only showed similarities of around 74% (Table 3).

We also retrieved COI gene sequences for each of the taxa from GenBank and compared them. Although most sequences grouped under the same species names were highly similar, some of them were clearly not, therefore indicating marine mussel specimen misidentifications.

COI sequences from *Brachidontes puniceus*, *B. rodriguezii* and *Perumytilus purpuratus* (Table 3) were intra-specifically similar both among them and with the corresponding sequences obtained in this work, therefore indicating that the taxonomic assignations of the specimens were correct. This was not the case for *B. exustus*, under which name were grouped rather different sequences; whilst some of them were coincident (99%) with those obtained in

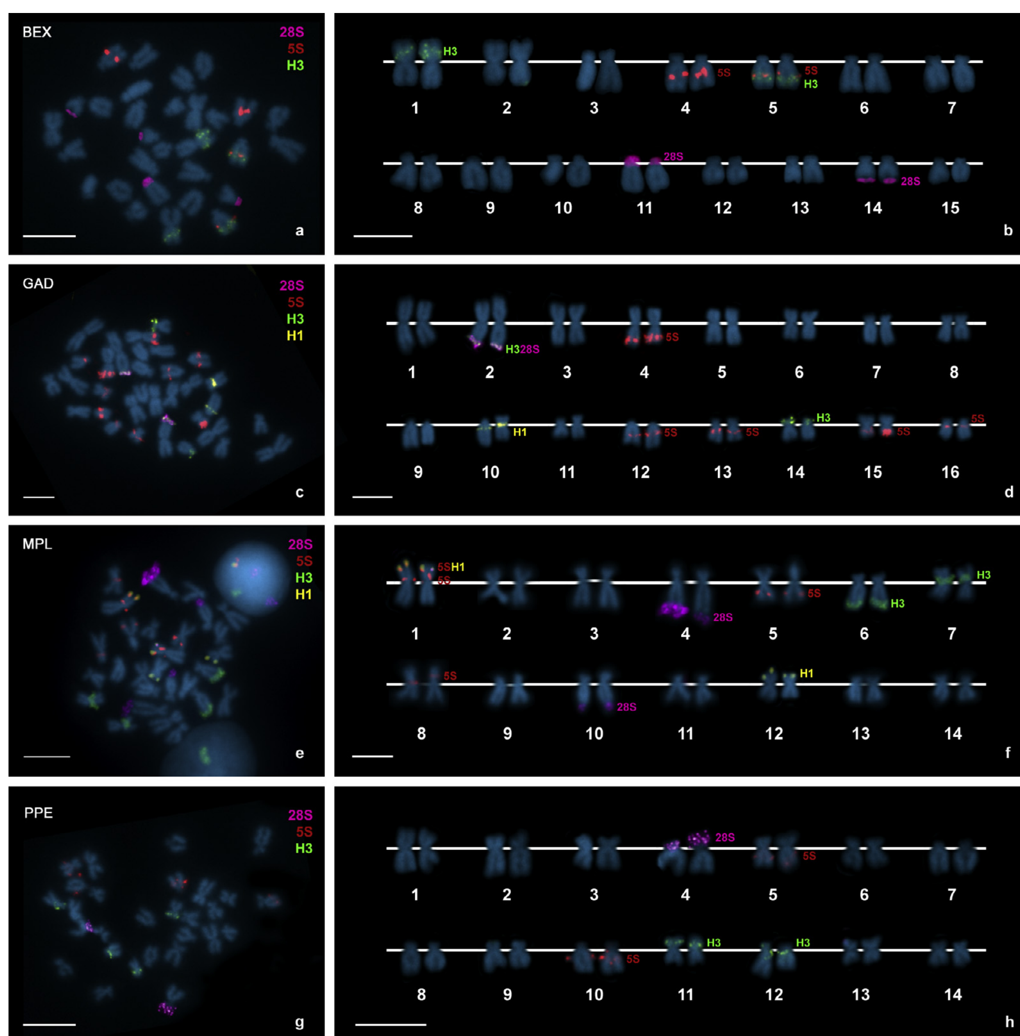


Fig. 2. FISH mapping of rDNA and histone gene probes to mitotic chromosomes of marine mussels counterstained with DAPI. Major rDNA (28S, purple) signals appear in a single chromosome pair in both *Gibbomodiola adriatica* (GAD) and *Perna perna* (PPE), and in two pairs in *Brachidontes exustus* (BEX) and *Mytilus platensis* (MPL). The number of minor rDNA (5S, red) clusters was two in BEX and PPE, four in three chromosome pairs in MPL and five in GAD. In contrast, the four marine mussel taxa show H3 histone gene signals (H3, green) on two chromosome pairs. H1 histone genes (H1, yellow) were only detected in two chromosome pairs in MPL and one chromosome pair in GAD. Overlapping signals were detected in GAD pair 2 (28S and H3) and in MPL pair 1 (5S and H1); besides, BEX pair 5 bears separated 5S rDNA and H3 histone gene signals. Scale bars, 5 μm.

this work, the remaining ones seem to correspond to *B. puniceus* (Table 3) and *Geukensia demissa* (Combosh et al., 2017).

All retrieved *Mytilus* sequences and those obtained in this work were intra-specifically similar (Table 3).

Perna perna COI gene sequences (Table 3) were also congruent among them and with our sequences, which perfectly matched those belonging to specimens of an Atlantic lineage (Cunha et al., 2014). In contrast, the mitogenome stored in GenBank as *P. perna* contains a COI gene sequence showing, on the one hand, low identity (67%) with the other *P. perna* sequences and, on the other, an almost perfect coincidence (99%) with those coming from another marine mussel, *Mytilaster solisianus* (Trovant et al., 2016).

Xenostrobus securis COI gene sequences (Table 3) were also similar among them and with our sequences.

3.3. Phylogenetic analysis

The phylogeny of the species of marine mussels constructed from the mitochondrial COI gene dataset obtained in this work is shown in Fig. 4. ML analysis reveals two mitochondrial clades sister to *Crassostrea virginica*, one formed by two taxa, *Gibbomodiola adriatica* and *Xenostrobus securis*, and the other by all the other species analyzed. In the latter clade the species of the genera *Mytilus* and *Perna* were clearly separated from the species of the genera *Perumytilus* and *Brachidontes*.

4. Discussion

4.1. Potential of chromosome analysis in marine mussel identification

The similar shell morphologies of many bivalves make their taxonomic identification complex (Huber, 2010, 2015). The introduction of non indigenous species in areas well outside their ranges of distribution further complicates the task and, as a consequence, specimen misidentifications increase. Though DNA barcoding was postulated as an alternative method in taxa identification (Hebert et al., 2003), in some cases DNA sequences are obtained from misidentified specimens, therefore boosting confusion and illustrating that further parameters have to be employed in species identification.

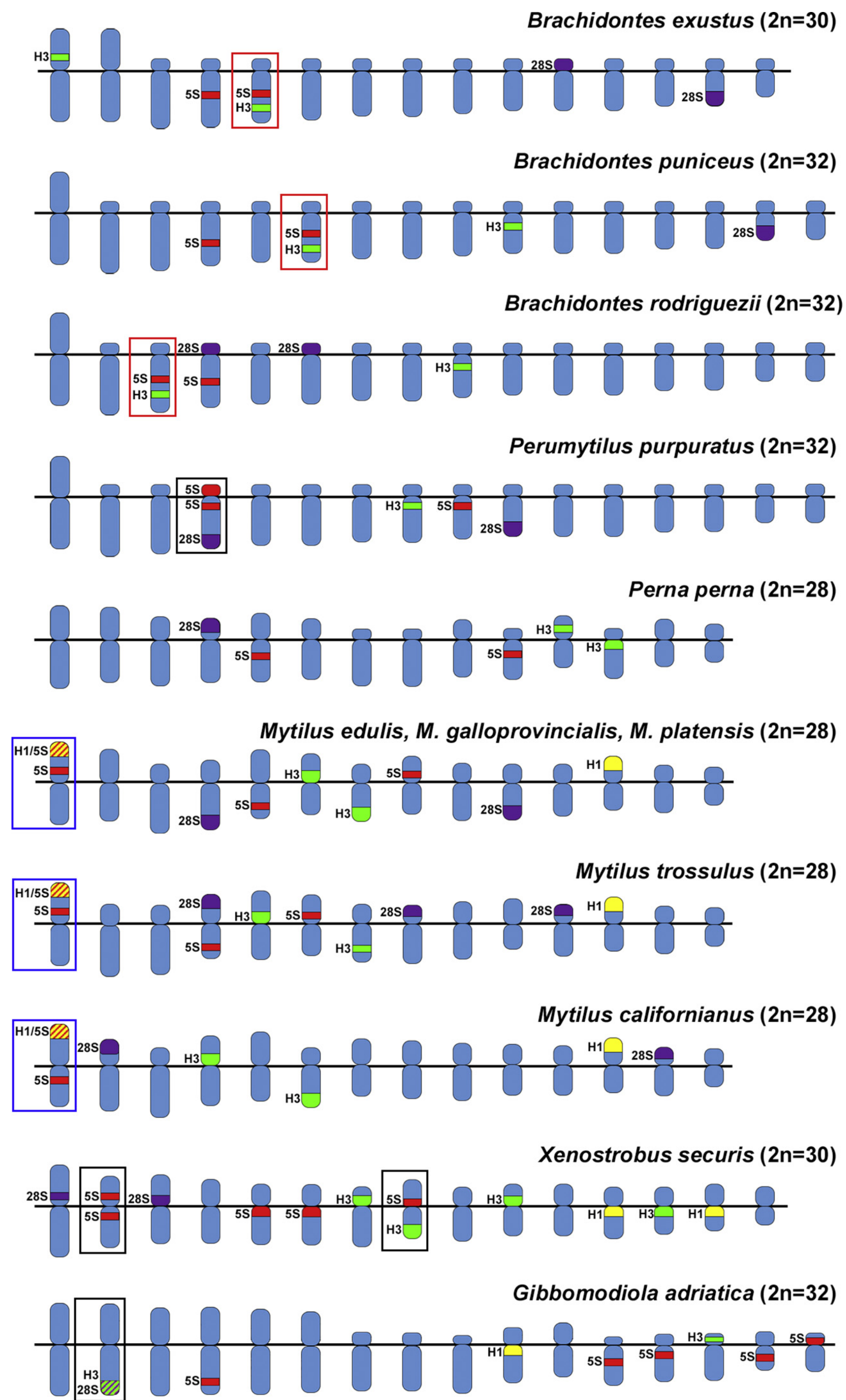


Fig. 3. Ideograms showing the chromosomal location of major rDNA (28S, magenta), 5S rDNA (5S, red), H3 histone gene (H3, green) and H1 histone gene (H1, yellow) clusters in twelve marine mussel taxa. Overlapping signals are represented as intercalated bars of the corresponding colors. Some of the chromosomes bearing different combinations of FISH signals are framed in different colors to highlight their conservation in the species of *Brachidontes* (red frames) and *Mytilus* (blue frames) and their differences with those present in *P. purpuratus*, *G. adriatica* and *X. securis* (black frames).

Although chromosome complements are characteristics of species and chromosome analysis can contribute to identify them, this kind of approach has been barely employed in marine mussels; to our knowledge, chromosome numbers have only been used as a taxonomic character in species of *Perna* (Ahmed, 1974; Holland, Gallagher, Hicks, & Davis, 1999). In spite of that, the demonstration that marine mussel species with identical diploid numbers and apparently similar karyotypes can show striking differences in the chromosomal distribution of rRNA and histone gene clusters (Pérez-García, Guerra-Varela, et al., 2010; Pérez-García et al., 2014) indicates that FISH karyotypes can be of great help in marine mussel identification. In that sense, the FISH mapping results presented here add significantly to what was previously known and further confirm the interest of FISH karyotypes as a taxonomic character in marine mussels.

In coincidence with *Brachidontes darwinianus* (Introini et al., 2004) and *B. mutabilis* (Ieyama, Kameoka, Tan, & Yamasaki, 1994) but differing from *B. pharaonis* (Vitturi, Gianguzza, Colomba, & Riggio, 2000), *B. puniceus* and *B. rodriguezii* (Pérez-García, Guerra-Varela, et al., 2010), *B. exustus* has a diploid chromosome number

Table 3
GenBank accession numbers of the COI gene sequences and highest sequence similarity (%) with sequences retrieved from GenBank.

Species	Accession numbers	Similarity	Accession numbers	Species	Reference
Brachidontes exustus	KY454042–KY454045	>99%	AY621861, AY621879	*Brachidontes exustus*	Lee and Ó Foighil (2005)
		99%	AY621901–AY621908	*Brachidontes exustus*	Lee and Ó Foighil (2005)
		99%	AY825204–AY825206	*Brachidontes exustus*	Lee and Ó Foighil (2005)
		99%	AY825139, AY825140	*Brachidontes exustus*	Lee and Ó Foighil (2005)
Brachidontes puniceus	KY454048–KY454051	>99%	HM999677, HM999676	*Brachidontes puniceus*	Cunha et al. (2011)
		>99%	**AY825105–AY825108**	***Brachidontes exustus***	Lee and Ó Foighil (2005)
Brachidontes rodriguezii	KY454052, KY454053	>99%	KT318192, KT318193	*Brachidontes rodriguezii*	Trovant et al. (2013)
Gibbomodiola adriatica	KY454028, KY454029	74%	KC509640	*Xenostrobus pulex*	Colgan and da Costa (2013)
		74%	KC509637	*Xenostrobus inconstans*	Colgan and da Costa (2013)
Mytilus edulis	KY454032, KY454033	98%	AY484747	*Mytilus edulis*	Boore, Medina, and Rosenberg (2004)
Mytilus galloprovincialis	KY454038, KY454039	99%	NC_006886	*Mytilus galloprovincialis*	Mizi, Zouros, Moschonas, and Rodakis (2005)
Mytilus platensis	KY454034, KY454035	99%	KP100301	*Mytilus edulis platensis*	Gaitán-Espitia, Quintero-Galvis, Mesas, and D'Elía (2016)
		99%	KP100300	*Mytilus chilensis*	Gaitán-Espitia et al. (2016)
Mytilus trossulus	KY454040, KY454041	>99%	KF931877, KF932000	*Mytilus trossulus*	Crego-Prieto et al. (2015)
Mytilus californianus	KY454036, KY454037	99%	NC_015993	*Mytilus californianus*	Cao et al. (2009)
Perna perna	KY454030, KY454031	>99%	KC691986, KC691989	*Perna perna*	Cunha et al. (2014)
		67%	**NC_026288.1** *	***Perna perna*** *	Uliano-Silva et al. (2015)
Perumytilus purpuratus	KY454046, KY454047	98%	KJ453867, KJ453884	*Perumytilus purpuratus*	Trovant, Orensanz, Ruzzante, Stotz, and Basso (2015)
Xenostrobus securis	KY454026, KY454027	>96%	FJ949111, FJ949111	*Xenostrobus securis*	Pascual et al. (2010)
		>97%	KC509723–KC509726	*Xenostrobus securis*	Colgan and da Costa (2013)

Discrepant results appear in bold. * Identity 99% with KT318216–KT318257 ascribed to *Mytilaster (Brachidontes) solisianus* (Trovant et al., 2016).

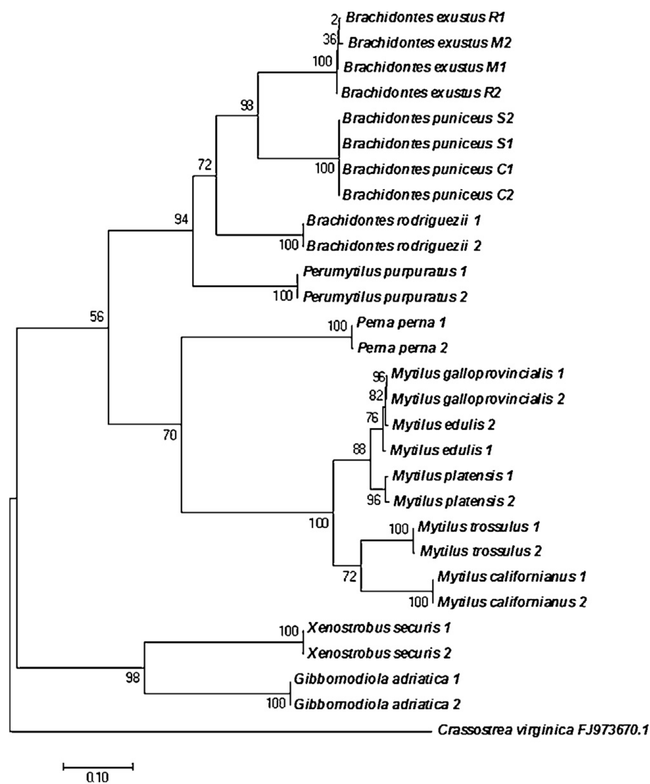


Fig. 4. Maximum likelihood tree based on mitochondrial COI gene sequences of the karyotyped marine mussels using the Eastern oyster *Crassostrea virginica* as outgroup. Numbers in internal nodes indicate maximum-likelihood bootstrap support values (500 replicates).

of 2n = 30. Although the karyotypes of *B. exustus*, *B. puniceus*, *B. rodriguezii* and *Perumytilus* (*Brachidontes*) *purpuratus* are roughly similar in morphology, they can be easily identified, and differentiated, on the basis of the distribution of rRNA and histone gene clusters on their chromosomes (Fig. 3). Taking this into account, chromosome analysis could shed light on the confused status of the *B. exustus* cryptic species complex (see below) in the western Atlantic (Lee & Ó Foighil, 2004, 2005).

The diploid chromosome number of 2n = 28 confirmed here for *M. platensis* and *P. perna* has also been reported in 12 more species of marine mussels (Table 1). Confirming that closely related species do not always show detectable karyotypic differences, the chromosomal distribution of rDNA and histone genes in *M. platensis* is identical to that previously reported for both *M. edulis* and *M. galloprovincialis* (Pérez-García et al., 2014) but clearly differs from that of *M. trossulus*, *M. californianus* (Pérez-García et al., 2014) and *P. perna*. These results suggest that although the chromosomal distribution of these sequences cannot discriminate mussel species when they are closely related (see below), they will do for most of them.

Although the chromosome number of *Gibbomodiola adriatica* (2n = 32) is also common to another nine marine mussel species, the FISH mapping data obtained further suggests that FISH karyotypes could become an essential tool to unequivocally identify most marine mussel species.

4.2. Misidentified marine mussel COI gene sequences

One major criticism to the employ of DNA barcodes as a method of species taxonomic assignation is the possibility of specimen misidentifications and the consequent accumulation of erroneous assigned sequences in DNA databases (i.e. Huber, 2015). Our results on marine mussel COI gene sequences indicate that whilst most sequences stored in GenBank are correctly assigned, some of them

are mistaken. The main errors were detected in *Perna perna* and *Brachidontes exustus*.

The COI gene sequences included in the whole mitogenome of *Perna perna* show low identity with all other *P. perna* sequences (Table 3), including ours, and are highly similar to those stored as *Mytilaster solisianus* (Trovant et al., 2016). The uncritical use of this erroneously assigned sequence led Uliano-Silva et al. (2015) to assume that the genus *Perna* is polyphyletic even if other published work on *Perna* phylogeny already demonstrated that this was not the case. Our results on karyotyped specimens confirm the correct assignation of the *P. perna* sequences used by Cunha et al. (2014).

The other mussel COI gene sequences seemingly erroneous were stored under the specific name *Brachidontes exutus*, a supposed species complex comprising of five (Lee & Ó Foighil, 2004) or four (Lee & Ó Foighil, 2005) cryptic species distributed in the western Atlantic (Caribbean Sea, Gulf of Mexico and Atlantic coast of Florida). Our results indicate that the situation is more complex and needs further analysis. The COI gene sequences and the karyotypes of *B. exustus* obtained in this work came from specimens collected both at the Atlantic (Miami) and the Gulf of Mexico (Rookery Bay) coasts of Florida. In spite of that, the karyotypes are identical and sequences highly similar and coincident with most of the western Atlantic sequences (*B. exustus* II, Atlantic clade in Lee & Ó Foighil, 2004, 2005) stored in GenBank. At the same time, other sequences (*B. exustus* III, Bahamas clade in Lee & Ó Foighil, 2004, 2005) coincide with those obtained from our karyotyped specimens of *B. puniceus*. Although much work is needed to wholly explain the real distribution of *B. exustus* in the western Atlantic, data showed in this work indicate that *B. puniceus* is also present in this area and that some specimens were mistakenly included into the presumed *B. exustus* species complex. Other sequences also assigned to *B. exustus* (Lee & Ó Foighil, 2004, 2005) show, on the one hand, low identity with both those in Cunha et al. (2011) and ours and, on the other, high similarity with those stored under the specific name *Geukensia demissa* (Combosch et al., 2017), therefore pointing to misidentification.

The validity of the marine mussel identification approach used in this work is reinforced by the phylogenetic tree obtained after using the COI gene sequences of twelve karyotyped marine mussel species, highly coherent with most proposed phylogenies for the family Mytilidae.

In conclusion, the results obtained in this work clearly indicate that the combined use of morphological, chromosomal and molecular characteristics in the identification of marine mussels can be a good approach for reducing misidentification when working with presumably invasive and/or cryptic species of Mytilidae.

Acknowledgements

This work was partly funded by grants from Xunta de Galicia, Ministerio de Economía y Competitividad and Fondos FEDER: "Unha maneira de facer Europa" (Axudas do programa de consolidación e estruturación de unidades de investigacións competitivas do SUG: ED431C 2016-037, AGL2014-52062-R). D. García-Souto was partially supported by a FPU fellowship from "Ministerio de Educación, Cultura y Deporte" (Spain).

References

Ahmed, M., & Sparks, A. K. (1970). Chromosome number: Structure and autosomal polymorphism in the marine mussels *Mytilus edulis* and *Mytilus californianus*. *Biological Bulletin*, *138*, 1–13.

Ahmed, M. (1974). Chromosomes of two species of the marine mussel *Perna* (Mytilidae: Pelecypoda). *Boletin del Instituto Oceanográfico de la Universidad de Oriente*, *13*, 17–22.

Baker, P., Fajans, J. S., Arnold, W. S., Ingrao, D. A., Marelli, D. C., & Baker, S. M. (2007). Range and dispersal of a tropical marine invader, the Asian green mussel, *Perna viridis* in subtropical waters of the Southeastern United States. *Journal of Shellfish Research*, *26*, 345–355. http://dx.doi.org/10.2983/0730-8000(2007)26[345:RADOAT]2.0.CO;2

Boltovskoy, D., Correa, N., Cataldo, D., & Sylvester, F. (2006). Dispersion and ecological impact of the invasive freshwater bivalve *Limnoperna fortunei* in the Río de la Plata watershed and beyond. *Biological Invasions*, *8*, 947–963. http://dx.doi.org/10.1007/s10530-005-5107-z

Boore, J. L., Medina, M., & Rosenberg, L. A. (2004). Complete sequences of the highly rearranged molluscan mitochondrial genomes of the scaphopod *Graptacme eborea* and the bivalve *Mytilus edulis*. *Molecular Biology and Evolution*, *21*, 1492–1503. http://dx.doi.org/10.1093/molbev/msh090

Branch, G. M., & Steffani, C. N. (2004). Can we predict the effects of alien species? A case-history of the invasion of South Africa by *Mytilus galloprovincialis* (Lamarck). *Journal of Experimental Marine Biology and Ecology*, *300*, 189–215. http://dx.doi.org/10.1016/j.jembe.2003.12.007

Cao, L., Ort, B. S., Mizi, A., Pogson, G., Kenchington, E., Zouros, E., et al. (2009). The control region of maternally and paternally inherited mitochondrial genomes of three species of the sea mussel genus *Mytilus*. *Genetics*, *181*, 1045–1056. http://dx.doi.org/10.1534/genetics.108.093229

Colgan, D. J., & da Costa, P. (2013). Invasive and non-invasive lineages in *Xenostrobus* (Bivalvia: Mytilidae). *Molluscan Research*, *33*, 272–280. http://dx.doi.org/10.1080/13235818.2013.826574

Combosch, D. J., Collins, T. M., Glover, E. A., Graf, D. L., Harper, E. M., Healy, J. M., et al. (2017). A family-level Tree of Life for bivalves based on a Sanger-sequencing approach. *Molecular Phylogenetics and Evolution*, *107*, 191–208. http://dx.doi.org/10.1016/j.ympev.2016.11.003

Crego-Prieto, V., Ardura, A., Juanes, F., Roca, A., Taylor, J. S., & Garcia-Vazquez, E. (2015). Aquaculture and the spread of introduced mussel genes in British Columbia. *Biological Invasions*, *17*, 2011–2026. http://dx.doi.org/10.1007/s10530-015-0853-z

Crooks, J. A., & Khim, H. S. (1999). Architectural vs. biological effects of a habitat-altering, exotic mussel, Musculista senhousia. *Journal of Experimental Marine Biology and Ecology*, *240*, 53–75. http://dx.doi.org/10.1016/s0022-0981(99)00041-6

Cunha, R. L., Lopes, E. P., Reis, D. M., & Castilho, R. (2011). Genetic structure of *Brachidontes puniceus* populations in Cape Verde archipelago shows signature of expansion during the last glacial maximum. *Journal of Molluscan Studies*, *77*, 175–181. http://dx.doi.org/10.1093/mollus/eyr001

Cunha, R. L., Nicastro, K. R., Costa, J., McQuaid, C. D., Serrao, E. A., & Zardi, G. I. (2014). Wider sampling reveals a non-sister relationship for geographically contiguous lineages of a marine mussel. *Ecology and Evolution*, *4*, 2070–2081. http://dx.doi.org/10.1002/ece3.1033

Diuportex-Chong, M. E., Rodríguez-Romero, F., Uribe-Alcocer, M., & Laguarda-Figueras, A. (1978). Karyotype characters of *Brachidontes recurvus* (Pelecipoda-Mytilidae). *Anuario del Centro de Ciencias Marinas y Limnología de la Universidad Nacional Autonónoma de Mexico*, *5*, 55–58.

Dixon, D. R., Jolly, M. T., Vevers, W. F., & Dixon, L. R. J. (2010). Chromosomes of Pacific hydrothermal vent invertebrates: Towards a greater understanding of the relationship between chromosome and molecular evolution. *Journal of the Marine Biological Association of the United Kingdom*, *90*, 15–31. http://dx.doi.org/10.1017/S0025315409000149

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–299.

Gaitán-Espitia, J. D., Quintero-Galvis, J. F., Mesas, A., & D'Elía, G. (2016). Mitogenomics of southern hemisphere blue mussels (Bivalvia: Pteriomorphia): Insights into the evolutionary characteristics of the *Mytilus edulis* complex. *Scientific Reports*, *6*, 26853. http://dx.doi.org/10.1038/srep26853

García-Souto, D., Pérez-García, C., Morán, P., & Pasantes, J. J. (2015). Divergent evolutionary behavior of H3 histone gene and rDNA clusters in venerid clams. *Molecular Cytogenetics*, *8*, 40. http://dx.doi.org/10.1186/s13039-015-0150-7

García-Souto, D., Kendall, J., Pérez-García, C., & Pasantes, J. J. (2016). Molecular cytogenetics in trough shells (Mactridae, Bivalvia): Divergent GC-rich heterochromatin content. *Genes*, *7*, 47. http://dx.doi.org/10.3390/genes7080047

Geller, J. B., Darling, J. A., & Carlton, J. T. (2010). Genetic perspectives on marine biological invasions. *Annual Review of Marine Science*, *2*, 367–393. http://dx.doi.org/10.1146/annurev.marine.010908.163745

Ghobashy, A. F. A., Mona, M. H., Yasseen, A. E., & Desouky, M. (1995). A chromosomic study of the marine bivalve *Modiolus arcuatulus* (Pteriomorphia: Mytilidae). *Journal of the Marine Biological Association of India*, *37*, 277–280.

Giribet, G., & Distel, D. (2003). Bivalve phylogeny and molecular data. In C. Lydeard, & D. R. Lindberg (Eds.), *Systematics and Phylogeography of Mollusks* (pp. 45–90). Washington, DC: Smithsonian Books.

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London (B)*, *270*, 313–332. http://dx.doi.org/10.1098/rspb.2002.2218

Holland, B. S., Gallagher, D. S., Hicks, D. W., & Davis, S. K. (1999). Cytotaxonomic verification of a non-indigenous marine mussel in the Gulf of Mexico. *The Veliger*, *42*, 280–282.

Holland, B. S. (2001). Invasion without a bottleneck: Microsatellite variation in natural and invasive populations of the brown mussel Perna perna (L). *Marine Biotechnology*, *3*, 407–415. http://dx.doi.org/10.1007/s1012601-0060-Z

Hopple, J. S., Jr., & Vilgalys, R. (1994). Phylogenetic relationships among coprinoid taxa and allies based on data from restriction site mapping of nuclear rDNA. *Mycologia*, *86*, 96–107. http://dx.doi.org/10.2307/3760723

Huber, M. (2010). *Compendium of bivalves. A full-color guide to 3300 of the world's marine bivalves. A status on Bivalvia after 250 years of research*. Hackenheim: ConchBooks.

Huber, M. (2015). *Compendium of Bivalves 2: A full-color guide to the remaining seven families. A systematic listing of 8'500 Bivalve species and 10'500 synonyms*. ConchBooks: Hackenheim.

Ieyama, H., Kameoka, O., Tan, T., & Yamasaki, J. (1994). Chromosomes and nuclear DNA contents of some species in Mytilidae. *Venus*, *53*, 327–331.

Ieyama, H. (1977). Studies on the chromosomes of two species in Mytilidae (Pteriomorphia, Bivalvia). *Venus*, *36*, 25–28.

Ieyama, H. (1983). Studies on the chromosomes of four species of the genus Septifer (Bivalvia, Mytilidae). *Venus*, *42*, 193–202.

Ieyama, H. (1984). Karyotype in eight species of the Mytilidae (Bivalvia: Pteriomorpha). *Venus*, *43*, 240–253.

Insua, A., Labat, J. P., & Thiriot-Quiévreux, C. (1994). Comparative analysis of karyotypes and nucleolar organizer regions in different populations of *Mytilus trossulus*, *Mytilus edulis* and *Mytilus galloprovincialis*. *Journal of Molluscan Studies*, *60*, 359–370.

Introini, G. O., De Magalhaes, C. A., & Recco-Pimentel, S. M. (2010). Chromosomal number of two species of bivalves: *Brachidontes darwinianus* (d'Orbigny, 1842) (Mytilidae) and *Isognomon bicolor* (CB Adams, 1845) (Isognomonidae). *The Nautilus*, *124*, 151–154.

Katsanevakis, S., Bogucarskis, K., Gatto, F., Vandekerkhove, J., Deriu, I., & Cardoso, A. S. (2012). Building the European alien species information network (EASIN): A novel approach for the exploration of distributed alien species data. *BioInvasions Records*, *1*, 235–245.

Knowlton, N. (1993). Sibling species in the sea. *Annual Review of Ecology and Systematics*, *24*, 189–216.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEG: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, http://dx.doi.org/10.1093/molbev/msw054, msw054

Lee, T., & Ó Foighil, D. (2004). Hidden Floridian biodiversity: Mitochondrial and nuclear gene trees reveal four cryptic species within the scorched mussel, *Brachidontes exustus*, species complex. *Molecular Ecology*, *13*, 3527–3542. http://dx.doi.org/10.1111/j.1365-294X.2004.02337.x

Lee, T., & Ó Foighil, D. (2005). Placing the Floridian marine genetic disjunction into a regional evolutionary context using the scorched mussel, *Brachidontes exustus*, species complex. *Evolution*, *59*, 2139–2158. http://dx.doi.org/10.1111/j.0014-3820.2005. tb00924.x

Levan, A., Fredga, K., & Sandberg, A. A. (1964). Nomenclature for centromeric position on chromosomes. *Hereditas*, *52*, 201–220. http://dx.doi.org/10.1111/j.1601-5223.1964.tb01953.x

Libertini, A., Boato, A., Panozzo, M., & Fogato, V. (1996). Karyotype and genome size in some species of Mytilidae (Bivalvia, Mollusca). *La kromosomo II*, *82*, 2819–2827.

Lourenço, C. R., Nicastro, K. R., Serrão, E. A., Castilho, R., & Zardi, G. I. (2015). Behind the mask: Cryptic genetic diversity of *Mytilus galloprovincialis* along southern European and northern African shores. *Journal of Molluscan Studies*, *81*, 380–387. http://dx.doi.org/10.1093/mollus/eyv004

Méndez, J., Pasantes, J. J., & Martínez-Expósito, M. J. (1990). Banding pattern of mussel (*Mytilus galloprovincialis*) chromosomes induced by 2xSSC/Giemsa-stain treatmen. *Marine Biology*, *106*, 375–377.

Martínez-Expósito, M. J., Pasantes, J. J., & Méndez, J. (1994a). NOR activity in larval and juvenile mussels (*Mytilus galloprovincialis* Lmk). *Journal of Experimental Marine Biology and Ecology*, *175*, 155–165. http://dx.doi.org/10.1016/0022-0981(94)90023-X

Martínez-Expósito, M. J., Pasantes, J. J., & Méndez, J. (1994b). Proliferation kinetics of mussel (*Mytilus galloprovincialis*) gill cells. *Marine Biology*, *120*, 41–45. http://dx.doi.org/10.1007/BF00381940

Mizi, A., Zouros, E., Moschonas, N., & Rodakis, G. C. (2005). The complete maternal and paternal mitochondrial genomes of the Mediterranean mussel *Mytilus galloprovincialis*: Implications for the doubly uniparental inheritance mode of mtDNA. *Molecular Biology and Evolution*, *22*, 952–967. http://dx.doi.org/10.1093/molbev/msi079

Nakamura, H. K. (1985). A review of molluscan cytogenetic information based on the CISMOH—Computerized Index System for Molluscan Chromosomes. Bivalvia, Polyplacophora and Cephalopoda. *Venus*, *44*, 193–225.

Pérez-García, C., Morán, P., & Pasantes, J. J. (2011). Cytogenetic characterization of the invasive mussel species *Xenostrobus securis* Lmk. (Bivalvia: Mytilidae). *Genome*, *54*, 771–778. http://dx.doi.org/10.1139/g11-040

Pérez-García, C., Morán, P., & Pasantes, J. J. (2014). Karyotypic diversification in *Mytilus* mussels (Bivalvia: Mytilidae) inferred from chromosomal mapping of rRNA and histone gene clusters. *BMC Genetics*, *15*, 84. http://dx.doi.org/10.1186/1471-2156-15-84

Pérez-García, C., Cambeiro, J. M., Morán, P., & Pasantes, J. J. (2010). Chromosomal mapping of rDNAs, core histone genes and telomeric sequences in *Perumytilus purpuratus* (Bivalvia: Mytilidae). *Journal of Experimental Marine Biology and Ecology*, *395*, 199–205. http://dx.doi.org/10.1016/j.jembe.2010.09.004

Pérez-García, C., Guerra-Varela, J., Morán, P., & Pasantes, J. J. (2010). Chromosomal mapping of rRNA genes, core histone genes and telomeric sequences in *Brachidontes puniceus* and *Brachidontes rodriguezii* (Bivalvia: Mytilidae). *BMC Genetics*, *11*, 109. http://dx.doi.org/10.1186/1471-2156-11-109

Palma-Rojas, C., Guerra, R., Brown, D., & Von Brand, E. (1997). Quantitative karyotype of *Choromytilus chorus* (Mollusca: Bivalvia: Mytilidae). *The Veliger*, *40*, 259–262.

Pasantes, J. J., Martínez-Expósito, M. J., Martínez-Lage, A., & Méndez, J. (1990). Chromosomes of Galician mussel. *Journal of Molluscan Studies*, *56*, 123–126.

Pascual, S., Villalba, A., Abollo, E., Garci, M., González, A. F., Nombela, M., et al. (2010). The mussel *Xenostrobus securis*: A well-established alien invader in the Ría de Vigo (Spain, NE Atlantic). *Biological Invasions*, *12*, 2091–2103. http://dx.doi.org/10.1007/s10530-009-9611-4

Rajagopal, S., Venugopalan, V. P., van der Velde, G., & Jenner, H. A. (2006). Greening of the coasts: A review of the *Perna viridis* success story. *Aquatic Ecology*, *40*, 273–297. http://dx.doi.org/10.1007/s10452-006-9032-8

Reeves, A., & Tear, J. (2000). *MicroMeasure for Windows, version 3.3.*. http://sites.biology.colostate.edu/MicroMeasure/ (Accessed 01 December 2016)

Rilov, G., & Crooks, J. A. (2009). *Biological Invasions in Marine Ecosystems*. Berlin: Springer-Verlag.

Smith, P. J., McVeagh, S. M., Won, Y., & Vrijenhoek, R. C. (2004). Genetic heterogeneity among New Zealand species of hydrothermal vent mussels (Mytilidae: *Bathymodiolus*). *Marine Biology*, *144*, 537–545. http://dx.doi.org/10.1007/s00227-003-1207-4

Sousa, R., Gutiérrez, J. L., & Aldridge, D. C. (2009). Non-indigenous invasive bivalves as ecosystem engineers. *Biological Invasions*, *11*, 2367–2385. http://dx.doi.org/10.1007/s10530-009-9422-7

Terranova, M. S., Lo Brutto, S., Arculeo, M., & Mitton, J. B. (2007). A mitochondrial phylogeography of Brachidontes variabilis (Bivalvia: Mytilidae) reveals three cryptic species. *Journal of Zoology, Systematics and Evolutionary Research*, *45*, 289–298. http://dx.doi.org/10.1111/j.1439-0469.2007.00421.x

Thiriot-Quiévreux, C., & Ayraud, N. (1982). Les caryotypes de quelques espéces de bivalves et de gastéropodes marins. *Marine Biology*, *70*, 165–172.

Thiriot-Quiévreux, C. (1984a). Le caryotype d'*Aulacomya ater regia* (Mollusca: Bivalvia: Mytilidae) des iles Kerguelen. *Marine Biology*, *79*, 89–91.

Thiriot-Quiévreux, C. (1984b). Chromosome analysis of three species of *Mytilus* (Bivalvia: Mytilidae). *Marine Biology Letters*, *5*, 65–73.

Thiriot-Quiévreux, C. (1994). Advances in cytogenetics of aquatic organisms. In A. Beaumont (Ed.), *Genetics and evolution of aquatic organisms* (pp. 369–388). London: Chapman and Hall.

Thiriot-Quiévreux, C. (2002). Review of the literature on bivalve cytogenetics in the last ten years. *Cahiers de Biologie Marine*, *43*, 17–26.

Torreiro, A., Martínez-Expósito, M. J., Trucco, M. I., & Pasantes, J. J. (1999). Cytogenetics in *Brachidontes rodriguezii* d'Orb (Bivalvia, Mytilidae). *Chromosome Research*, *7*, 49–55.

Trovant, B., Ruzzante, D. E., Basso, N. G., & Orensanz, J. L. (2013). Distinctness, phylogenetic relations and biogeography of intertidal mussels (*Brachidontes*, Mytilidae) from the south-western Atlantic. *Journal of the Marine Biological Association of the United Kingdom*, *93*, 1843–1855. http://dx.doi.org/10.1017/S0025315413000477

Trovant, B., Orensanz, J. L., Ruzzante, D. E., Stotz, W., & Basso, N. G. (2015). Scorched mussels (Bivalvia: Mytilidae: Brachidontinae) from the temperate coasts of South America: Phylogenetic relationships, trans-Pacific connections and the footprints of Quaternary glaciations. *Molecular Phylogenetics and Evolution*, *82*, 60–74. http://dx.doi.org/10.1016/j.ympev.2014.10.002

Trovant, B., Basso, N. G., Orensanz, J. M., Lessa, E. P., Dincao, F., & Ruzzante, D. E. (2016). Scorched mussels (*Brachidontes* spp., Bivalvia: Mytilidae) from the tropical and warm-temperate southwestern Atlantic: The role of the Amazon River in their speciation. *Ecology and Evolution*, *6*, 1778–1798. http://dx.doi.org/10.1002/ece3.2016

Uliano-Silva, M., Americo, J. A., Costa, I., Schomaker-Bastos, A., de Freitas Rebelo, M., & Prosdocimi, F. (2015). The complete mitochondrial genome of the golden mussel *Limnoperna fortunei* and comparative mitogenomics of Mytilidae. *Gene*, *577*, 202–208. http://dx.doi.org/10.1016/j.gene.2015.11.043

Vilgalys, R., & Hester, M. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology*, *172*, 4238–4246. http://dx.doi.org/10.1128/jb.172.8.4238-4246

Vitturi, R., Gianguzza, P., Colomba, M. S., & Riggio, S. (2000). Cytogenetic characterization of *Brachidontes pharaonis* (Fisher P, 1870): Karyotype, banding and fluorescent in situ hybridization (FISH) (Mollusca: Bivalvia: Mytilidae). *Ophelia*, *52*, 213–220.

WRIMS Editorial Board World Register of Introduced Marine Species. http://www.marinespecies.org/introduced/ (Accessed 09 January 2017).

WoRMS Editorial Board World Register of Marine Species. http://www.marinespecies.org/ (Accessed 09 January 2017).