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Phylogeny and phylogeography of Atlantic oyster species: evolutionary history, limited genetic connectivity and isolation by distance

C. Lazoski[1], J. Gusmão[1,2], P. Boudry[3], A. M. Solé-Cava[1,*]

[1]Laboratório de Biodiversidade Molecular – Departamento de Genética, Instituto de Biologia, UFRJ, CCS, Bloco A, sala A2-98, Ilha do Fundão, 21941-490 Rio de Janeiro, Brazil

[2]Laboratório de Genética Marinha — Departamento de Genética, Instituto de Biologia Roberto Alcântara Gomes, UERJ, Maracanã, 20550-900 Rio de Janeiro, Brazil

[3]Ifremer, UMR M100 Physiologie et Ecophysiologie des Mollusques Marins, 29280 Plouzané, France

ABSTRACT: The phylogenetic relationships between naturally occurring Atlantic *Crassostrea* oyster species were inferred through analyses of mitochondrial (cytochrome oxidase subunit I and 16S) and nuclear (second internal transcribed spacer) sequences. We also scored 15 allozyme loci on 422 oysters to study population structuring of *C. rhizophorae* and *C. brasiliana* along 9000 km of the Western Atlantic coastline. Despite morphological similarities, *C. virginica* was genetically more closely related to *C. rhizophorae* than to *C. brasiliana*. In contrast, *C. paraibanensis* was genetically indistinguishable from *C. brasiliana*, which is probably a junior synonym of the African *C. gasar*. Significant genetic differentiation between populations of *C. rhizophorae* and *C. gasar* were found along the Western Atlantic coast, supporting an isolation-by-distance pattern.

KEY WORDS: Population genetics · Biogeography · Allozymes · Cytochrome *c* oxidase subunit I · COI · 16S · Internal transcribed spacer 2 · ITS-2

INTRODUCTION

Oysters are the world's most economically important mollusks, with a global average production of 4.6 million tons yr^{-1} (FAO 2010). In the Western Atlantic, oysters of the genus *Crassostrea* are the fourth largest marine fishery resource (average of 200 000 tons yr^{-1}; FAO 2010). The most heavily exploited species in the region are *C. virginica* (Gmelin, 1791), *C. rhizophorae* (Guilding, 1828) and the exotic *C. gigas* (Thünberg, 1793).

Phylogenetic studies have demonstrated the existence of 2 evolutionary lineages within the *Crassostrea* genus, one from the Atlantic and another from the Pacific, which could have been formed during the closure of the Tethys Seaway (Ó Foighil & Taylor 2000). Molecular phylogenies of both nuclear and mitochondrial genomes have helped to clarify the taxonomic confusion of the Pacific *Crassostrea* species (Reece et al. 2008, Wu et al. 2010). On the other hand, the taxonomic status of Atlantic *Crassostrea* oysters is still confused: there is clear evidence for the existence of at least 2 oyster species along the Brazilian coast (Absher 1989, Ignacio et al. 2000), but 5 other nominal species have been cited for the area, regarded as native (*C. virginica*, Carriker & Gaffney 1996; *C. paraibanensis*, Singarajah 1980) or invasive (*C. gigas*, Melo et al. 2010b; *C. gasar* Adanson, 1757, Lapègue et al. 2002; *Crassostrea* sp., Varela et al. 2007, Melo et al. 2010a) species. The correct identification of those oyster species is of paramount importance, because they are used in aquaculture and in biomonitoring studies (e.g. Rebelo et al. 2003), and may be under environmental threat (Carranza et al. 2009).

The uncertainty in the classification of Atlantic oyster species may have been caused in part by the great

*Corresponding author. Email: sole@biologia.ufrj.br

morphological and ecological similarities between *Crassostrea brasiliana* (Lamarck, 1819) and *C. virginica*. *C. rhizophorae* and *C. brasiliana* are also morphologically quite similar and have been considered synonyms in monographic studies of Brazilian mollusks (Rios 1994). This may have led some systematists to suggest that *C. rhizophorae* and *C. brasiliana* were morphotypes of a single species (*C. virginica*) (Harry 1985, Carriker & Gaffney 1996).

Crassostrea rhizophorae is found in the intertidal zone, either attached to *Rhizophora mangle* roots or on rocks, from the Caribbean to the southern Atlantic (Santa Catarina State, Brazil) (Carriker & Gaffney 1996). *C. brasiliana* usually attaches to rocks in infralittoral zones, and it is most abundant from southeastern Brazil (Espírito Santo State) to the more temperate regions in southern Brazil (Absher 1989). The species has also been observed in French Guyana (Lapègue et al. 2002) and in Venezuela (Hoover & Gaffney 2005).

Besides the ecological differences, these oyster species can be distinguished by shell size, which is much larger in *Crassostrea brasiliana* (50 to 190 mm) than in *C. rhizophorae* (20 to 65 mm) (Absher 1989). However, this only holds true for adult specimens. Based on Lamarck's small-shelled holotype for *C. brasiliana*, Singarajah (1980) considered it a synonym of *C. rhizophorae*. Hence, he described the large oysters found in Paraíba State (northeastern Brazil) as a new species, *C. paraibanensis* (Singarajah 1980). However, other authors considered that this species might be synonymous with *C. brasiliana* (Rios 1994, Carriker & Gaffney 1996).

Those taxonomic problems can also result from differing views on the degree of differentiation required to define a species in *Crassostrea*. Since the late 1970s, many studies have employed molecular methods to address the systematics and population genetics of *Crassostrea* species. For example, consistent genetic differences have been found between *C. rhizophorae* and *C. virginica* using allozyme (Buroker et al. 1979, Hedgecock & Okazaki 1984) and ribosomal DNA data (Littlewood 1994), as well as between *C. rhizophorae* and *C. brasiliana* using morphology (Absher 1989), allozymes (Ignacio et al. 2000, Lazoski 2004) and mitochondrial 16S or cytochrome *c* oxidase subunit I (COI) data (Lapègue et al. 2002, Boudry et al. 2003, Lazoski 2004, Varela et al. 2007). In contrast, Lapègue et al. (2002) did not find any differences between 16S sequences of West African *C. gasar* and South American *Crassostrea* specimens, which led them to suggest that *C. brasiliana* and *C. gasar* might be the same species (with *C. brasiliana* being a junior synonym of *C. gasar*). However, no molecular studies have compared *C. virginica* and *C. brasiliana* populations.

Although marine species with potentially high dispersal capability are believed to have high levels of gene flow, there are many examples showing the opposite (e.g. Palumbi 2003, Johansson et al. 2008), including studies of *Crassostrea* species (Hedgecock & Okazaki 1984, Hare & Avise 1998, Xiao et al. 2010). Population genetic studies have unveiled patterns of isolation by distance among oyster populations (Launey et al. 2002, Rose et al. 2006, Xiao et al. 2010), and are of great interest not only when addressing evolutionary and ecological processes but also as a basis for management and conservation of commercially important marine species.

In spite of the economic importance of *Crassostrea* oysters, no study has been carried out on the degree of genetic connectivity of their populations along the Western Atlantic. Moreover, the unresolved systematics of those species has confounded the compiled fishery statistics data (FAO 2010) for the Latin American coast, and the true geographic distribution of oyster species along this area is still uncertain (Singarajah 1980, Carriker & Gaffney 1996, Ignacio et al. 2000, Lapègue et al. 2002, Varela et al. 2007).

In this paper, we compare Atlantic populations of *Crassostrea* by means of allozyme electrophoresis and analyses of nuclear (internal transcribed spacer 2, ITS-2) and mitochondrial (COI, 16S) sequences. Our objectives were to define the taxonomic boundaries among the Western Atlantic oysters, *C. brasiliana*, *C. virginica*, *C. rhizophorae* and *C. paraibanensis*, and to verify their actual distribution. We also compared the nuclear and mitochondrial gene sequences from specimens of *C. brasiliana* and the African *C. gasar* to resolve their taxonomic ambiguity. Additionally, we investigated the levels of genetic variation and the population structures in *C. brasiliana* and *C. rhizophorae* along 9000 km of the Western Atlantic coastline.

MATERIALS AND METHODS

Sampling. A total of 419 individuals, presumed to be either *Crassostrea rhizophorae* or *C. brasiliana*, were collected between May 1996 and February 2003 from 21 localities in Brazil and Panama (Table 1). Sixty-five samples of other *Crassostrea* species were also collected in the Gulf of Mexico (USA; 29° 03′ N; 95° 07′ W: *C. virginica*, N = 6), Africa (Senegal; 14° 10′ N; 16° 51′ W: *C. gasar*, N = 7; Lapègue et al. 2002) and Brazil (Itajaí; 26° 55′ S; 48° 38′ W: hatchery of *C. gigas*, N = 31; and Mamanguape, PB1, 07° 06′ S; 34° 54′ W: *C. paraibanensis*, N = 21) (Table 1, Fig. 1). Twenty-two individuals of *Ostrea* sp. from Brazil (Sepetiba, RJ1, 22° 58′ S; 43° 42′ W; Picinguaba, SP1, 23° 22′ S; 44° 50′ W) were used as an outgroup for the allozyme analysis. After collection, the oysters were transported (alive, in liquid N_2 or in ethanol) to the laboratory, where they were identified morpholog-

Table 1. *Crassostrea rhizophorae* and *C. brasiliana*. Sampling sites, coordinates and habitats for the putative *Crassostrea rhizophorae* and *C. brasiliana* collected. (i): intertidal zone; (s): subtidal zone. *Samples available only for DNA analysis

Sites	Coordinates	Habitat	*C. brasiliana*	*C. rhizophorae*
Panama				
Panama (PAN)	09°10′N; 80°17′W	Rocks (i)	–	14
Brazil				
Belém (PA)	01°25′S; 48°28′W	Mangrove (i)	12	–
Araioses (MA)	02°53′S; 41°54′W	Mangrove (i)	12*	–
Parnaíba (PI)	02°51′S; 41°45′W	Rocks (i)	5*	–
Fortaleza (CE)	03°46′S; 38°26′W	Rocks (i)	–	12*
Natal (RN1)	05°45′S; 35°11′W	Mangrove (i)	–	35
Natal (RN2)	05°52′S; 35°09′W	Rocks (i)	–	20
Mamanguape (PB1)	07°06′S; 34°54′W	Mangrove (i)	7	10
Cabo Branco (PB2)	08°07′S; 34°52′W	Mangrove (i)	–	24
Recife (PE)	08°46′S; 34°56′W	Mangrove (i)	24(1*)	1(3*)
Salvador (BA1)	13°00′S; 38°26′W	Rocks (i)	20	–
Caravelas (BA2)	17°44′S; 39°15′W	Mangrove (i)	–	30
Vitória (ES)	20°18′S; 40°17′W	Rocks (i)	–	12*
Sepetiba (RJ1)	22°58′S; 43°42′W	Rocks (i)	–	22
Guaratiba (RJ2)	22°58′S; 43°40′W	Mangrove (i)	–	20*
Rio das Ostras (RJ3)	22°31′S; 41°56′W	Rocks (s)	5*	–
Picinguaba (SP1)	23°22′S; 44°50′W	Rocks (i)	–	10
Itanhaém (SP2)	24°11′S; 46°47′W	Mangrove (i)	4	–
Cananéia (SP3)	25°00′S; 47°56′W	Rocks (s)	7*	–
Paranaguá (PR1)	25°32′S; 48°22′W	Rocks (s)	40	–
Paranaguá (PR1)	25°32′S; 48°22′W	Rocks (i)	–	35
Guaratuba (PR2)	25°51′S; 48°35′W	Rocks (i)	2	32
Total			**139**	**280**

ically (Absher 1989; Fig. 2), measured (shell height, length and depth), dissected, and stored in either liquid nitrogen or ethanol until required for genetic analyses.

Allozymes. Horizontal 12.5% starch gel electrophoresis was carried out as previously described (Ignacio et al. 2000). The 11 enzyme systems investigated and the 3 buffer systems used are summarized in Table 2. The enzyme systems were stained according to standard procedures (Manchenko 1994) and corresponded to a total of 15 allozyme loci.

DNA purification, amplification and sequencing. Total DNA purification was performed using a modified 1% N-cetyl N,N,N-trimethylammonium bromide (CTAB) protocol (2% CTAB, 20 mM EDTA, 0.2% β-mercaptoethanol, 0.1 M Tris, 1.4 mM NaCl), followed by a sodium acetate- and isopropanol-induced precipitation step (Gusmão & Solé-Cava 2002). Polymerase chain reactions (PCR) used approximately 10 ng of template DNA, 1 unit of *Taq* polymerase (GE Life Sciences), 200 µM each of the 4 dinucleo-

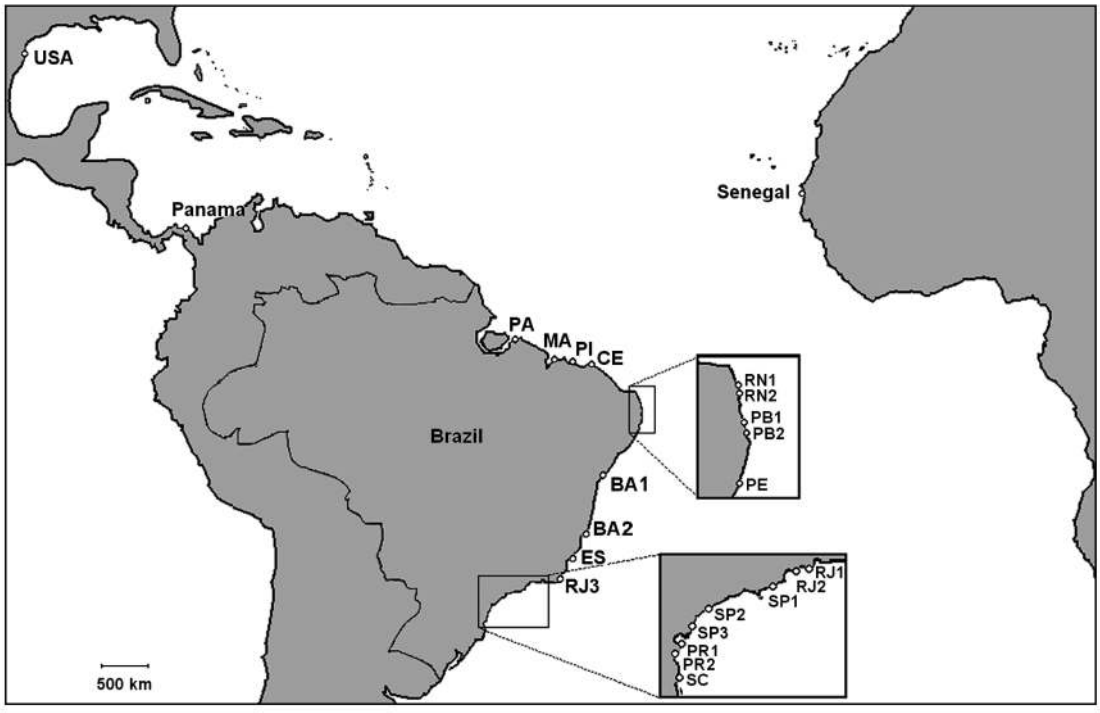

Fig. 1. *Crassostrea* spp. and *Ostrea* sp. Collection sites for specimens

Fig. 2. *Crassostrea* spp. and *Ostrea* sp. Shells from Western Atlantic populations. *C. brasiliana* (Brazil): **1** (SC); **2, 17** (PR1); **3** (PR2); **9, 18** (PE); **11** (PA); *C. rhizophorae* (Brazil): **4, 5** (RJ1); **6, 8** (RN2); **7** (SP1); **10, 20** (PR1); *C. virginica* (USA): **19** (Gulf of Mexico); *C. gigas* (Brazil): **16** (SC); *Ostrea* sp. (Brazil): **12, 13** (RJ1); **14, 15** (SP1). Unit scale: cm

Table 2. Enzymes studied (and abbreviations), Enzyme Commission numbers (EC), and buffer systems analyzed. TEM: 0.10 M Tris, 0.01 M EDTA, 0.10 M maleate, pH 7.4; TC8: 0.25 M Tris, 0.06 M citrate, pH 8.0; LI: 0.005 M citrate, 0.03 M Tris (gel), 0.06 M LiOH, 0.30 M borate (buffer tank), pH 8.5/8.1 (Manchenko 1994)

Enzymes	Abbrev	EC	Buffers
Adenylate kinase	AK	2.7.4.3	TEM
Catalase	CAT	1.11.1.6	TC8
Phosphoglucomutase	PGM	2.7.5.1	TC8
Phosphogluconate dehydrogenase	PGD	1.1.4.4	TEM
Phosphoglucose isomerase	PGI	5.3.1.9	TC8
Glutamate oxaloacetate transaminase	GOT	2.6.1.1	LI
Isocitrate dehydrogenase	IDH	1.1.1.42	TEM
Leucine amino peptidase	LAP	3.4.1.1	LI
Malate dehydrogenase	MDH	1.1.1.37	TC8
Mannose phosphate isomerase	MPI	5.3.1.8	TC8
Proline-phenylalanine dipeptidase	PEP-A	3.4.13.18	TC8

tides, 500 nM of each primer, and 1.5 mM $MgCl_2$ in 20 µl of 1× PCR buffer (GE Life Sciences). Amplifications were performed in a mini-cycler (Sprint) programmed to begin with a denaturing step of 3 min at 95°C, which was followed by 30 cycles consisting of the following steps: 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 5 min. Negative controls, involving template-free reactions, were included in all PCR amplifications. The primers used for the amplification of each specific genomic region were (1) 16SAR (5′-CGC CTG TTT ATCA AAA AC A T-3′) and 16SBR (5′-CCG GTC TGAACTCAG ATC ACG T-3′) (Palumbi 1996) for the amplification of a 560 bp fragment of the mitochondrial large ribosomal subunit (16S); (2) PH19 (5′-CAT CGA CAC TT(T/C)GAA CGC A-3′) and ITS2 (5′-AAT CCT GGTTAG TTT CTT TTC CTC CGC T-3′) (Dixon et al. 1995) for the amplification of about 650 bp of the ITS-2; (3) LCO (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO (5′-TAA ACT TCA GGGTGA CCA AAAAAT CA-3′) (Folmer et al. 1994) for the amplification of a 700 bp fragment of the COI mitochondrial gene; and (4)

COIbrF (5′-GGG TTT TGAGCA GTT TTA GCCGGG-3′) and COIbrR (5′-GGT CAT CCA GAA GTGTAC GTC C-3′), developed in this work, for the *C. brasiliana*-specific amplification of a 661 bp fragment of COI (annealing temperature of 60°C).

DNA sequencing was carried out as previously described (Gusmão et al. 2006). The purification of PCR products was performed with a GFX™ PCR DNA and Gel Band Purification Kit (GE Life Sciences) according to the manufacturer's instructions. We directly sequenced 47 ITS-2, 167 COI, and 24 16S DNA fragments from *Crassostrea* species originating in 24 populations. Sequences were deposited in GenBank (accession numbers FJ544267–FJ544312, FJ717606–FJ717651). Additionally, *Crassostrea* sequences from GenBank were included in our phylogenetic analyses (accession numbers EU007484, EU007485, EU007509, EU007511, Reece et al. 2008; NC_007175, Milbury & Gaffney 2005; AJ312937, Lapègue et al. 2002; DQ839413, DQ839414, DQ839415, Pie et al. 2006). Sequences were aligned using the Clustal X multiple alignment program version 1.83 (Thompson et al. 1997), and alignments were confirmed by visual inspection.

DNA sequence analysis. Phylogenetic analyses were conducted using MEGA 4.0 (Tamura et al. 2007) and PAUP 4.0 (Swofford 1998) programs. Pairwise Kimura 2-parameter distances (K2P; Kimura 1980) were used to build neighbor-joining trees (Saitou & Nei 1987). The MODELTEST 3.06 program (Posada & Crandall 1998) was used to evaluate the most appropriate model of DNA substitution for maximum-likelihood analyses (Felsenstein 1981) of the data set. The best-fit models, which were chosen after comparisons between likelihood scores from different DNA substitution models, were the HKY model (HKY+G: A = 0.2233; C = 0.1764; G = 0.1993; T = 0.4010; gamma = 0.1600) for the COI analyses, and the K80 model (K80+G: A = 0.2233; C = 0.1764; G = 0.1993; T = 0.4010; gamma = 0.7191) for the ITS-2 analyses. Starting tree(s) were obtained via neighbor-joining, from which a heuristic search was employed using the branch-swapping algorithm (TBR, tree-bisection-reconnection). Branch support was assessed by bootstrapping the original data set using 1000 replicates.

Divergence times, population subdivision, isolation by distance and historical demography. Allozyme genotype frequencies were used to estimate allele frequencies, level of genetic variation (heterozygosity, H), fits to Hardy-Weinberg equilibrium (F_{IS}; Wright 1978), inbreeding indices (F_{ST}; Wright 1978), and pairwise unbiased genetic distances (D_{Nei}; Nei 1978) using the programs BIOSYS-1 version 1.7 (Swofford & Selander 1981) and GENETIX version 4.05 (Belkhir et al. 2002). The significance of F_{IS} (H_o: $F_{IS} = 0$) was estimated using a χ^2 test (Waples 1987). Divergence times were estimated from allozyme data using the relationship $t = 5D_{Nei} \times 10^6$ (Nei 1987).

Spatial analysis of molecular variance (SAMOVA; Dupanloup et al. 2002) was conducted with both allozyme and COI markers to determine hierarchical genetic structures. The significance of the fixation indices against the null hypothesis of panmixis was also evaluated using a Markov-Chain procedure with 10 000 permutations, using the software ARLEQUIN version 3.11 (Excoffier et al. 2005). To test for nonrandom associations between genetic and geographic distances in *Crassostrea rhizophorae* and *C. brasiliana* populations, we used the web service IBDWS version 3.11 (Jensen et al. 2005, http://ibdws.sdsu.edu), testing the significance of pairwise correlations between Φ_{ST} (or F_{ST}, for allozyme data) and geographic distances (in km) between all populations, through a Mantel test (10 000 permutations). Geographic coordinates and distances between sampling sites, measured as the shortest spherical distance by sea, were obtained using Google Earth.

Haplotype (h) and nucleotide (π) diversity estimates, and 2 tests of neutrality, Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997), were obtained from ARLEQUIN. For the COI data, we constructed a haplotype network using the statistical parsimony procedure of Templeton et al. (1992) implemented in the TCS version 1.21 program (Clement et al. 2000). The time of divergence between species was estimated, for mitochondrial sequences, using an evolutionary rate of 1.21% sequence divergence (K2P) per million years for COI (Marko 2002).

We used mismatch distribution analysis (Rogers & Harpending 1992), in the ARLEQUIN software, to test for historical population expansion events within oyster populations. If the sudden expansion model was not rejected, then the parameter tau (τ) was converted to time since expansion ($t = \tau/2\mu$) in years before present (for 665 bp, $\mu = 8.05 \times 10^{-6}$ substitutions per locus per year, assuming a 1 yr generation time and a 1.21% substitution rate; Marko 2002).

We used the coalescent-based program MDIV (Nielsen & Wakeley 2001), on CBSU Web Computing Resources (http://cbsuapps.tc.cornell.edu/mdiv.aspx), to estimate the maximum likelihood values of theta ($\theta = 2N_{ef}\mu$), scaled migration rate ($M = N_{ef}m$), scaled time of divergence ($T = t_1/N_{ef}$), and time to most recent common ancestor (TMRCA = t_2/N_{ef}) (N_{ef} = effective female population size, m = migration rate, t_1 = population divergence time, t_2 = gene coalescence time, and μ = mutation rate). The coalescent-scaled parameter T was converted to T_{div} (time in years since 2 populations diverged) according to the formula: $T_{div} = T\theta/(2\mu)$. For each pairwise comparison, a minimum of 3 chains were run using the finite

sites model (HKY, Hasegawa et al. 1985), with a 2 000 000 generation Markov chain Monte Carlo (MCMC) for each simulation and a 500 000 generation burn-in time, with different random seeds (set T_{max} and M_{max} to 10). Standard coalescent models may not be applicable to species with extremely high fecundities (Eldon & Wakeley 2006) and high variance in the cross-generation contribution to the gene pool (the sweepstakes hypothesis; Hedgecock 1994, Hedgecock et al. 2007), like those observed in oysters. However, estimates of effective population size through different approaches can give results that are fairly well correlated even under extreme fluctuations in reproductive effort and overlapping generations (Cenik & Wakeley 2010).

RESULTS

Allozymes

We analyzed 422 individuals from 21 Western Atlantic *Crassostrea* and *Ostrea* populations with 15 allozyme loci. Allele frequency estimates are presented in Table 3.

Heterozygosity levels were high, as is often observed in oysters (Hedgecock & Okazaki 1984, Ignacio et al. 2000), and no significant deviations from Hardy-Weinberg expectations were found at the species level (*Crassostrea brasiliana*/*C. paraibanensis*: $F_{IS} = -0.07$, $\chi^2 = 1.08$, df = 2.52, $p > 0.70$; *C. rhizophorae*: $F_{IS} = 0.06$, $\chi^2 = 1.93$, df = 5.69, $p > 0.80$; *C. virginica*: $F_{IS} = 0.07$, $\chi^2 =$

Table 3. *Crassostrea* spp. and *Ostrea* sp. Allele frequencies and sample sizes (N) at 15 allozyme loci in 21 populations of *Crassostrea* and *Ostrea* species. *C.p.*: *C. paraibanensis*; *C.v.*: *C. virginica*; *C.g.*: *C. gigas*

Locus	*C. rhizophorae*										*C. brasiliana*						*C.p.*	*C.v.*	*C.g.*	*Ostrea* sp.	
	PAN	RN1	RN2	PB1	PB2	BA2	RJ1	SP1	PR1	PR2	PA	PB1	PE	BA1	SP2	PR1	PB1	USA	SC	RJ1	SP1
Ak																					
(N)	(10)	(15)	(20)	(10)	(23)	(24)	(22)	(10)	(19)	(32)	(12)	(7)	(23)	(20)	(4)	(34)	(21)	(6)	(29)	(12)	(10)
A	0.15	–	–	–	–	0.04	0.02	–	0.13	0.22	–	–	–	–	–	–	–	–	–	–	–
B	0.85	1.00	1.00	1.00	1.00	0.96	0.98	1.00	0.87	0.78	–	–	–	–	–	–	–	–	–	–	–
C	–	–	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.67	–	–
D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.21	0.83	0.90
E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.12	0.17	0.10
Cat																					
(N)	(13)	(15)	(16)	(10)	(12)	(20)	(18)	(4)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(30)	(21)	(6)	(7)	(8)	(8)
A	–	–	–	–	–	–	–	–	–	–	–	0.07	–	–	–	0.03	–	–	–	–	–
B	–	–	–	–	–	–	–	–	0.03	–	0.96	0.93	1.00	1.00	1.00	0.97	0.98	–	–	0.06	–
C	–	–	–	–	–	–	–	0.12	0.13	0.20	0.04	–	–	–	–	–	0.02	–	–	0.94	1.00
D	0.15	0.67	0.66	0.65	0.67	0.53	0.53	0.50	0.74	0.64	–	–	–	–	–	–	–	–	0.93	–	–
E	0.85	0.33	0.34	0.35	0.33	0.42	0.47	0.38	0.10	0.16	–	–	–	–	–	–	–	1.00	0.07	–	–
F	–	–	–	–	–	0.05	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Got																					
(N)	(14)	(15)	(20)	(10)	(23)	(27)	(22)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(31)	(12)	(10)
A	–	–	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	0.96	1.00	–	–	–	–
B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.04	–	–	–	1.00	1.00
C	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	1.00	1.00	–	–
Idh-1																					
(N)	(14)	(15)	(20)	(10)	(23)	(27)	(22)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(29)	(12)	(10)
A	–	–	–	–	–	–	–	–	–	–	0.96	1.00	1.00	1.00	1.00	0.99	0.98	–	–	–	–
B	–	–	–	–	0.02	–	0.02	–	0.03	–	0.04	–	–	–	–	0.02	0.02	–	–	–	–
C	0.86	1.00	1.00	0.95	0.96	0.91	0.96	1.00	0.89	0.94	–	–	–	–	–	–	–	–	0.02	–	–
D	–	–	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–	0.74	1.00	1.00
E	0.14	–	–	0.05	0.02	0.09	–	–	0.08	0.06	–	–	–	–	–	–	–	1.00	–	–	–
F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.24	–	–
Idh-2																					
(N)	(14)	(15)	(20)	(10)	(23)	(27)	(22)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(29)	(12)	(10)
A	–	–	–	–	0.02	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–
B	1.00	1.00	1.00	1.00	0.96	0.98	1.00	1.00	1.00	0.98	–	–	–	–	–	–	–	–	–	–	0.10
C	–	–	–	–	0.02	0.02	–	–	–	–	–	–	–	–	–	–	–	–	1.00	1.00	0.90
D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.00	–	–	–
E	–	–	–	–	–	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–	–
Lap-1																					
(N)	(14)	(15)	(20)	(9)	(23)	(27)	(22)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(29)	(12)	(10)
A	–	–	–	–	–	–	–	–	0.05	0.02	–	–	–	–	–	–	–	–	–	1.00	1.00
B	0.11	0.03	0.15	–	–	0.02	–	–	0.29	0.22	–	0.07	–	–	–	0.04	0.02	–	1.00	–	–
C	0.89	0.97	0.85	1.00	0.98	0.98	0.89	1.00	0.66	0.75	0.88	0.79	1.00	1.00	1.00	0.93	0.96	1.00	–	–	–
D	–	–	–	–	0.02	–	0.11	–	–	0.01	0.12	0.07	–	–	–	0.03	0.02	–	–	–	–
E	–	–	–	–	–	–	–	–	–	–	–	0.07	–	–	–	–	–	–	–	–	–

(Table continued on next page)

Table 3 (continued)

Locus	*C. rhizophorae*										*C. brasiliana*						*C.p.*	*C.v.*	*C.g.*	*Ostrea* sp.	
	PAN	RN1	RN2	PB1	PB2	BA2	RJ1	SP1	PR1	PR2	PA	PB1	PE	BA1	SP2	PR1	PB1	USA	SC	RJ1	SP1
Lap-2																					
(N)	(14)	(15)	(20)	(10)	(23)	(27)	(22)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(31)	(12)	(10)
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	–
B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.00	–	–
C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.00	1.00
Mdh-1																					
(N)	(14)	(15)	(20)	(10)	(23)	(27)	(22)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(31)	(10)	(10)
A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.00	–	–	–
B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.30	0.30
C	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	–	–	0.70	0.65
D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.00	–	0.05
Mdh-2																					
(N)	(14)	(15)	(20)	(10)	(23)	(27)	(22)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(31)	(10)	(10)
A	0.25	0.03	0.02	–	0.02	0.06	0.09	–	–	0.06	0.08	0.14	0.17	0.15	–	0.01	0.14	–	–	–	–
B	–	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–	0.03	–	–	–	–	–
C	0.75	0.97	0.98	1.00	0.98	0.94	0.91	1.00	0.97	0.94	0.92	0.86	0.83	0.85	1.00	0.96	0.86	1.00	1.00	–	–
D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.75	1.00
E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.25	–
Mpi																					
(N)	(13)	(15)	(20)	(9)	(21)	(25)	(22)	(10)	(19)	(32)	(12)	(7)	(23)	(20)	(4)	(34)	(21)	(6)	(31)	(12)	(10)
A	–	–	–	–	–	–	–	–	–	–	–	–	0.02	0.03	–	–	–	–	0.31	–	0.05
B	0.04	–	–	–	–	–	–	–	–	–	0.33	0.50	0.46	0.52	0.13	0.13	0.29	–	0.68	1.00	0.95
C	0.15	0.10	–	–	0.07	0.20	0.11	–	0.18	0.09	0.59	0.43	0.48	0.45	0.87	0.84	0.62	0.33	0.01	–	–
D	0.27	0.67	0.55	0.56	0.50	0.40	0.46	0.35	0.58	0.81	0.08	0.07	0.04	–	–	0.03	0.09	0.33	–	–	–
E	0.50	0.23	0.40	0.39	0.38	0.24	0.41	0.55	0.19	0.10	–	–	–	–	–	–	–	0.33	–	–	–
F	0.04	–	0.05	0.05	0.05	0.12	0.02	0.10	0.05	–	–	–	–	–	–	–	–	–	–	–	–
G	–	–	–	–	–	0.04	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Pep-1																					
(N)	(14)	(15)	(20)	(10)	(23)	(25)	(21)	(6)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(31)	(12)	(3)
A	0.03	–	–	–	–	0.12	–	–	–	–	–	–	–	–	–	–	–	–	–	0.08	–
B	0.18	0.33	0.05	0.25	0.28	0.12	0.45	0.25	0.60	0.33	–	–	–	–	–	–	–	–	0.29	0.25	0.50
C	0.79	0.60	0.92	0.50	0.59	0.48	0.55	0.75	0.40	0.67	–	–	–	–	–	–	–	–	0.32	0.13	–
D	–	0.07	0.03	0.25	0.11	0.26	–	–	–	–	–	0.07	0.02	–	–	–	0.02	1.00	–	0.17	–
E	–	–	–	–	–	0.02	–	–	–	–	0.83	0.50	0.48	0.30	0.25	0.31	0.50	–	0.18	0.21	0.50
F	–	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–	0.18	–	–
G	–	–	–	–	–	–	–	–	–	–	0.17	0.43	0.50	0.70	0.75	0.69	0.48	–	0.02	0.08	–
H	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.01	0.08	–
Pep–2																					
(N)	(6)	(15)	(20)	(7)	(12)	(27)	(13)	(10)	(19)	(32)	(12)	(5)	(24)	(13)	(4)	(27)	(15)	(6)	(27)	(8)	(8)
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pgd																					
(N)	(13)	(15)	(20)	(10)	(23)	(26)	(22)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(31)	(12)	(10)
A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.08	–	–	–
B	–	–	–	–	–	0.08	–	–	–	–	0.04	–	–	–	0.13	0.02	0.02	0.17	–	–	–
C	0.23	–	0.02	–	0.02	0.23	0.07	0.20	0.05	0.09	0.96	0.93	0.96	0.95	0.87	0.85	0.86	0.58	0.21	–	–
D	0.77	1.00	0.98	1.00	0.98	0.69	0.93	0.80	0.95	0.91	–	0.07	0.04	0.05	–	0.13	0.12	0.17	0.79	–	0.05
E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.00	0.95
Pgi																					
(N)	(14)	(15)	(20)	(10)	(23)	(26)	(21)	(10)	(19)	(32)	(12)	(7)	(23)	(20)	(4)	(34)	(21)	(6)	(31)	(12)	(10)
A	–	–	–	–	–	0.02	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B	0.28	0.10	0.07	0.10	0.13	0.13	0.14	0.15	0.05	0.08	–	–	–	–	–	–	–	–	–	–	–
C	–	–	–	–	–	0.13	–	–	0.03	0.12	–	–	–	–	0.13	–	–	–	–	0.08	–
D	0.68	0.87	0.88	0.90	0.78	0.64	0.83	0.85	0.89	0.75	–	–	0.02	0.10	–	0.04	0.07	0.92	0.18	–	–
E	0.04	0.03	0.05	–	0.09	0.06	–	–	0.03	0.05	1.00	1.00	0.96	0.87	0.87	0.96	0.93	0.08	0.11	0.92	1.00
F	–	–	–	–	–	0.02	–	–	–	–	–	–	0.02	0.03	–	–	–	–	0.71	–	–
Pgm																					
(N)	(11)	(15)	(20)	(6)	(16)	(25)	(15)	(10)	(19)	(32)	(12)	(7)	(24)	(20)	(4)	(34)	(21)	(6)	(25)	(12)	(10)
A	0.09	0.03	–	–	–	0.02	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B	–	0.07	–	0.08	0.06	0.08	0.07	–	–	0.09	–	–	–	–	–	–	–	–	–	–	–
C	0.14	0.30	0.20	0.25	0.25	0.36	0.10	0.20	0.16	0.39	–	–	–	–	–	–	–	–	–	–	–
D	0.32	0.23	0.55	0.67	0.47	0.32	0.27	0.10	0.39	0.46	–	–	–	–	–	–	–	–	–	–	–
E	0.45	0.37	0.20	–	0.22	0.16	0.56	0.70	0.32	0.06	–	–	–	–	–	–	–	–	–	–	–
F	–	–	0.05	–	–	0.06	–	–	0.13	–	–	–	–	–	–	–	–	0.25	–	1.00	1.00
G	–	–	–	–	–	–	–	–	–	–	0.88	1.00	0.96	1.00	0.87	0.91	0.88	0.75	0.02	–	–
H	–	–	–	–	–	–	–	–	–	–	0.12	–	0.04	–	0.13	0.09	0.12	–	0.06	–	–
I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.92	–	–
H_o	0.23	0.16	0.15	0.17	0.19	0.24	0.18	0.16	0.21	0.23	0.11	0.11	0.13	0.11	0.10	0.10	0.14	0.11	0.20	0.16	0.14
H_e	0.26	0.17	0.16	0.17	0.20	0.28	0.21	0.18	0.24	0.24	0.11	0.14	0.11	0.10	0.09	0.11	0.14	0.13	0.21	0.15	0.11

0.05, df = 2.41, p > 0.90; *C. gigas*: F_{IS} = 0.04, χ^2 = 0.12, df = 3.32, p > 0.90; *Ostrea* sp.: F_{IS} = –0.22, χ^2 = 0.47, df = 0.32, p > 0.40).

We found 3 diagnostic loci that distinguished *Crassostrea rhizophorae* from *C. virginica*, 5 that distinguished *C. rhizophorae* from *C. brasiliana*, 6 that distinguished *C. brasiliana* from *C. virginica*, and 6 that distinguished *C. virginica* from *C. gigas* (Table 3). Although specimens of *C. virginica* and *C. brasiliana* are morphologically very similar, the former seems to be genetically more closely related to *C. rhizophorae* (Fig. 3). *C. brasiliana* and *C. paraibanensis* were genetically indistinguishable (D_{Nei} = 0).

Intraspecific genetic distances were short among Brazilian populations of *Crassostrea brasiliana* (D_{Nei} = 0 to 0.03) and of *C. rhizophorae* (D_{Nei} = 0 to 0.08) (Fig. 3). Despite the large geographic distances involved (up to 9000 km), the genetic distances separating *C. rhizophorae* populations from Panama and Brazil were also small (D_{Nei} = 0.02 to 0.08).

Populations of both species were found to be genetically structured in the Western Atlantic. Eight genetically different groups could be detected in *Crassostrea rhizophorae*: (1) Panama; (2) RN1; (3) RN2; (4) PB1, PB2; (5) BA2; (6) RJ1, SP1; (7) PR1; and (8) PR2 (SAMOVA: F_{CT} = 0.09; p < 0.001). Three genetic stocks were observed in *C. brasiliana*: (1) PA; (2) PB1, PE, BA1; and (3) SP2, PR1 (SAMOVA: F_{CT} = 0.08, p < 0.01). Mantel tests for both species showed significant isolation by distance (IBD) relationships (*C. rhizophorae*: r = 0.53, p < 0.05; *C. brasiliana*: r = 0.77, p < 0.01; Fig. 4), even after excluding the very divergent Panamanian *C. rhizophorae* population (r = 0.36, p < 0.05).

DNA markers

Analyses with the 3 DNA markers showed congruent results regarding interspecific relationships. ITS-2 intraspecific variation was low in all species (*Crassostrea rhizophorae*: 4 haplotypes, h = 0.271, π = 0.001; *C. virginica*: 1 haplotype; *C. brasiliana*/*C. paraibanensis*: 1 haplotype; and *C. gasar*: 2 haplotypes, h = 0.400, π = 0.003). *C. brasiliana*, *C. paraibanensis* and *C. gasar* were genetically extremely similar (K2P < 0.008), whereas the other species could be readily distinguished by their ITS-2 sequences (Fig. 5).

We found 28 different COI haplotypes in the Western Atlantic populations of *Crassostrea rhizophorae* (h = 0.866, π = 0.007), 10 haplotypes in the South American populations of *C. brasiliana* (h = 0.590, π = 0.003), 3 haplotypes in the Brazilian population of *C. paraibanensis* (h = 0.524, π = 0.001), and only 1 haplotype in the *C. gasar* population from Joal-Fadiouth (Senegal, Africa). As observed with the ITS-2 sequences, *C. brasiliana*, *C. paraibanensis* and *C. gasar* were very

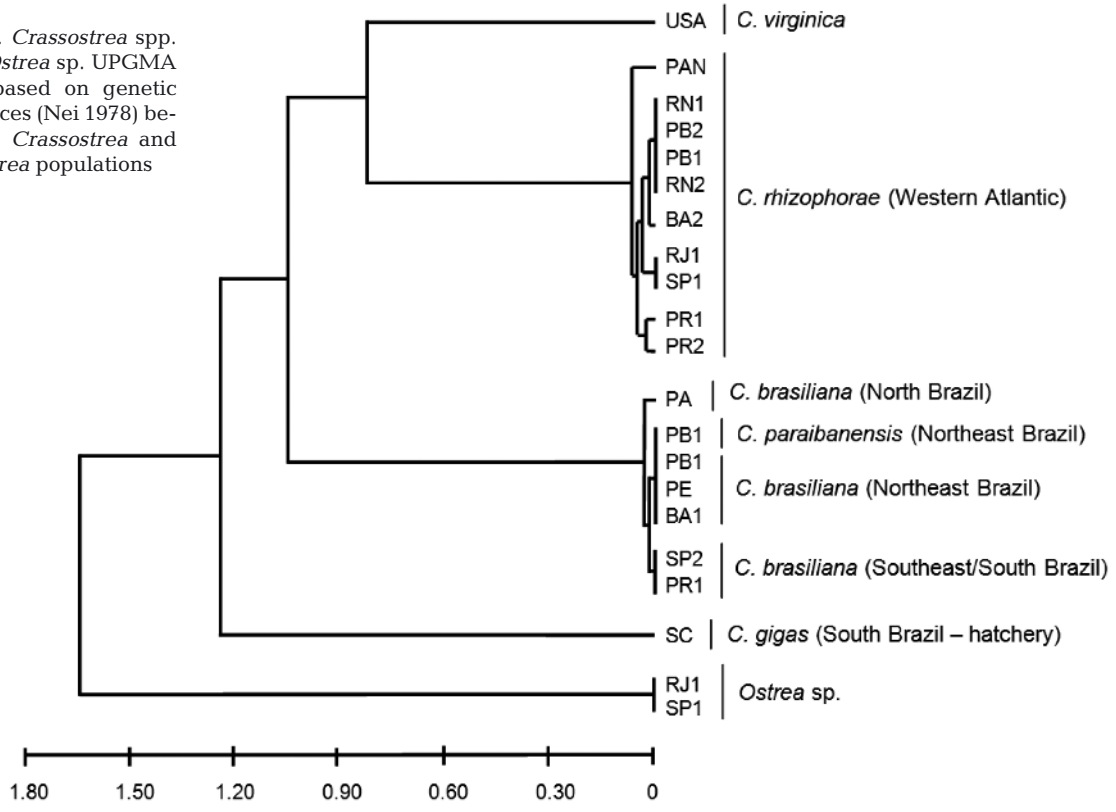

Fig. 3. *Crassostrea* spp. and *Ostrea* sp. UPGMA tree based on genetic distances (Nei 1978) between *Crassostrea* and *Ostrea* populations

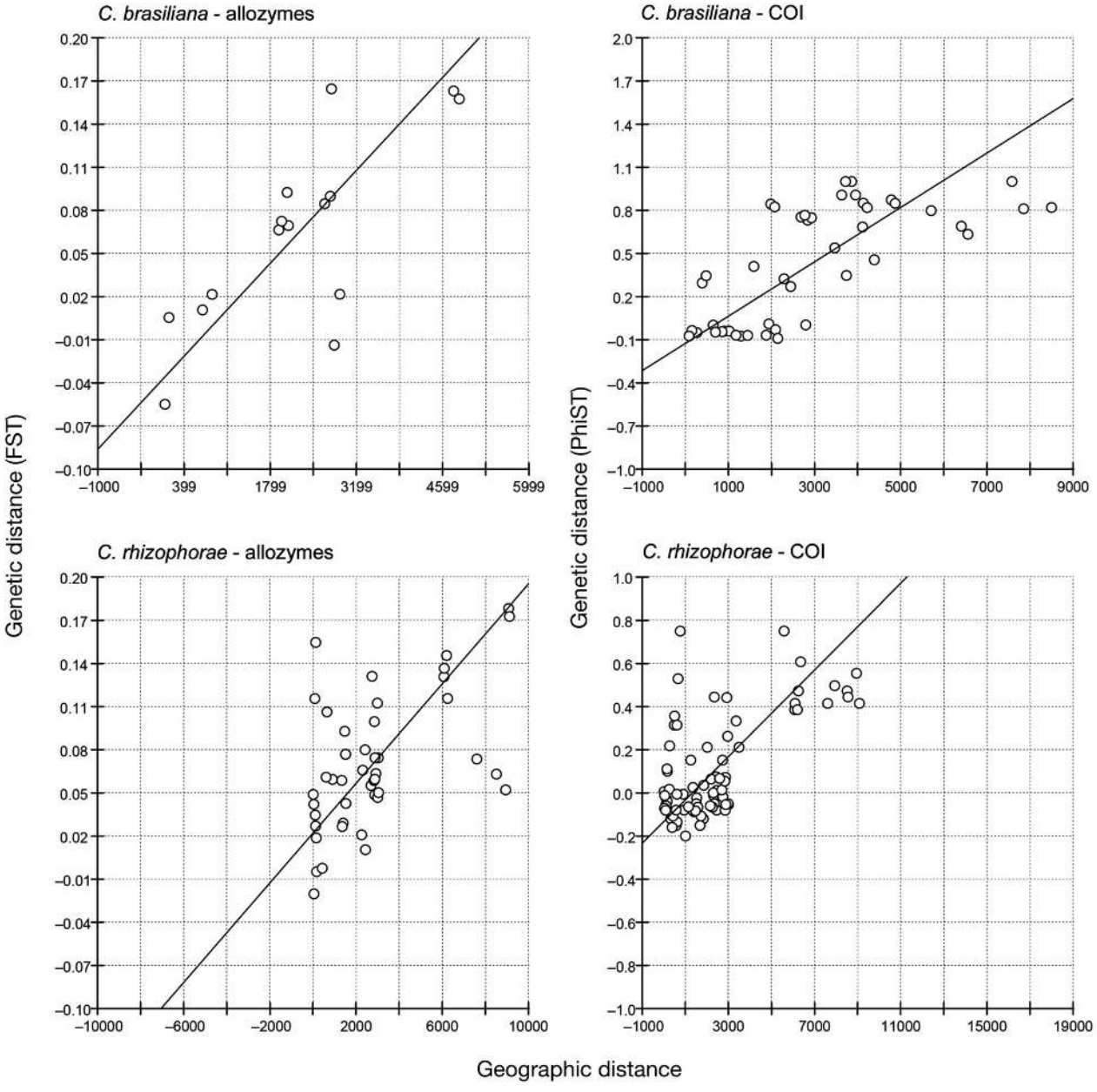

Fig. 4. *Crassostrea* spp. Pairwise comparison between genetic (linearized F_{ST}/Φ_{ST}) and geographical (linearized km) distances based on nuclear (allozymes) and mitochondrial (COI) markers

similar in their COI sequences (K2P < 0.008). Again, COI sequences clearly differentiated the other analyzed *Crassostrea* species (K2P varied between 0.16 and 0.27; Fig. 6). Intraspecific pairwise divergences of *C. rhizophorae* were low both for the Brazilian (K2P < 0.008) and the Caribbean (Panama, K2P < 0.003) populations. In contrast, a large differentiation (K2P = 0.018 to 0.025) was found between sequences from Brazilian and Panamanian specimens, which clustered in separate clades with high bootstrap support in the neighbor-joining and maximum likelihood trees (Fig. 6).

With the exception of *Crassostrea brasiliana*/*C. paraibanensis*/*C. gasar*, which had identical 16S sequences, the other *Crassostrea* species were well differentiated (Fig. 7). Interspecific sequence differentiation was 0.036 between *C. rhizophorae* and *C. virginica* and 0.118 between *C. rhizophorae* and *C. brasiliana*. No intraspecific variation was found in the 16S sequences analyzed.

Populations of both *Crassostrea rhizophorae* and *C. brasiliana* were found to be genetically structured in the Western Atlantic. Two highly divergent genetic

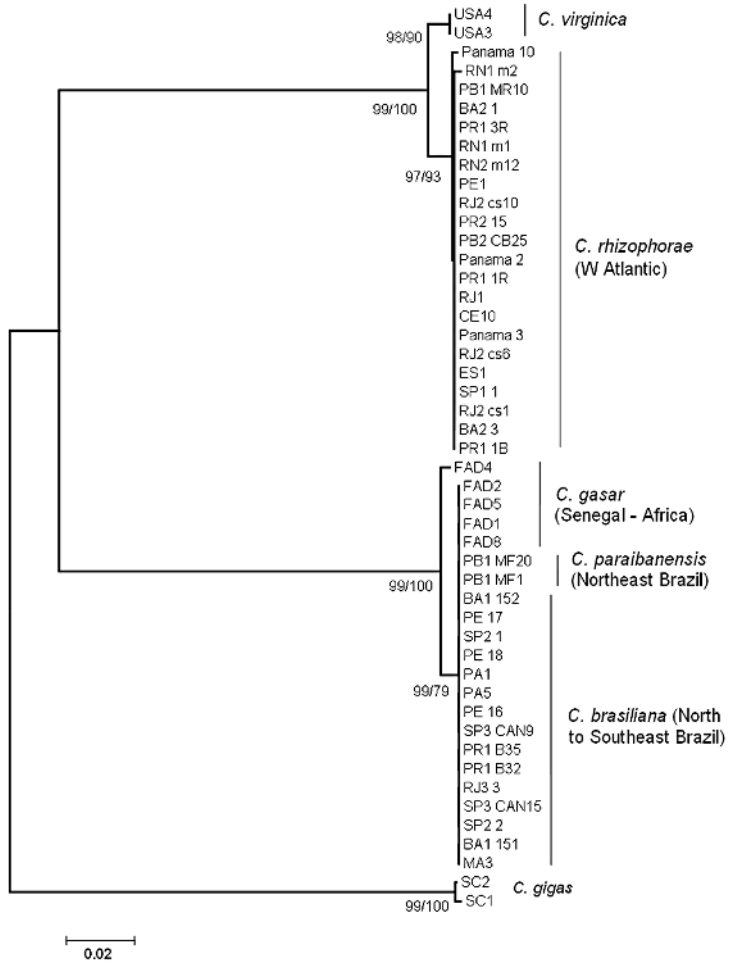

Fig. 5. *Crassostrea* spp. ITS-2-based (521 bp) tree. The numbers below the branches are bootstrap values (1000 replicates) for neighbor-joining and maximum likelihood trees, respectively

groups could be detected in *C. rhizophorae*: (1) Panama and (2) Brazil (SAMOVA COI data: F_{CT} = 0.894, p < 0.0001; Fig. 8). Excluding Panama from the analysis, 10 genetic stocks could be detected in *C. rhizophorae*: (1) CE; (2) RN1; (3) RN2; (4) PB1; (5) PB2; (6) PE; (7) BA2; (8) ES, RJ2, SP1; (9) RJ1; and (10) PR1, PR2 (SAMOVA COI data: F_{CT} = 0.156, p < 0.001). These population groupings are similar to those observed in the allozyme analyses. Three genetic stocks were observed in *C. brasiliana*: (1) PA, PB1, PE, BA1; (2) RJ3; and (3) SP2, PR1, PR2 (SAMOVA COI data: F_{CT} = 0.724, p < 0.001; Fig. 8). The *C. gasar* population from Joal-Fadiouth formed a fourth group when included in the analyses (SAMOVA COI data: *C. brasiliana*/*C. gasar*: F_{CT} = 0.738, p < 0.001; Fig. 8). As seen with allozymes, a significant correlation between geographic and genetic distances was observed in the COI analyses for *C. brasiliana* (Mantel test: r = 0.713, p < 0.0001; Fig. 4), whereas in *C. rhizophorae* the correlation was only significant when including the highly differentiated Panamanian population (Fig. 4).

A significant departure from neutrality was observed for the *Crassostrea rhizophorae* populations from Panama (Tajima's D = –1.43, p = 0.03; Fu's F_S = –26.30, p = 0) and Brazil (D = –2.35, p < 0.001; F_S = –27.84, p = 0), and for *C. brasiliana* from northern and northeastern (D = –2.09, p = 0.002; F_S = –6.62, p = 0.001) and southeastern and southern (D = –1.67, p = 0.026; F_S = –0.60, p = 0.209) Brazilian populations. A unimodal distribution was observed in the mismatch analyses both for *C. rhizophorae* and *C. brasiliana*, closely matching the expected distributions under the sudden expansion model. Time estimates for population expansion were 27 thousand years ago (kya) for *C. rhizophorae* from Panama (τ = 0.44) and 107 kya for *C. rhizophorae* from Brazil (τ = 1.72). For *C. brasiliana*, estimates of time since expansion were 171 kya for the N/NE population (τ = 2.75) and 186 kya for the SE/S population (τ = 3.00).

DISCUSSION

Synonymy of *Crassostrea brasiliana*, *C. paraibanensis* and *C. gasar*

The 3 Western Atlantic *Crassostrea* species studied in this work, *C. rhizophorae*, *C. brasiliana* and *C. virginica*, could be readily distinguished by both nuclear (allozymes and ITS-2) and mitochondrial (COI and 16S) markers. On the other hand, *C. brasiliana* and the African *C. gasar* clustered together in all DNA analyses, which indicates that they are conspecific, as previously suggested (Verdon 2000, Lapègue et al. 2002) based on smaller data sets. Additionally, oysters found on rocks in the subtidal zone of the same shore where *C. paraibanensis* was described (Singarajah 1980) were genetically indistinguishable from *C. brasiliana*/ *C. gasar*. We can safely conclude, therefore, that the nominal species *C. paraibanensis* and *C. brasiliana* are junior synonyms of *C. gasar*, which, henceforth, will be the name used to denominate both the South American and African oysters.

Moreau (2001) used restriction fragment length polymorphism (RFLP) analyses of ITS-2 to compare African and South American oysters identified as *Crassostrea gasar* and reported 2 different restriction patterns, H1 and H2, in the African specimens, but only H2 in the South American specimens. Because the African populations of *C. gasar* were more polymorphic, the author suggested that oysters from South America might have originated from African oysters.

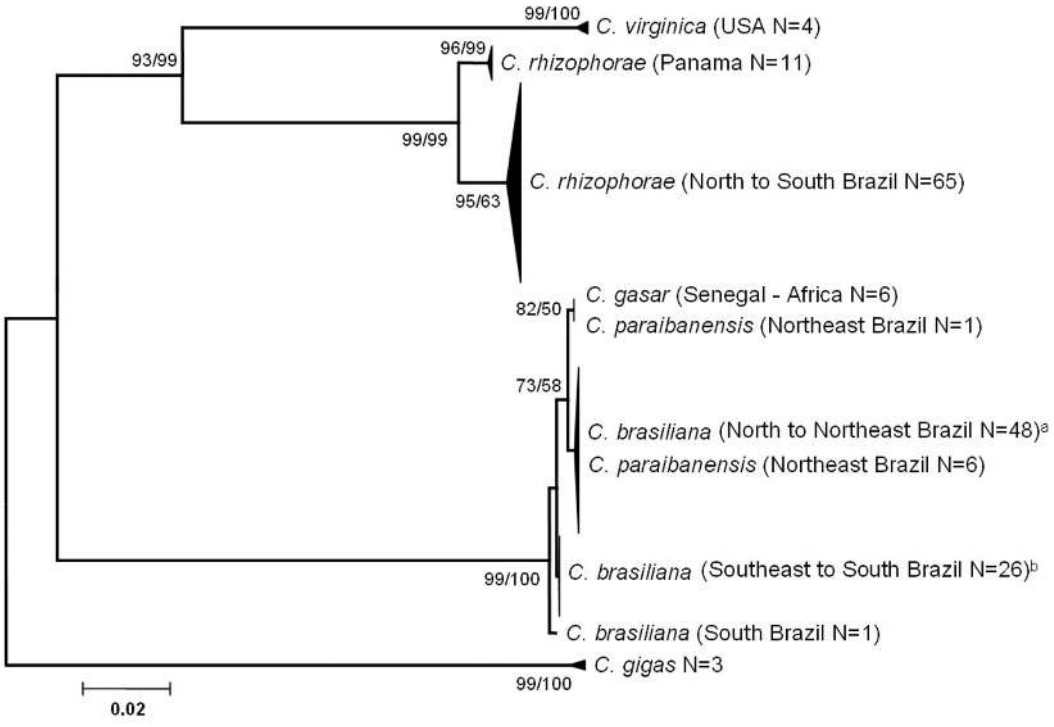

Fig. 6. *Crassostrea* spp. Cytochrome *c* oxidase subunit I-based (648 bp) tree. The numbers on the branches are bootstrap values (1000 replicates) for neighbor-joining and maximum likelihood trees, respectively. 'a' and 'b' indicate the presence of haplotypes with individuals from more than one region (a: Southeast N = 2; b: Northeast N = 4)

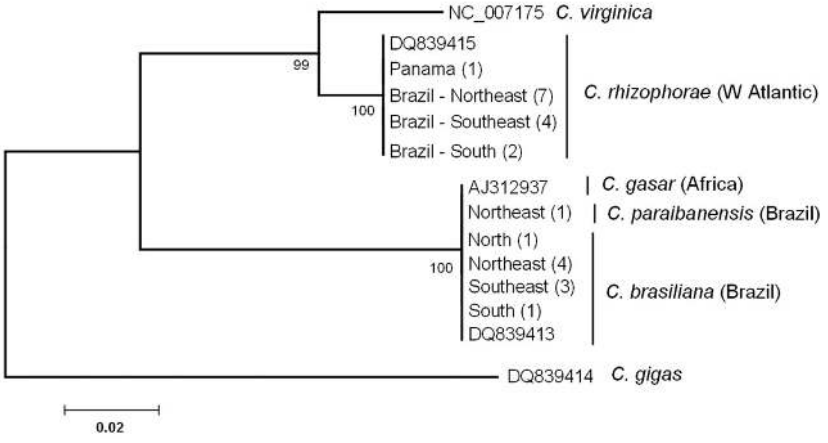

Fig. 7. *Crassostrea* spp. 16S-based (495 bp) neighbor-joining tree. The numbers below the branches are bootstrap values (1000 replicates). The numbers in parentheses represent the total number of individuals sequenced with each haplotype

The high similarity between populations of the same oyster species on both sides of the South Atlantic indicates a recent dispersal event. This could have been either a naturally occurring event, such as the dispersal of larvae by the equatorial current (Lapègue et al. 2002) or of adults by rafting (Ó Foighil et al. 1999). Alternatively, it might be the result of the anthropogenic transport of larvae in ballast water or of adults incrusting ship hulls as suggested by Lapègue et al. (2002).

The fossil records identified as *Crassostrea gasar* from Senegal (near Bassoul) (Demarcq & Demarcq 1992) and as *C.* '*brasiliana*' from Brazil (S/SE regions) (Fairbridge 1976), date from the Holocene period (4000 to 6000 yr ago), thus refuting the anthropogenic transport hypothesis for the occurrence of *C. gasar* on both continents. In any case, the very high genetic similarity, across nuclear and mitochondrial genes, rules out vicariance as the origin of the African and South American oyster populations, since the 2 continents have been separated for over 16 million yr.

Cytochrome oxidase gene sequences from African *Crassostrea gasar* specimens were more similar to those from *C. gasar* from N/NE Brazil than to sequences from other parts of the Brazilian coast. This is compatible with colonization by natural dispersal from Africa through the westerly flowing Equatorial Current, which arrives in Brazil on its northeastern

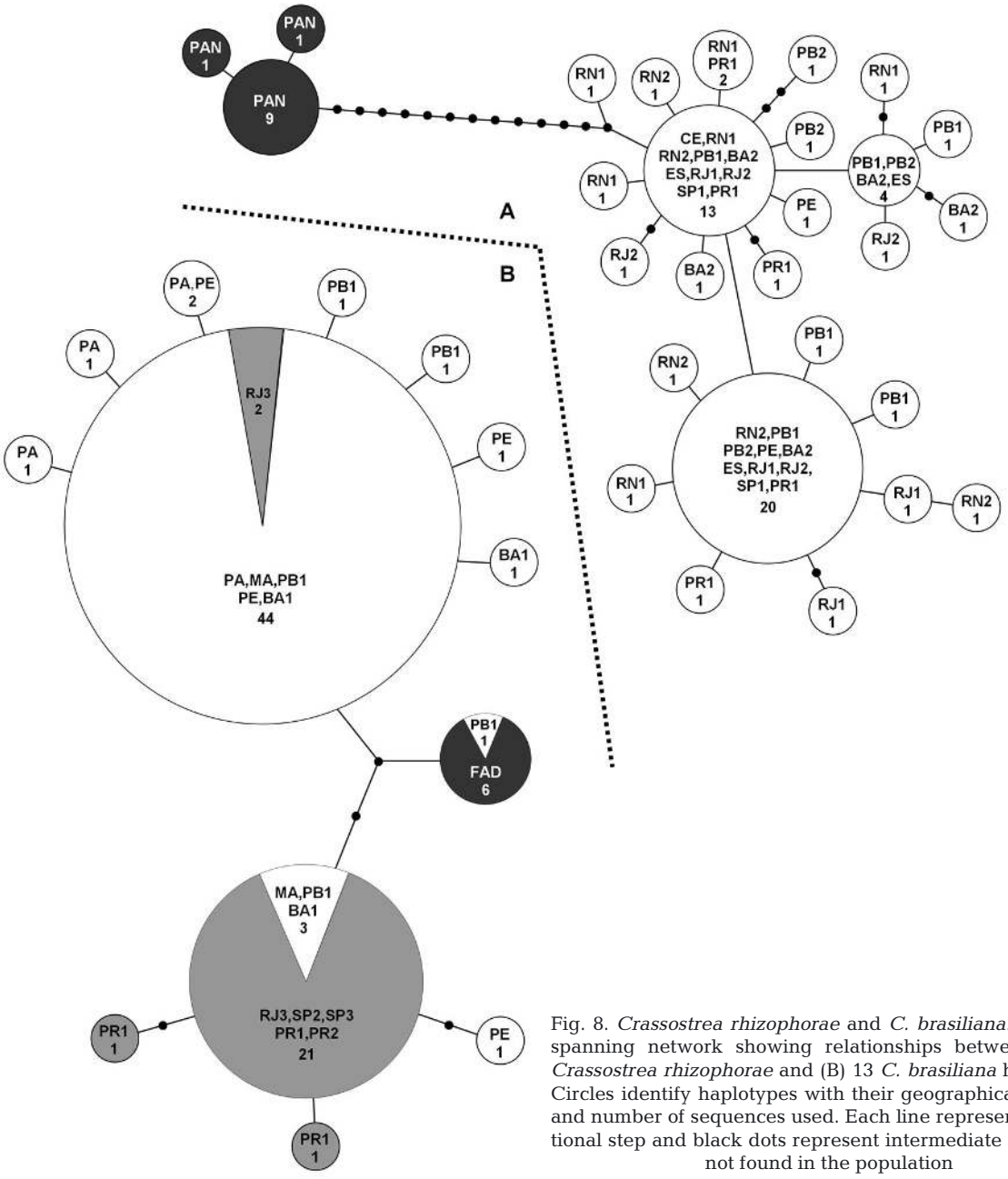

Fig. 8. *Crassostrea rhizophorae* and *C. brasiliana*. Minimum spanning network showing relationships between (A) 28 *Crassostrea rhizophorae* and (B) 13 *C. brasiliana* haplotypes. Circles identify haplotypes with their geographical locations and number of sequences used. Each line represents 1 mutational step and black dots represent intermediate haplotypes not found in the population

coast. The higher levels of polymorphism of RFLP ITS-2 patterns of African samples were also interpreted as evidence for an African origin of the species (Moreau 2001). Contrastingly, we found a higher nucleotide diversity in Brazilian than in African *C. gasar*, which might indicate a South American origin for the species, but which might also result from the maintenance of a larger effective population size of the species on the Brazilian coast, contradicting the conclusion of Moreau (2001) based on RFLP ITS-2 patterns.

The genetic similarity observed between *Crassostrea gasar* and *C.* '*brasiliana*' is higher than that found between Pacific (*C. gigas*) and Portuguese (*C. angulata*) oysters (Batista et al. 2006). Until recently, these 2 species were considered distinct based on fossil records and geographic distribution. Nevertheless, there are strong physiological, morphological, reproductive and genetic similarities between them, which suggest they may be closely related species or even the same Pacific species (Boudry et al. 1998, Leitão et al. 2007), probably transported anthropogenically from Taiwan.

Phylogenetic relationships among Atlantic *Crassostrea* species and fossil records

All phylogenetic analyses demonstrated that the morphologically highly similar species, *Crassostrea rhizophorae* and *C. virginica*, are sister species genetically very different from *C. gasar*. However, *C. gasar* still groups with the other Atlantic oysters. The small genetic distances (allozymes: D_{Nei} = 0.02 to 0.08) found between *C. rhizophorae* populations from Panama and Brazil (6000 to 9000 km apart) are similar to the genetic distances observed in comparisons of other marine organisms in those regions (Lazoski et al. 2001, Williams et al. 2001, Gusmão et al. 2006). This similarity provides additional support for the hypothesized close relationship of Caribbean and Brazilian faunas (Briggs 1974).

Crassostrea oysters, like other ostreids, are believed to have originated during the Late Cretaceous, like e.g. *C. soleniscus* (Meek, 1871) and *C. cusseta* Sohl & Kauffman, 1964 (Stenzel 1971), or earlier, in the Mid Jurassic period, like *C. tetoriensis* Komatsu & Chinzei, 2002 (Komatsu et al. 2002). During the Oligocene and Miocene periods, there was a great variety of oyster species, most of which had large and thick shells and inhabited littoral areas in the Western Atlantic (Kirby 2001). Some of these oysters probably gave rise to the Atlantic Crassostreini lineage after the Tethys Seaway closure (Ó Foighil & Taylor 2000).

Fossil records from the eastern coast of the USA (from the Priaborian period, Late Eocene, 37 million years ago, Ma) show the presence of a very large oyster, *Crassostrea gigantissima* (Finch, 1824), which was replaced by oysters similar to *C. virginica* in the Early Miocene strata (Aquitanian period, 23 Ma; Sohl & Kauffman 1964, Lawrence 1995, Kirby 2001). As a result, *C. virginica* is usually seen as a direct descendant of *C. gigantissima* or of an extinct common ancestor (Sohl & Kauffman 1964, Lawrence 1995, Kirby 2000). The American fossils identified as *C. virginica*, both from the Miocene and from the Holocene periods, had thin shells, whereas the Pliocene and Pleistocene '*C.* aff. *virginica*' specimens from the USA and Caribbean are thick-shelled (Kirby 2000, 2001). If this is correct, it means that *C. virginica* changed back from thin to thick shells during the Miocene-Holocene period. However, this conjecture is not consistent with the reported tendency of large and thick-shelled oysters to evolve into smaller and thinner-shelled species (Kirby 2000). This raises doubt as to the presence of *C. virginica* in the Miocene strata.

The divergence times estimated through allozymes and mitochondrial data indicate that *Crassostrea virginica* and *C. rhizophorae* diverged in the Pliocene period (6.8 to 3.2 Ma), after the evolutionary split in the lineages leading to *C. gasar* and the ancestor of *C. virginica* and *C. rhizophorae*, which happened between the Miocene and the early Pliocene periods (9.5 to 4.6 Ma).

Based on the morphological and ecological similarities of *Crassostrea gasar* and *C. virginica* (Sandison & Hill 1966), it is important to consider the presence of *C. gasar* in the fossil record. This has not been done previously, because *C. gasar* was not considered a valid species in the Western Atlantic, so its morphological features (like the presence of thick and large shells) were only considered a part of the phenotypic space occupied by *C. virginica* and *C. rhizophorae*.

Pleistocene and Pliocene thick- and large-shelled oysters from the Atlantic and Caribbean (also identified as '*Crassostrea virginica*' and '*C.* aff. *virginica*'; Kirby 2000, 2001, Kirby & Jackson 2004) can be either *C. virginica* or *C. gasar*. This casts doubt on the identification of thick-shelled fossil oyster specimens as *C. virginica*, which means that the identification of fossil oysters, at least in the USA and Caribbean, should be revised.

Fossils identified as *Crassostrea virginica* date from the same period as those of *C. titan* (Conrad, 1853) from the state of California (USA) and *C. cahobasensis* (Pilsbry & Brown, 1917) from the Caribbean, which have large and thick shells (Kirby 2001) like those of *C. gasar*. Those shell features have been used to infer the habitat and other ecological characteristics of fossil oysters (Kirby 2000), and to aid in their classification (Sohl & Kauffman 1964, Stenzel 1971). However, the use of shell characteristics to distinguish crassostreine genera has been criticized (Lawrence 1995). As the identification of extant oyster species based solely on their external shell features can clearly be misleading, correctly identifying and classifying fossil oysters based on those same shell characteristics is a difficult task (Lawrence 1995, Valentine et al. 2006).

Geographical distribution, isolation by distance, and historical demography of Western Atlantic *Crassostrea rhizophorae* and *C. gasar*

Crassostrea rhizophorae was found in 13 of 21 locations studied, from the Caribbean (Panama) to southern Brazil (Paraná State, 25° 51′ S; 48° 35′ W), and *C. gasar* was found in 12 sites along the Brazilian coast ranging from the northern (Pará State, 01° 25′ S; 48° 28′ W) to the southern (Paraná State, 25° 51′ S; 48° 35′ W) regions. *C. rhizophorae* was only found in the intertidal environments. In contrast, *C. gasar*, which is known to live mainly in the subtidal zone, was also found in 8 intertidal sites, and in 2 of them (Paraíba State, northeastern Brazil, and Paranaguá Bay, southern Brazil) it was attached alongside *C. rhizophorae* on mangrove tree roots. Due to their large phenotypic plasticity, it is very difficult to correctly identify even adult specimens of

C. gasar and *C. rhizophorae* where they coexist. Hence, the confirmation, here, of the specific status of *C. rhizophorae* and *C. gasar*, the expansion of the known distribution of *C. gasar* to northern and northeastern Brazil, and the evidence of their occurrence in different habitats have important implications for biological and ecological studies of Latin American oysters. Due to their economic importance, the taxonomic separation of these oysters has consequences for biomonitoring and aquaculture programs (Ignacio et al. 2000, Rebelo et al. 2003).

A genetic study of allozymes from *Crassostrea rhizophorae* populations showed a weak genetic heterogeneity between populations near Rio de Janeiro (Southeast Brazil) and Paranaguá Bay (South Brazil) (Ignacio et al. 2000). Similar results were observed for other *Crassostrea* species (Buroker et al. 1979). However, the present analyses using a larger number of populations have unveiled significant intraspecific genetic differences both within *C. rhizophorae* and within *C. gasar.* Almost the same genetic partitioning detected by allozyme analyses of *C. rhizophorae* (one group in Panama and various in Brazil) and of *C. gasar* (at least one group each in northern/northeastern and south/southeastern Brazil) were also found by COI sequencing analyses.

There are many factors that can influence genetic population structure in marine organisms, including limited dispersal capability and the existence of extrinsic barriers to gene flow, such as temperature, salinity, and ocean currents (Launey et al. 2002), as well as historical factors, such as climate and marine current oscillations, and sea level changes (Rocha et al. 2008). Sampling strategies and genetic patchiness must also be taken into account (Arnaud-Haond et al. 2008).

Some intraspecific genetic differences have been observed in *Crassostrea rhizophorae* from the Caribbean (Hedgecock & Okazaki 1984) and between Atlantic and Gulf of Mexico *C. virginica* (Hare & Avise 1998, Varney et al. 2009). As the larvae of *Crassostrea* are planktonic and have great dispersal capability (for instance, *C. virginica* larvae can spend 2 to 3 wk in the plankton; Kennedy 1996), the high levels of genetic differentiation found in these works and in the present study are surprising.

The isolation by distance observed here for *Crassostrea rhizophorae* and *C. gasar* indicates that restricted larval dispersal may be one of the factors responsible for the genetic structuring of these species. Isolation by distance has also been detected in other oyster species, such as *Ostrea edulis* (Launey et al. 2002), *C. virginica* (Rose et al. 2006), and *C. ariakensis* (Xiao et al. 2010).

Mismatch distribution analyses suggest that the population history of both species is characterized by expansion events, which are congruent with changes (ocean currents and sea levels) in the Late Pleistocene. Unfortunately, information about the biology of South American oyster species is still very scarce, which makes discussing the possible causes of the observed population heterogeneity difficult. Nevertheless, despite the factors responsible for the observed structuring patterns, our results show that those genetically distinct *Crassostrea rhizophorae* and *C. gasar* groups should be considered as discrete management units and this should be taken into account for future aquaculture programs and fisheries management of both resources, especially considering that these species usually display different growth rates and tolerances for temperature and salinity (Absher 1989, Melo et al. 2010b).

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