Marine Biology (2000) 136: 987–991

B. L. Ignacio · T. M. Absher · C. Lazoski
A. M. Solé-Cava

Genetic evidence of the presence of two species of *Crassostrea* (Bivalvia: Ostreidae) on the coast of Brazil

Received: 14 January 1999 / Accepted: 8 December 1999

Abstract Although oysters are commercially very important in Brazil, there is still much dispute about the number of *Crassostrea* species occurring on the Brazilian coast. The dispute is centered around *C. brasiliana*, considered by some authors to be a junior synonym of *C. rhizophorae*. In this paper we compared, by allozyme electrophoresis, sympatric and allopatric populations of the two putative species. Of the 17 loci analysed, five were diagnostic for the two species in sympatry (gene identity = 0.46 to 0.47), clearly demonstrating that they are distinct biological species. Heterozygosity (h) levels were high for both species (h = 0.24 to 0.28), and no heterozygote deficiencies were observed in any population (local inbreeding, F_{IS} = 0.141; $P > 0.70$). Levels of population structure in *C. rhizophorae* along 1300 km of coast were very low (population inbreeding, F_{ST} = 0.026; $P > 0.15$), indicating that the planktonic, planktotrophic larvae of these species are capable of long-range dispersal.

Introduction

Oyster morphology can be strongly influenced by environmental conditions, to the point that identification based on shell characteristics such as colour, form, structure and muscle scar is extremely prone to error. This large phenotypic variance has also hindered the classification of oyster species, and only recently has ordination of species into groups with characteristics in common become possible (Gunter 1951). This classification, combined with reproductive data, the presence/absence of a promial chamber and the morphology of the adult shell hinge, group the principal species of oysters of economical interest in the Western Atlantic Ocean into the genera *Ostrea* and *Crassostrea*. Within the genus *Crassostrea*, there is still much debate as to the actual number of native species that occur on the eastern coast of South America (Morretes 1949; Santos 1978; Absher 1989). Some authors (e.g. Wakamatsu 1973; Absher 1989; Nascimento 1991) have used the binomen *C. brasiliana* (Lamarck, 1819) for the subtidal rocky-shore form of *Crassostrea*, regarded as distinct mainly because of its large size. However, size is considered unreliable for taxonomic purposes by many authors (McLean 1941; Abbott 1974; Rios 1994), since it may be influenced by environmental factors, and *C. brasiliana* was held by Rios (1994) to be synonymous with the generally smaller *C. rhizophorae* (Guilding, 1828), a common Caribbean species that occurs among the roots of mangrove trees in Brazil (Lamy 1929).

Recently, large differences in growth rates and larval morphology have been described between *Crassostrea rhizophorae* and *C. brasiliana*, indicating that they may indeed be distinct biological species (Absher 1989). Given the economic importance of these two putative species, it is important to ascertain their specific status using characters that can establish if they interbreed in the field. Because they are independent of morphological characters, and because of the objectivity of the "biological species concept" in the detection of sibling species, molecular methods are highly suitable for establishing specific status (Knowlton 1993; Thorpe and Solé-Cava 1994). Such methods have been used to raise putative morphs of *C. gigas* from Japan to species level (Buroker et al. 1979), to reject the possible conspecificity

Communicated by J. P. Thorpe, Port Erin

B. L. Ignacio · C. Lazoski · A. M. Solé-Cava (✉)
Laboratório de Biodiversidade Molecular
Departamento de Genética,
Instituto de Biologia,
Universidade Federal do Rio de Janeiro, CCS, Bloco A,
Ilha do Fundão, 21.941-490,
Rio de Janeiro, Brazil

Fax: 005 (0)21 2800 994
e-mail: sole@biologia.ufrj.br

T. M. Absher
Centro de Estudos do Mar,
UFPR,
83255-000 Pontal do Sul,
Paraná, Brazil

of *C. virginica* and *C. rhizophorae* (Hedgecock and Okazaki 1984) and of *C. gigas* and *C. angulata* (Boudry et al. 1998), to discriminate closely related *Crassostrea* species from the Pacific (Banks et al. 1993), and to demonstrate the Asian origin of *C. angulata* from Portugal (O'Foighil et al. 1998). The aim of the present paper was to study, by allozyme electrophoresis, sympatric and allopatric populations of *C.* cf. *rhizophorae* and *C.* cf. *brasiliana*, to estimate their levels of genetic variation and population structure, and to establish whether they are reproductively isolated and, hence, whether they are distinct biological species.

Materials and methods

Forty-nine samples each of *Crassostrea* cf. *rhizophorae* and *C.* cf. *brasiliana* were collected in January 1996 and April 1999 in Pontal do Sul, Paranaguá Bay, Brazil (25°30′S; 48°30′W). Additionally, 50 samples of *C.* cf. *rhizophorae* were collected in February and March 1996 and November 1998 at two further intertidal sites along the Brazilian coast: Guaratiba (23°00′S; 43°40′W) and Itacuruçá (22°55′S; 43°55′W). *C.* cf. *rhizophorae* was found either attached to mangrove (*Rhizophora mangle*) roots or on rocks in the intertidal zone; *C.* cf. *brasiliana* was found attached to rocks in the subtidal zone. The oysters were kept alive until arrival at the laboratory (Rio de Janeiro), where they were frozen at −20 °C or in liquid nitrogen until electrophoresis.

Allozymes from the adductor muscle were analysed by 12.5% starch-gel electrophoresis as previously described by Solé-Cava et al. (1985) and Murphy et al. (1990), using three buffer systems: 0.10 M Tris, 0.01 M EDTA, 0.10 M maleate, pH 7.4 (TEM); 0.25 M Tris, 0.06 M citrate, pH 8.0 (TC8); and 0.005 M citrate, 0.03 M Tris (gel), 0.06 M LiOH, 0.30 M borate (buffer tank), pH 8.5/8.1 (LI). Of the 20 enzyme systems analysed, 13 provided consistent and reproducible results in all populations. Standard enzyme stains (Manchenko 1994) were used for the visualisation of allozymes. The 13 enzymes, along with their abbreviations, Enzyme Commission Numbers, and the buffer systems used are listed in Table 1.

Genotype frequencies were used to estimate gene frequencies, heterozygosities, unbiased genetic identities (Nei 1978), and the sub-population (F_{IS})- and population (F_{ST})-level inbreeding indices (Nei and Chesser 1983), using the BIOSYS-1 programme (Swofford and Selander 1981). The significance of F_{IS} (null hypothesis, H_0: $F_{IS} = 0$) and F_{ST} (H_0: $F_{ST} = 0$) were tested as:

$$\chi^2 = NF_{IS}^2(k-1); \quad df = k(k-1)/2 \ ,$$

and

$$\chi^2 = 2NF_{ST}(k-1); \quad df = (k-1)(s-1) \ ,$$

where N = total number of individuals analysed, k = number of alleles sampled per locus, and s = number of sub-populations analysed (Waples 1987).

Mean effective number of migrants (N_em) between populations was estimated as:

$$N_em = ((1/F_{ST}) - 1)/4 \quad \text{(Wright 1978)}.$$

Specimens of the two sympatric populations of *Crassostrea* were deposited at the Centro de Estudos do Mar Museum, Paranaguá, Brazil (*C.* cf. *rhizophorae* from mangroves = No. 623; *C.* cf. *rhizophorae* from intertidal rocks = No. 624; *C.* cf. *brasiliana* = No. 625).

Table 1 *Crassostrea* spp. Enzymes studied (and abbreviations), Enzyme Commission numbers, and buffer systems used

Enzyme	E.C.#	Buffer
Adenylate kinase (*Ak*)	2.7.4.3	TEM
Catalase (*Cat*)	1.11.1.6	TC8
α-Esterases (α*Est*)	3.1.1.X	TEM
Glutamate oxaloacetate transaminase (*Got*)	2.6.1.1	LI
Isocitrate dehydrogenase (*Idh*)	1.1.1.42	TEM
Leucine aminopeptidase (*Lap*)	3.4.1.1	LI
Malate dehydrogenase (*Mdh*)	1.1.1.37	TC8
Mannose 6-phosphate isomerase (*Mpi*)	5.3.1.8	TC8
Peptidases (PRO-PHE) (*Pep*)	3.4.1.1	TC8
Phosphogluconate dehydrogenase (*Pgd*)	1.1.4.4	TC8
Phosphoglucose isomerase (*Pgi*)	5.3.1.9	TC8
Phosphoglucomutase (*Pgm*)	2.7.5.1	TC8
Superoxide dismutase (*Sod*)	1.15.1.1	TEM

Results

Seventeen loci were resolved from samples from each of the four populations of *Crassostrea* spp. analysed. Gene frequencies are given in Table 2. As observed in many marine invertebrates (Nevo 1978; Solé-Cava and Thorpe 1991), including other oyster species (Buroker et al. 1979; Hedgecock and Okazaki 1984; Michinina and Rebordinos 1997), heterozygosity levels were high (0.24 to 0.28: Table 2). A moderate, but not significant, heterozygote deficiency was observed in the populations analysed ($F_{IS} = 0.141$; $\chi^2 = 4.08$, $df = 6$; $P > 0.70$), and no significant deviations from Hardy–Weinberg expectations were found for any locus ($P > 0.05$; Fisher's exact-test corrected with a Bonferroni series: Lessios 1992).

Fixed allele differences were found at 5 (*Ak*, *Got-2*, *Idh-1*, *Idh-2*, *Pgm*: Table 2) of the 17 loci analysed for the two putative species of *Crassostrea*. Unbiased genetic identity, I (Nei 1978), levels were high between populations of *C.* cf. *rhizophorae* ($I = 0.993$ to 0.999), but very low between those and *C.* cf. *brasiliana* ($I = 0.456$ to 0.469). The levels of genetic structure of *C.* cf. *rhizophorae* populations were low ($F_{ST} = 0.026$; $\chi^2 = 10.6$; $df = 7$; $P > 0.15$).

Discussion

The very low levels of genetic identity and the presence of five diagnostic loci (sensu Ayala 1983) between sympatric samples of *Crassostrea* cf. *rhizophorae* and *C.* cf. *brasiliana* clearly demonstrate that they are indeed distinct biological species, as suggested by Absher (1989). The gene-identity values observed in the comparison between *C. rhizophorae* and *C. brasiliana* were as low as those found for different species of other invertebrates (Thorpe 1982; Knowlton 1993; Solé-Cava and Boury-Esnault 1999). The contrast between these differences and the high similarity observed between populations of *C.* cf. *rhizophorae* 1300 km apart further

Table 2 *Crassostrea* spp. Gene frequencies of 17 loci analysed

Locus	*C. rhizophorae*			*C. brasiliana*
	Itacuruçá (N = 15.3)	Pontal do Sul (N = 21.6)	Guaratiba (N = 18.6)	Pontal do Sul (N = 23.6)
Ak	(10)	(13)	(10)	(12)
A	0.20	0.00	0.00	0.00
B	0.80	1.00	1.00	0.00
C	0.00	0.00	0.00	0.92
D	0.00	0.00	0.00	0.08
Cat	(15)	(38)	(31)	(39)
A	0.00	0.00	0.00	0.15
B	0.23	0.35	0.37	0.85
C	0.77	0.65	0.63	0.00
Est-1	(7)	(11)	(9)	(12)
A	1.00	1.00	1.00	1.00
Est-2	(11)	(8)	(11)	(5)
A	0.18	0.25	0.36	0.00
B	0.59	0.63	0.46	0.00
C	0.23	0.12	0.18	0.40
D	0.00	0.00	0.00	0.60
Got-1	(12)	(19)	(15)	(17)
A	0.08	0.16	0.07	0.09
B	0.92	0.84	0.93	0.82
C	0.00	0.00	0.00	0.09
Got-2	(7)	(9)	(9)	(8)
A	0.00	0.00	0.00	1.00
B	1.00	1.00	1.00	0.00
Idh-1	(17)	(21)	(17)	(21)
A	0.00	0.00	0.00	0.05
B	0.00	0.00	0.00	0.86
C	0.00	0.00	0.00	0.09
D	1.00	1.00	1.00	0.00
Idh-2	(17)	(21)	(17)	(21)
A	1.00	0.95	1.00	0.00
B	0.00	0.05	0.00	0.00
C	0.00	0.00	0.00	1.00
Lap	(13)	(20)	(15)	(20)
A	0.27	0.20	0.10	0.02
B	0.65	0.70	0.73	0.91
C	0.08	0.10	0.10	0.07
D	0.00	0.00	0.07	0.00
Mdh-1	(31)	(26)	(33)	(42)
A	1.00	1.00	1.00	1.00
Mdh-2	(24)	(34)	(33)	(36)
A	0.08	0.03	0.03	0.10
B	0.92	0.96	0.94	0.54
C	0.00	0.01	0.03	0.36
Mpi	(20)	(23)	(19)	(27)
A	0.00	0.00	0.00	0.30
B	0.10	0.20	0.08	0.70
C	0.50	0.45	0.66	0.00
D	0.40	0.35	0.26	0.00
Pep	(10)	(23)	(15)	(18)
A	0.00	0.13	0.03	0.00
B	0.65	0.56	0.61	0.00
C	0.35	0.31	0.33	0.08
D	0.00	0.00	0.03	0.64
E	0.00	0.00	0.00	0.28
Pgd	(17)	(28)	(25)	(32)
A	0.00	0.00	0.00	0.14
B	0.21	0.18	0.04	0.70
C	0.79	0.82	0.96	0.16
Pgi	(31)	(39)	(32)	(42)
A	0.00	0.01	0.00	0.00
B	0.19	0.17	0.03	0.00
C	0.74	0.73	0.89	0.12
D	0.07	0.09	0.08	0.88
Pgm	(16)	(25)	(22)	(30)
A	0.16	0.06	0.02	0.00
B	0.06	0.08	0.07	0.00
C	0.03	0.24	0.16	0.00
D	0.13	0.18	0.11	0.00
E	0.34	0.20	0.16	0.00
F	0.06	0.14	0.19	0.00
G	0.19	0.02	0.16	0.00
H	0.03	0.06	0.11	0.00
I	0.00	0.02	0.02	0.00
J	0.00	0.00	0.00	0.22
K	0.00	0.00	0.00	0.55
L	0.00	0.00	0.00	0.23
Sod	(2)	(9)	(4)	(9)
A	1.00	1.00	1.00	1.00
Heterozygosity	0.28	0.28	0.24	0.27

confirms that *C. rhizophorae* and *C. brasiliana* must be regarded as different species.

This is the first paper on the levels of population structure of *Crassostrea rhizophorae*. The population of this species in the area studied (along 1300 km of Brazilian coast) does not seem to be structured ($P > 0.15$; N_em was not calculated, since the F_{ST} did not differ significantly from zero: Avise 1994). Similarly, low levels of population structure (inferred both from F_{ST} and gene-identity values) have been found in conspecific populations of other species of *Crassostrea* (Buroker et al. 1979; Hedgecock and Okazaki 1984; King et al. 1994; Michinina and Rebordinos 1997). In *C. virginica*, a large homogeneity of allozyme allele frequencies (Buroker 1983; Karl and Avise 1992) and of 16S ribosomal gene-sequences (Small and Chapman 1997) was observed along the Florida and Gulf of Mexico coasts, at odds with the mtDNA and scnDNA data, which showed a clear phylogeographic division of the populations of *C. virginica* around Cape Canaveral (Hare and Avise 1996, 1998; Hare et al. 1996). The difference was interpreted as evidence of balancing selection acting on the allozyme loci (Karl and Avise 1992, but see McDonald et al. 1996) and of functional constraints that had prevented divergence between the 16S sequences (Small and Chapman 1997). This indicates the need for caution regarding the use of allozymes or conserved genes for inferring population structure. In the absence of further evidence, however, it is not unreasonable to interpret intra-population allozyme homogeneity as an indication of high levels of gene flow (Wright 1978; Avise 1994). The high population homogeneity of *C. rhizophorae*

along 1300 km of Brazilian coast is not surprising, considering that *Crassostrea* species have long-lived planktotrophic larvae that are potentially capable of long-distance dispersal (Mackie 1984).

The revalidation of *Crassostrea brasiliana* as a distinct biological species has important consequences not only for the systematics of the genus, but also for studies of the ecology and biology of oysters in South America. Given their economic importance, the taxonomic separation of these species will also have important implications in oyster fisheries and aquaculture programs [*C. rhizophorae* and *C. brasiliana* may also have different growth rates and tolerances to salinity variation (Absher 1989; Nascimento 1991)], and it is thus important that the species in this region are clearly identifiable. Beside the subtle morphological differences between these two species, their ecological preferences provide a further indication to their identity: the smaller mangrove and intertidal rock oysters are usually *C. rhizophorae*, whereas the large subtidal oysters, were found, in this study, invariably to be *C. brasiliana*.

Acknowledgements We thank C. Russo, M. Rebelo and S.W. Christo for help with sample collecting. This work was supported by grants from CNPq, PADCT, FUJB and FAPERJ.

References

Abbott RT (1974) American seashells. Van Nostrand Reinhold Co, New York

Absher TM (1989) Populações naturais de ostras do gênero *Crassostrea* do litoral do Paraná – desenvolvimento larval, recrutamento e crescimento. PhD thesis. Oceanography Institute, Universidade de São Paulo, São Paulo

Avise JC (1994) Molecular markers, natural history and evolution. Chapman & Hall, London

Ayala FJ (1983) Enzymes as taxonomic characters. In: Oxford GS, Rollinson D (eds) Protein polymorphism: adaptive and taxonomic significance. Academic Press, London, pp 3–26

Banks MA, Hedgecock D, Waters C (1993) Discrimination between closely related Pacific oyster species (*Crassostrea*) via mitochondrial DNA sequences coding for large subunit rRNA. Molec mar Biol Biotechnol 2: 129–136

Boudry P, Heurtebise S, Collet B, Cornette F, Gerard A (1998) Differentiation between populations of the Portuguese oyster, *Crassostrea angulata* (Lamark) and the Pacific oyster, *Crassostrea gigas* (Thunberg), revealed by mtDNA RFLP analysis. J exp mar Biol Ecol 226: 279–291

Buroker NE (1983) Population genetics of the American oyster *Crassostrea virginica* along the Atlantic coast and the Gulf of Mexico. Mar Biol 75: 99–112

Buroker NE, Hershberger WK, Chew KK (1979) Population genetics of the family Ostreidae. I. Intraspecific studies of *Crassostrea gigas* and *Saccostrea commercialis*. Mar Biol 54: 157–169

Gunter G (1951) The species of oysters of Gulf Caribbean and West Indian Region. Bull mar Sci 1: 40–45

Hare MP, Avise JC (1996) Molecular genetic analysis of a stepped multilocus cline in the American oyster (*Crassostrea virginica*). Evolution 50: 2305–2315

Hare MP, Avise JC (1998) Population structure in the American oyster as inferred by nuclear gene genealogies. Molec Biol Evolut 15: 119–128

Hare MP, Karl SA, Avise JC (1996) Anonymous nuclear DNA markers in the American oyster and their implications for the heterozygote deficiency phenomenon in marine bivalves. Molec Biol Evolut 13: 334–345

Hedgecock D, Okazaki NB (1984) Genetic diversity within and between populations of American oysters (*Crassostrea*). Malacologia 25: 535–549

Karl SA, Avise JC (1992) Balancing selection at allozyme loci in oysters – implications from nuclear RFLPS. Science, NY 256: 100–102

King TL, Ward R, Zimmerman EG (1994) Population structure of eastern oysters (*Crassostrea virginica*) inhabiting the Laguna Madre, Texas, and adjacent bay systems. Can J Fish aquat Sciences 51: 215–222

Knowlton N (1993) Sibling species in the sea. A Rev Ecol Syst 24: 189–216

Lamy EP (1929) Notes sur quelques lamellibranches de la Martinique. Bull Mus natn Hist nat, Paris 1: 201–108

Lessios HA (1992) Testing electrophoretic data for agreement with Hardy–Weinberg expectations. Mar Biol 112: 517–523

Mackie GL (1984) Bivalve reproduction. In: Tompa AS, Verdonk NH, Van Der Bigelaar JAM (eds) The Mollusca. Vol. 7. Academic Press, New York, pp 351–418

Manchenko GP (1994) A handbook of detection of enzymes on electrophoretic gels. CRC Press, London

McDonald JH, Verrelli BC, Geyer LB (1996) Lack of geographic variation in anonymous nuclear polymorphisms in the American oyster, *Crassostrea virginica*. Molec Biol Evolut 13: 1114–1118

McLean RA (1941) The oysters of the Western Atlantic. Notul Nat 67: 1–13

Michinina SR, Rebordinos L (1997) Genetic differentiation in marine and estuarine natural populations of *Crassostrea angulata*. Mar Ecol Prog Ser 154: 167–174

Morretes FL (1949) Ensaio de catálogo dos moluscos do Brasil. Archos Mus parana 7: 1–226

Murphy RW, Sites JW, Buth DG, Haufler CH (1990) Proteins. I. Isozyme electrophoresis. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer Associates, Sunderland, Massachusetts, pp 45–126

Nascimento IA (1991) *Crassostrea rhizophorae* (Guilding) and *C. brasiliana* (Lamarck) in South and Central America. In: Menzel W (ed) Estuarine and marine bivalve mollusk culture. CRC Press, Boston, pp 125–134

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, Austin, Tex 89: 583–590

Nei M, Chesser RK (1983) Estimation of fixation indices and gene diversity. Ann hum Genet 47: 253–259

Nevo E (1978) Genetic variation in natural populations: patterns and theory. Theor Popul Biol 13: 121–177

O'Foighil D, Gaffney PM, Wilbur AE, Hilbish TJ (1998) Mitochondrial cytochrome oxidase I gene sequences support an Asian origin for the Portuguese oyster *Crassostrea angulata*. Mar Biol 131: 497–503

Rios EC (1994) Seashells of Brazil. Fundação Universidade do Rio Grande, Rio Grande, Brazil

Santos AE (1978) Desenvolvimento embrionário das ostras de mangue *Crassostrea rhizophorae* (Guilding, 1828) (Mollusca – Lamellibranchia) em laboratório. Universidade Federal do Paraná, Curitiba, pp 1–86

Small MP, Chapman RW (1997) Intraspecific variation in the 16S ribosomal gene of *Crassostrea virginica*. Molec mar Biol Biotechnol 6: 189

Solé-Cava AM, Boury-Esnault N (1999) Levels of inter and intraspecific differentiation in marine sponges. Mem Qd Mus 44: 591–602

Solé-Cava AM, Thorpe JP (1991) High levels of genetic variation in natural populations of marine lower invertebrates. Biol J Linn Soc 44: 65–80

Solé-Cava AM, Thorpe JP, Kaye JG (1985) Reproductive isolation with little genetic divergence between *Urticina* (= *Tealia*) *felina* and *U. eques* (Anthozoa: Actiniaria). Mar Biol 85: 279–284

Swofford DL, Selander RB (1981) BIOSYS-1, a FORTRAN programme for the comprehensive analysis of electrophoretic data in population genetics and systematics. J Hered 72: 281–283

Thorpe JP (1982) The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematics. A Rev Ecol Syst 13: 139–168

Thorpe JP, Solé-Cava AM (1994) The use of allozyme electrophoresis in invertebrate systematics. Zoologica Scr 23: 3–18

Wakamatsu T (1973) A ostra de Cananéia e seu cultivo. Superintendência do Desenvolvimento do Litoral Paulista/Instituto Oceanográfico, São Paulo

Waples RS (1987) A multispecies approach to the analysis of gene flow in marine shore fishes. Evolution 41: 385–400

Wright S (1978) Evolution and the genetics of populations. Vol 4. The University of Chicago Press, London