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INVASION NOTE

Crassostrea gigas in natural oyster banks in southern Brazil

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Abstract We report on the invasion of Brazil by the Pacific oyster *Crassostrea gigas*, and discuss the likely routes of invasion. Because this phenotypically diverse oyster sometimes resembles the native species *C. brasiliana* and *C. rhizophorae*, its invasion went unnoticed until it was detected through the analysis of DNA sequences for ribosomal 16S and the ribosomal second internal transcribed spacer. *C. gigas* was found amongst the native species in oyster banks up to 100 km south of oyster farms in South Brazil. Under most circumstances, water temperatures in the coastal southerly Brazil current would be too high to allow for the establishment of stable populations of *C. gigas*, but the production of spat in oyster farm laboratories has probably selected for resistance to warmer temperatures, which would promote invasion by *C. gigas*.

Keywords Bioinvasion · 16S · Ribosomal · Molecular markers · Ostreidae

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Introduction

Worldwide, oyster farms rarely culture native species. Ruesink et al. (2005) reported that there have been 168 introductions and 14 transplantations of 18 oyster species in 73 countries or regions; 24 of these introductions and transplantations have resulted in bioinvasions by the introduced oysters. The Pacific oyster, *Crassostrea gigas*, is the most commonly introduced oyster species (66 out of 168 introductions = 39%; Ruesink et al. 2005), followed by *C. virginica*, *Ostrea edulis* and *Saccostrea commercialis*. Invasive populations of *C. gigas* were established in 15 of the 57 countries, where it was introduced (Ruesink et al. 2005), including the coastlines of the northeastern Atlantic (Andrews 1980; Diederich et al. 2005), Tasmania, Australia (Ayres 1991; Shatkin et al. 1997), many European countries (Chew 1990), New Zealand (Dinamani 1991), and, recently, South Africa (Robinson et al. 2005). Invasive populations of *C. gigas* have also been reported in Argentina from Patagonia (Orensanz et al. 2002; Escapa et al. 2004) to the mouth of the Plata River (Penchaszadeh 2005), but no invasions have so far been reported in Brazil. Invasive species are capable of influencing many ecological processes, causing complex changes in coastal ecosystems (Ruesink et al. 2005; Thieltges et al. 2006; Hollebone and Hay 2008 and examples therein). Oyster introductions can impact habitat structure (at least on soft-sediment environments), influencing trophic dynamics and

water quality, thereby greatly reducing or even leading to the extinction of native populations by introducing disease-causing organisms (NRC 2004; Ruesink et al. 2005; McKindsey et al. 2007), facilitating other bioinvasions (Mineur et al. 2007) or outcompeting native oyster species (Krassoi et al. 2008).

The first cultivation of *C. gigas* seeds in Brazil occurred in 1974, when the Marine Research Institute in Cabo Frio, Rio de Janeiro State, imported oysters from Great Britain (Muniz et al. 1986; Silveira 1989; Poli et al. 1990; Poli 2004). In 1975, the São Paulo State Fisheries Institute imported seeds from Japan and started growth trials in Cananéia, São Paulo (Akaboshi 1979; Akaboshi et al. 1983). In 1981, the Bahia Biology Institute imported new seeds from Great Britain to start oyster cultures in north-eastern Brazil (Ramos et al. 1986). In the following year, the Fazenda Jacostra oyster farm (formerly Sostramar), in Cananéia, imported seeds from France and became the first farm in Brazil to produce *C. gigas* oyster seeds (Jacques Debeauvais, personal communication). In 1987, Pacific oyster seeds from the Cabo Frio Marine Research Institute were introduced to Santa Catarina State to assess their performance. In the following years, the culturing of oysters in Santa Catarina continued to use seeds from Sostramar Laboratory, seeds imported from laboratories in Chile and the USA, and seeds produced by the Laboratory of Marine Mollusks (LMM) at the Federal University of Santa Catarina. On October 30, 1998, however, the introduction of juvenile and adult oysters was prohibited in Brazil for sanitary reasons (IBAMA Administrative Edict n. 145-n, of October 29, 1998).

Of the oyster culture trials mentioned above, success was achieved only in Santa Catarina, where oyster culture continues with seeds produced by the LMM. The state of Santa Catarina is Brazil's main oyster producer (3,152 tonnes/year), with cultivation occurring mostly in the North and South bays of Florianópolis Island and in the area around Palhoça (around 27°69′S; 48°57′W). Together, these areas produce about 91% of all oysters reared in Brazil (Oliveira Neto 2008).

Molecular markers have been used successfully in systematic studies of oysters (Hare et al. 1996; Ignacio et al. 2000; Lazoski 2004; Reece et al. 2008). For example, they were used to show partial differentiation between the Portuguese oyster (*C. angulata*) and the Pacific oyster (Boudry et al. 1998), to detect the occurrence of *C. sikamea* in Japan (Hedgecock et al. 1999), and to discriminate between the three important cultivated oysters in Thailand (*C. belcheri*, *C. iredalei*, and *Saccostrea cucullata*; Klinbunga et al. 2003). In Brazil, genetic studies helped to revalidate *C. brasiliana*, which was formerly considered as a junior synonym of *C. rhizophorae* (Ignacio et al. 2000). Molecular markers are also very useful for the identification of invasive species, which is of crucial importance because of the increase of invasive species in coastal areas worldwide (McGlashan et al. 2008). Recently, while using molecular markers to discriminate spat from the two oyster species used as broodstock for experimental culture of native oysters in Brazil, we observed oysters caught in natural banks that presented genetic patterns different from those of *C. rhizophorae* and *C. brasiliana*. Preliminary analysis of genetic sequences from those specimens indicated that the exotic species *C. gigas* might be establishing populations in natural oyster banks in southern Brazil (unpublished results).

The aims of this work were to use genetic markers to verify the presence of *C. gigas* oysters in natural environments in South Brazil, to identify the possible origins of these *C. gigas* populations, and to investigate the ecological factors that have facilitated the invasion of these areas by *C. gigas*.

Materials and methods

Collection of samples

A total of 116 individuals of adult *Crassostrea* spp. were collected in February 2006 at ten sampling points from three localities in Santa Catarina State, Brazil—the North Bay (around 27°27′S; 48°30′W; $N = 38$) and the South Bay (around 27°46′S; 48°34′W; $N = 67$) of Florianópolis Island and in the Laguna littoral (28°30′S; 48°40′W; southern Santa Catarina; $N = 11$), 100 km south of Florianópolis (Fig. 1; Table 1).

Some oysters had shell characteristics that were common to both *C. brasiliana* and *C. gigas*, confounding correct taxonomic separation of these species based on morphology alone. Shells of the two species are similarly coarse, solid, thick, laminated, with broad and slightly undulating ventral margins. Their left valves are deeply cupped and right valves are flat or

Fig. 1 Distributions of *C. gigas* (★), *C. brasiliana* (○), and *C. rhizophorae* (●) at the collection sites (North Bay, sites 1–4; South Bay, sites 5–9; Laguna, site 10). * Areas of occurrence of oyster farms

Table 1 Number of oyster individuals identified through PCR/RFLP of the ITS-2 and 16S regions from three sites in Santa Catarina State, Brazil

Sites	*C. rhizophorae*	*C. brasiliana*	*C. gigas*	Total
North Bay	26	12	–	38
South Bay	22	–	45	67
Laguna	–	5	6	11
Total	48	17	51	116

slightly convex. The main difference is a deeper undulation of the ventral margins of the shell of *C. gigas*, but that was also observed in some specimens of *C. brasiliana*. The colour of *C. gigas* was usually whitish with many purple streaks and spots radiating away from the umbo, whereas *C. brasiliana* was usually brown or pale grey. However, it was not uncommon for specimens of *C. brasiliana* and *C. rhizophorae* to present the colour pattern of *C. gigas* and vice versa (Fig. 2). Oysters with the external morphological characteristics of *C. gigas* were found at all three of the study sites.

After collection, oysters were transported alive to the laboratory, where preliminary species identification was conducted on the basis of their external morphology (Absher 1989; Nascimento 1991). Sections of muscle or mantle from all collected oysters (*C. rhizophorae* = 35, *C. brasiliana* = 33, *C. gigas* = 48) were stored in 75% ethanol until required for genetic analyses.

DNA extraction and PCR/RFLP analysis

Total DNA purification was performed using a modified CTAB protocol as previously described in Gusmão and Solé-Cava (2002).

Species differentiation was achieved using a PCR/RFLP diagnostic system for the identification of adults and larvae of *Crassostrea* species based on restriction digestions of nuclear and mitochondrial markers (Lazoski 2004). Individuals of western Atlantic *Crassostrea* species (*C. rhizophorae* and *C. brasiliana* from Brazil) and *C. gigas* from aquaculture (Santa Catarina State, Brazil) were used as positive controls in all restriction gel analyses.

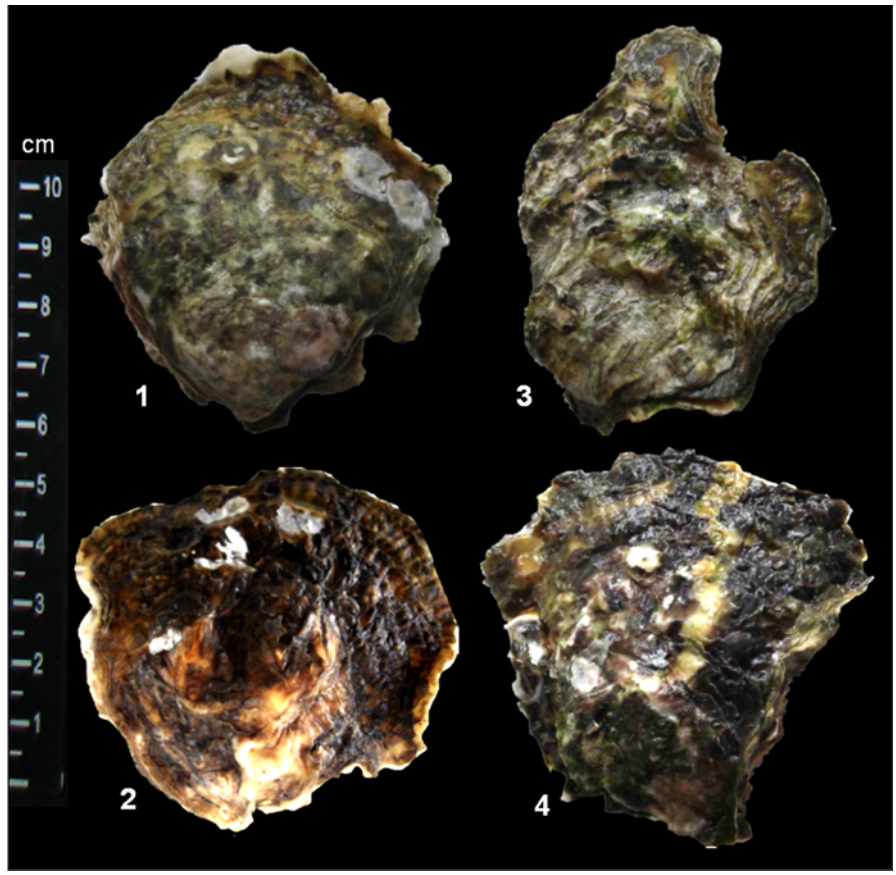

Fig. 2 Specimens of *Crassostrea* spp. collected in Florianópolis Island. (*1*) *Crassostrea rhizophorae*; (*2*) *C. brasiliana*; and (*3*, *4*) *C. gigas*

We used primers 16SAR (5′-CGCCTGTTTATCA AAAACAT-3′) and 16SBR (5′-CCGGTCTGAACTC AGATCACGT-3′) (Kessing et al. 1989) to amplify a 560 bp fragment of the mitochondrial large ribosomal subunit (16S) and primers PH19 (5′-CATCGACAC TT(T/C)GAACGCA-3′) and ITS2 (5′-AATCCTGGT TAGTTTCTTTTCCTCCGCT-3′) (Dixon et al. 1995) to amplify an approximately 650 bp fragment of the second internal transcribed ribosomal spacer (ITS-2).

Polymerase chain reaction (PCR) was performed in a mini-cycler (Sprint) with the following programme—a denaturing step at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 52°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min. PCR reactions used 10 ng of template DNA, 1 unit of *Taq* polymerase (GE Life Sciences), 200 μM each of the four dinucleotides, 200 nM of each primer, and 1.5 mM of $MgCl_2$, in 20 μL of 1× PCR buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris HCl, pH 9.0, GE Life Sciences). Negative controls, consisting of template-free reactions, were included in all PCR amplifications.

RFLP analyses of the amplified products followed standard procedures (Chow et al. 1993), using unpurified products from the PCR reactions (5 μL of PCR reaction per 15 μL of restriction reaction). Restriction reactions were conducted as recommended by the supplier (GibcoBRL®) for 4 h at 37°C, and the results were analysed on 2% agarose gels, after staining with ethidium bromide. Visualisation of fragments was carried out under UV light and recorded with a digital camera.

Sequencing

DNA sequencing was carried out using standard procedures (Hoelzel and Green 1992). Purification of PCR products was performed with a GFX™ PCR DNA and Gel Band Purification Kit (GE Life Sciences), following the manufacturer's instructions.

Direct sequencing of both fragment strands was conducted through the use of a fluorescent dye-terminator cycle sequencing reaction (Thermo Sequenase™ Dye Terminator Cycle Sequencing Kit), using an ABI (377/3100) automatic sequencer. We sequenced the ITS-2 and 16S regions of 10 oysters (3 *C. rhizophorae*, 3 *C. brasiliana*, and 4 *C. gigas*). Sequences were deposited in GenBank (Accession Numbers FJ478027-FJ478036 for 16S and FJ478037-FJ478046 for ITS-2). Additional *Crassostrea* sequences from GenBank were also included in the analysis (Accession Numbers AY905542, Milbury and Gaffney 2005; DQ839413, DQ839414, and DQ839415, Pie et al. 2006; EU072458 and EU072460, Kim et al. unpublished; FJ544284 and FJ544304, Lazoski et al. unpublished).

Data analysis

Sequences were aligned using the Clustal X multiple alignment program, version 1.83 (Thompson et al. 1997), and alignments were confirmed through visual inspection. Phylogenetic analyses were conducted using the MEGA 4 programme (Tamura et al. 2007). For neighbour-joining analysis (Saitou and Nei 1987), sequence divergence between pairs of populations was calculated using Kimura 2-parameter distances (Kimura 1980).

Results

Using the ITS-2 and 16S diagnostic system, we found three distinct genetic patterns that corresponded to the three *Crassostrea* species in the 116 oysters analysed (Fig. 3; Table 1). Our results genetically confirmed the presence of the exotic species *C. gigas* in natural environments at two sampling sites (South Bay and Laguna).

Analyses of ITS-2 and 16S sequences clearly distinguished among *Crassostrea* species (Fig. 4). Each PCR/RFLP pattern corresponded to one of the three *Crassostrea* species, confirming the performance of the diagnostic system.

Intraspecific variation was low in all species. *C. rhizophorae* and *C. brasiliana* were monomorphic for both ITS-2 and 16S, whereas *C. gigas* presented two haplotypes each of ITS-2 ($h = 0.500$, $\pi = 0.003$)

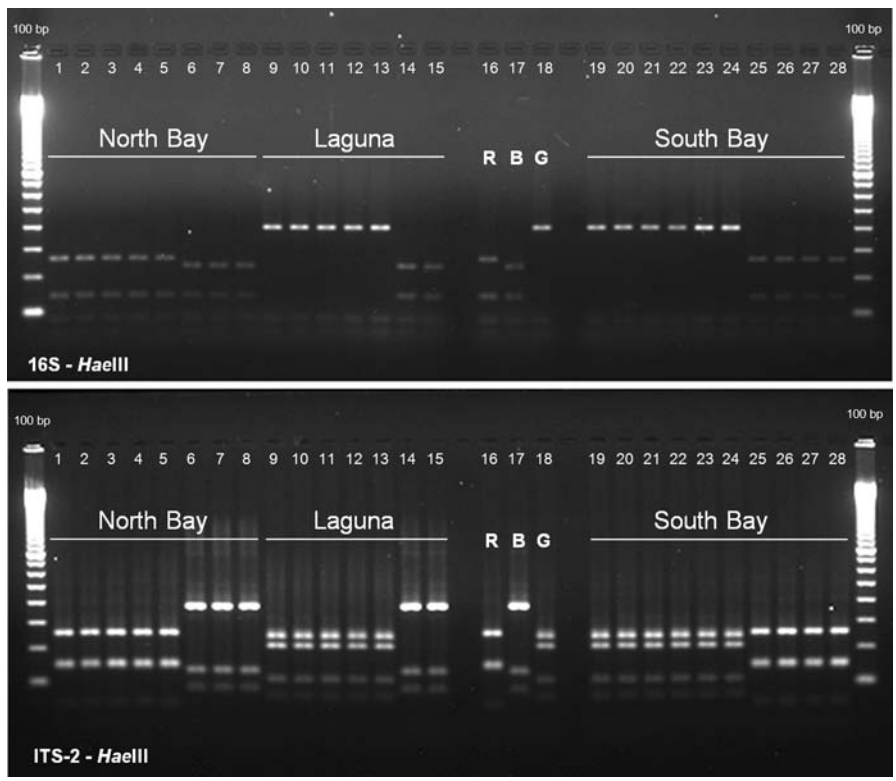

Fig. 3 Restriction fragment length polymorphisms of *Crassostrea* spp. from southern Brazil based on *Hae*III digestion of 16S and ITS-2 DNA fragments. *Lanes 1–5, 25–28*: *C. rhizophorae*. *Lanes 6–8, 14, 15*: *C. brasiliana*. *Lanes 9–13, 19–24*: *C. gigas*. *Lanes 16–18*: positive controls of *C. rhizophorae* (*R*), *C. brasiliana* (*B*), and *C. gigas* (*G*)

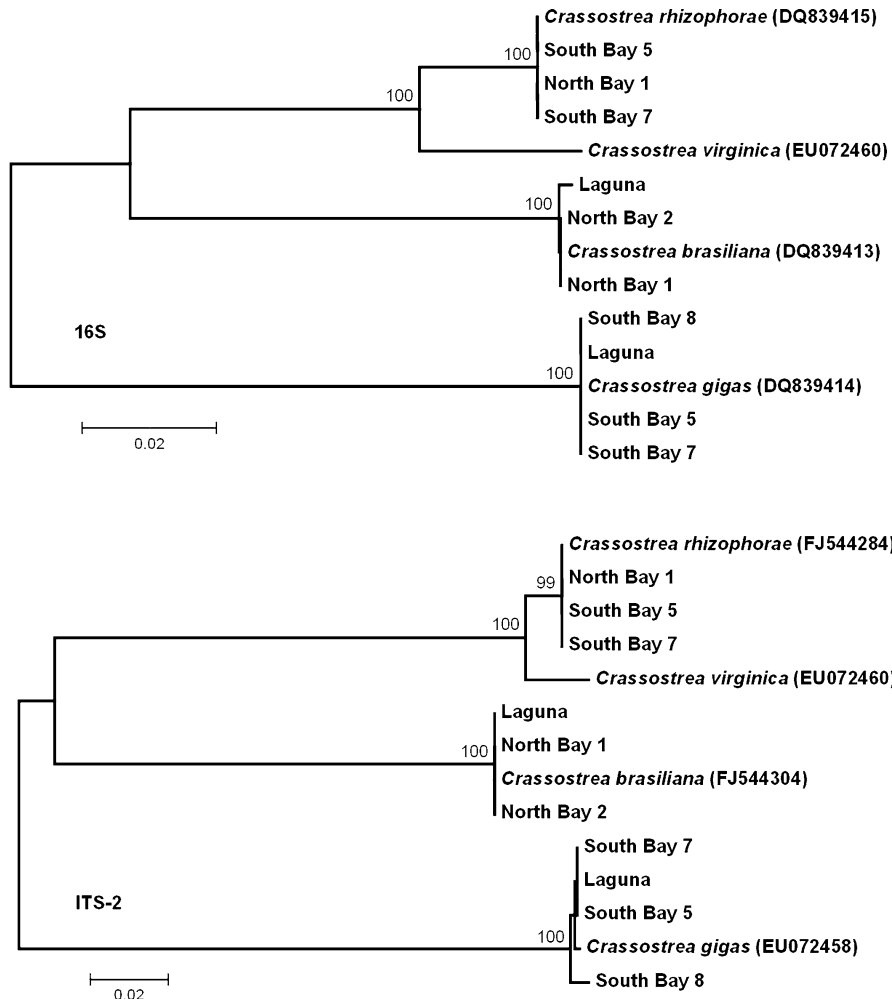

Fig. 4 Mitochondrial 16S and nuclear ITS-2 neighbour-joining (K2P) trees of *Crassostrea* specimens collected from natural oyster beds (North Bay, sites 1 and 2; South Bay, sites 5, 7, 8; Laguna, site 10). Specimens with complete species names are from GenBank. *Numbers* on branches are *bootstrap* values

and 16S ($h = 0.667$, $\pi = 0.001$). Interspecific sequence divergences (pairwise K2P distances) were high for ITS-2 sequences (*C. gigas–C. brasiliana* = 0.265–0.267; *C. gigas–C. rhizophorae* = 0.281–0.284; *C. rhizophorae–C. brasiliana* = 0.244; Fig. 4) and somewhat smaller for 16S sequences (*C. gigas–C. brasiliana* = 0.170–0.172; *C. gigas–C. rhizophorae* = 0.168; *C. rhizophorae–C. brasiliana* = 0.125–0.127; Fig. 4).

Discussion

Based on morphology, PCR/RFLP and sequencing of nuclear and mitochondrial markers, it is clear that the exotic oyster *Crassostrea gigas* occurs in natural banks on the south coast of Brazil.

Crassostrea gigas was found not only on Florianópolis Island, where there are aquaculture farms of *C. gigas* and *Perna perna* mussels, but also in Laguna (southern Santa Catarina), 100 km south of the Florianópolis Island *C. gigas* farms.

In the South Bay of Florianópolis Island, where rocky shores and more exposed zones predominate, *C. rhizophorae* and *C. gigas* were found in the shaded and protected areas of the intertidal zone (salinity of 33.7 ± 2.9‰). In the North Bay, *C. rhizophorae* and *C. brasiliana* were found in mangroves of the intertidal zone and in the estuary of the Ratones and Barra rivers, including sites in the river mouth where large salinity variations occur daily (5–34‰) and where wave action is the highest. But they were also found along the calmer and shadier river margins. No *C. gigas* oysters were found in Sambaqui

Beach, where *C. rhizophorae* and *C. brasiliana* are commonly found in exposed rocky shores with little variation in salinity (mean of 34.1 ± 2.2‰).

In Laguna, *C. brasiliana* and *C. gigas* settled on rocks in the low-tide zone of channels that supplied water to shrimp farms. The presence of *C. gigas* in Laguna and in the South Bay could be explained by the environmental characteristics of those sites, which have seawater temperatures ranging from 13 to 30°C during the year, conditions that are suitable for *C. gigas* reproduction. Additionally, when oyster farms were established in Florianópolis in 1987, the LMM started a process of selecting seeds for growth rate and survival. Only oysters that had survived to at least one summer period (temperature of 27.8 ± 1.03°C), were used in subsequent crosses. The reported high heritability of survival over summer mortalities (over 89% narrow sense heritability; Dégremont et al. 2007), and the large number of generations of selection (about 15) at the LMM may have resulted in an increase of tolerance to higher temperatures, thereby favouring the reproduction and settling of *C. gigas* in natural environments. The absence of *C. gigas* in the Ratones and Barra rivers is probably associated with the wide fluctuation in the river's salinity.

Currently, there are many countries where *C. gigas* was introduced and became a successfully established invader species, e.g., the United States, Australia, New Zealand, Denmark, Germany, Italy, and South Africa (Korringa 1976; Chew 1990; Menzel 1991). The environmental agencies of the governments of those countries have serious concerns about the environmental impact on indigenous species. Some researchers predict that functional similarity will increase the intensity of competition between native and exotic species (Baker 1995; Bando 2006). Competition is expected between indigenous and exotic species when they cohabit, and the successful establishment of an oyster species is particularly affected by temperature, salinity and desiccation (Diederich et al. 2005). In many cases, indigenous and exotic oyster species differ fundamentally in their tolerances to those factors (Krassoi et al. 2008). Minimum and maximum water temperatures were 13 and 30°C in the North and South bays, 12 and 28°C in Laguna, respectively. The temperatures required by *C. gigas* for gametogenesis (9–16°C), gamete release (16–20°C), and for normal larval development (24–28°C) (Perdue and Erickson 1984; Ruiz et al. 1992), are compatible with those found in the studied area, although high mortalities are often observed in warm summer months (personal observation). On the other hand, the higher water temperatures found further north along the coast of Brazil (26–30°C) probably prevent the establishment of *C. gigas* in those areas.

There are large invasive banks of *C. gigas* in Argentina, so it might be argued that the oysters found in South Brazil originated from larval dispersal from that area. However, this is unlikely, because the prevailing currents in the area are from north to south (the Brazil Current), and the Plata River may represent an effective turbidity and salinity barrier to dispersal. Hence, *C. gigas* populations found in the wild around southern Brazil have probably originated from oyster farms in the area, and the invasion was facilitated by the colder waters found in South Brazil and the acclimation of the species through selection for higher temperatures in the oyster farms. The approaches to species identification used in this paper may be helpful in establishing the geographical extent of this new invasion and its ecological consequences.

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