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Research Article

Molecular evidence of introgression between the oysters *Magallana gigas* (= *Crassostrea gigas*) and *Saccostrea palmula* in a coastal lagoon of the Gulf of California

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ABSTRACT. The El Soldado coastal lagoon in Sonora, Mexico, is a protected natural area and a Ramsar site; hence, fishing and aquaculture activities have been prohibited there since 2006. Considering its excellent state of conservation, it serves as a model for comparative studies with other coastal lagoons of the Gulf of California that are more heavily impacted by human activity. Therefore, it is essential to describe its biodiversity and emphasize the importance of its conservation. El Soldado has several species of mollusks; however, only two oyster species have previously been reported, which underestimates the diversity of the Ostreidae family in that area. This study was conducted to determine the species of ostreids inhabiting this coastal lagoon and to corroborate anatomical identification using molecular tools. The following four oyster species were found: the mangrove oyster *Saccostrea palmula*, the pleasure oyster *Crassostrea corteziensis*, the flat oyster *Ostrea angelica*, and the rock oyster *Striostrea prismatica*. However, some *S. palmula* specimens showed mitochondrial sequences and a nuclear region of *Magallana gigas* (= *Crassostrea gigas*), representing a threat to the evolutionary legacy of the *S. palmula* genome, and the magnitude of the impact at the population level and in other nearby coastal lagoons where *M. gigas* is cultured remains to be addressed.

Keywords: hybridization; introgression; native oysters; phylogenetic analysis; Gulf of California

INTRODUCTION

The bivalve mollusks of the Ostreidae family are among the most consumed worldwide (Ruesink et al. 2005), with approximately 78 recognized living species (WoRMS 2025) distributed along continental coasts (except Antarctica) and on some oceanic islands (Gunter 1950, Liu et al. 2011). In this family, the most representative genera are *Ostrea*, *Magallana*, and *Crassostrea*, which include numerous edible species and are of significant interest to fisheries and aquaculture (Keen 1971). Among Ostreidae species, at least 18 have been used for

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human consumption due to their flavor and ease of access, thereby contributing to the overexploitation of several natural populations worldwide (Menzel 1991, Carriker & Gaffney 1996). The Pacific oyster, *Magallana* (*Crassostrea*) *gigas*, is of great interest to aquaculture due to its high consumption and investment in oyster farms. Due to its potential for rapid growth and tolerance to environmental conditions, it is cultivated in various regions of the world, making it cosmopolitan. It has been introduced into several countries, particularly on the western coasts of the USA since the 1920s and in France since 1966 (FAO 2009). To date, this species has been introduced into 64 countries and 10 territories, establishing feral populations in 32 countries and contributing to oyster production in 36 countries (Martínez-García et al. 2022).

The Pacific oyster was introduced to the northwest region of Mexico in the 1970s to initiate oyster farming in the state of Baja California; subsequently, oyster farming spread to the states of Baja California Sur, Sinaloa, and Sonora (Barbosa-Solomieu 2004). In Sonora, in the El Soldado coastal lagoon, a commercial culture of *M. gigas* had been operated until 20 years ago, when the lagoon was declared a Natural Protected Area (BOGES 2006). After this, no further aquaculture activities were permitted. The lagoon has a mangrove ecosystem that provides significant environmental services, including refuge and breeding for species, protection against storms and surges, food provision, and places for recreation and education. Due to its excellent state of conservation, it has been used as a model for the study of other coastal lagoons in the region that are subject to greater anthropogenic impact (Vargas-González et al. 2017).

The decree establishing protection for El Soldado promotes a thorough understanding of the site's ecological environment, thereby strengthening conservation efforts. Studies have been conducted on malacological diversity (Martínez-Córdova 1996), sediment composition (Vargas-González et al. 2017), sediment biogeochemical function (Medina-Galván et al. 2021), and fish diversity (Salas-Mejía et al. 2024).

The malacological study by Martínez-Córdova (1996) was conducted in four coastal lagoons of Sonora, including El Soldado, which had 76 species of mollusks, but only two oyster species, viz., *Ostrea angelica* and *Saccostrea palmula*. Considering that in the Mexican Pacific, including the Gulf of California, there are nine native and two exotic oyster species, *M. gigas* and *Magallana sikamea* (Lodeiros et al. 2020), we considered that the number of oyster species in El Soldado could be underestimated. Therefore, the objective of this study was to conduct an exhaustive search and identify the oyster species inhabiting El Soldado to contribute to knowledge of its biodiversity, without the intention of determining the total or relative abundance of the species, leading us to document evidence of the discovery of introgression of the Pacific oyster towards the native oyster *S. palmula*.

MATERIALS AND METHODS

Study location

The El Soldado coastal lagoon is located 20 km north of Guaymas and less than 10 km east of San Carlos in the state of Sonora, Mexico (27°57’10”N, 110°58’40”W; Fig. 1). Salas-Mejía et al. (2024) conducted their study on fish species diversity simultaneously with the present study, and reported that the waters of El Soldado have an average depth of <2 m, an annual water temperature range of 16-32°C, and a salinity range of 30-35, in addition to a range of sedimentary grain size from coarse in the mouth area to fine in the inner part of the lagoon. The lagoon covers 1.85 km^2 and is permanently connected to the adjacent sea via a 50-m-wide mouth (Medina-Galván et al. 2021). In 2006, the lagoon was declared a Natural Protected Area (BOGES 2006), and since 2011, it has been a Ramsar site due to its importance for the conservation of aquatic plants and animals. Some parts of the lagoon have mangroves, with the following three species present: *Avicennia germinans*, *Rhizophora mangle*, and *Laguncularia racemosa* (Salas-Mejía et al. 2024).

Sampling

Three visits were made in 2019 (October-December), and four in 2022 (February, May-June, and October) to search for and collect organisms. All sampling was conducted under collection permits issued by the Comisión de Ecología y Desarrollo Sustentable del Estado de Sonora (CEDES), Hermosillo, Sonora (Of. No. UG-COORD-027-22 and Of. No. UG-COORD-035-22). Each visit was made with three to four people covering the perimeter of the lagoon on foot. A small boat was also used to approach the mangrove areas within the lagoon. The organisms were collected manually. Moreover, to increase the likelihood of finding oyster species in El Soldado, spat collectors were constructed using nylon monofilament bags containing 10 empty shells from various mollusk species, including *M. gigas*. Each unit was kept afloat with a buoy on the surface and a small anchor on the bottom and had two collection bags, one 40 cm below the sea surface and another 1 m deep. The collectors

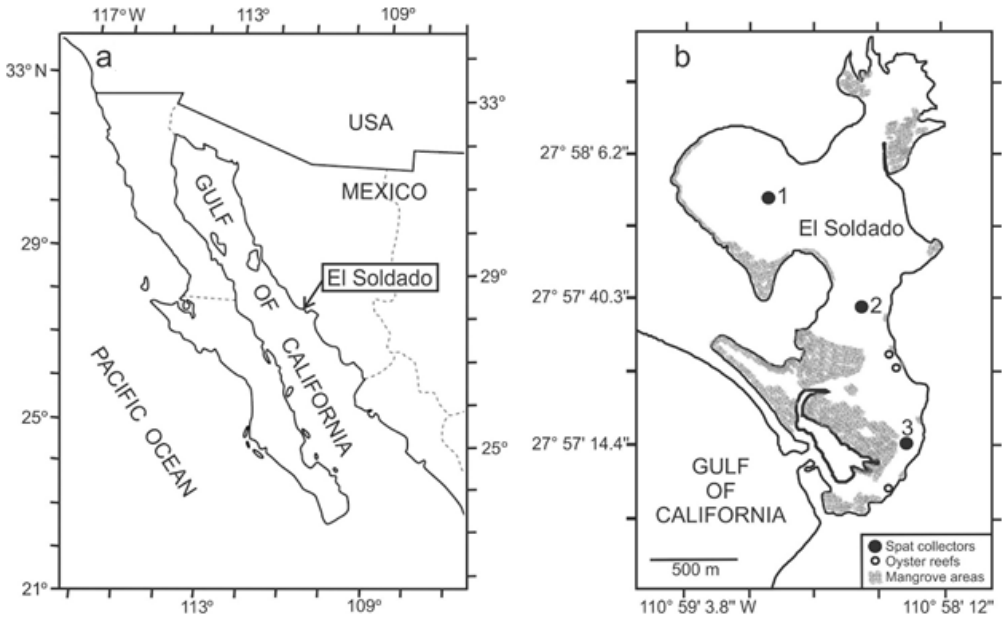

Figure 1. a) Location of the El Soldado coastal lagoon in the Gulf of California. b) Location of the oyster reefs, mangrove areas, and spat collector sites within El Soldado.

were placed in three sites and were replaced every three months between January 2020 and October 2022. The collected organisms were transported to the Molecular Ecology Laboratory of the University of Sonora.

Species identification

The specimens were morphologically identified according to Keen (1971), Fischer (1995), and Lodeiros et al. (2020). Once morphological identification was completed, tissue samples (typically mantle and gills) were obtained, preserved in ethanol (>95%), and stored at -4°C for subsequent molecular verification in some individuals. This molecular verification was performed with sequences of the mitochondrial genes *cytochrome c oxidase subunit I* (*COI*) and *16S ribosomal RNA* (*16S*) since both have proven their efficiency for phylogenetic, systematic, and species diversity analyses in the Ostreidae family (Liu et al. 2011, Sekino & Yamashita 2013, Trivedi et al. 2013, Salvi et al. 2014).

Ostreid DNA was obtained from a 25 mg mixture of portions of the mantle and gill tissue. As a precautionary measure, portions of the same tissues from each organism were preserved in ethanol (>95%) and frozen (-70°C). DNA extraction was performed using the QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN). The DNA concentration was estimated using a NanoDrop 1000 spectrophotometer, and the A260/A280 absorbance ratio was used to determine its purity.

To rule out contamination, all samples were processed independently, separated by species and genes, following procedures that minimize cross-contamination. For *M. gigas*, we used DNA extracts previously employed in an earlier study, which had been properly verified and preserved, obtained from specimens collected at Laguna La Cruz, Bahía de Kino, on July 2, 2018, allowing us to confirm the species' genetic identity and improve the reliability of the sequences obtained. Additionally, the consistent morphology of the analyzed specimens further reduces the misidentification.

The amplification reactions were conducted in a total volume of 20 µL containing 10 µL of Crystal Taq Master solution (Jena Bioscience), 50 ng of DNA, 5 pmol of each oligonucleotide, and PCR-grade water. Two partial mitochondrial fragments, *COI* and *16S*, and the Nuclear Ribosomal Internal Transcribed Spacer 1 (ITS1) region were amplified using the oligos and PCR conditions detailed in Table 1.

All PCR products were visualized on 2% agarose gels and stained with GelRed Nucleic Acid Gel Stain (Biotium). Digital images of the gels were acquired using the DNR MiniBis Pro system. The PCR products were purified using the QIAquick PCR Purification kit (QIAGEN) and sent to the two-way sequencing service of Macrogen (South Korea) and to the Institute of Biotechnology of the Universidad Nacional Autónoma de México.

Table 1. Oligonucleotides and PCR conditions for the amplification of *COI*, *16S*, and ITS1 from ostreids collected in the El Soldado coastal lagoon, Sonora, Mexico.

DNA region	Oligonucleotides	PCR conditions	Expected amplicon
COI	Folmer et al. (1994) LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Liu et al. (2011) Initial denaturation for 3 min at 94°C Thirty cycles of 94°C for 60 s 50°C for 90 s 72°C for 60 s A final extension at 72°C for 10 min	~700
16S	Palumbi (1996) 16Sar: 5′-CGCCTGTTTATCAAAAACAT-3′ 16Sbr: 5′-CCGGTCTGAACTCAGATCACGT-3′	Liu et al. (2011) Initial denaturation for 3 min at 94°C Thirty cycles of 94 °C for 60 s 56 °C for 90 s 72 °C for 60 s A final extension at 72°C for 10 min	~455
ITS1	Hedgecock et al. (1999) ITS-A: 5′-GGTTTCTGTAGGTGAACCTGC-3′ ITS-B: 5′-CTGCGTTCTTCATCGACCC-3′	Pagenkopp-Lohan et al. (2015) Initial denaturation for 10 min at 94°C Thirty-five cycles of 94°C for 30 s 55°C for 60 s 72°C for 45 s A final extension at 72°C for 5 min	~491-611

All PCR analyses included negative controls to monitor for potential contamination, and each amplification was performed according to consistent replication procedures. Sequencing quality control was conducted by manually inspecting all chromatograms; chromatogram evaluation was performed independently by two people. Any sequence that did not meet our quality criteria -such as the presence of ambiguous base calls, background noise, or irregular peak morphology was re-amplified and re-sequenced to ensure data integrity. Only sequences that passed these quality filters were used in the downstream alignment and phylogenetic analyses.

Sequence analysis

The sequences were edited using ChromasPro v.2.1.10.1 (Technelysium) to remove ambiguities and were subsequently deposited in GenBank. All gene sequences were aligned together with Ostreidae sequences obtained from GenBank using Clustal X (Thompson et al. 1997). Phylogenetic analyses were conducted using a gene-partitioned scheme, treating the mitochondrial markers *16S* and *COI* as separate partitions, and no additional codon-based partitioning was applied to the *COI* dataset. The concatenated dataset (*16S*+*COI*) maintained these same gene-based partitions.

Evolutionary models for each partition were selected using the Akaike information criterion (AIC) implemented in PAUP*. For the mitochondrial *16S* and *COI* partitions, the GTR+Γ model (nst = 6) was identified as the best-fit model, assuming variable base frequencies and a symmetric substitution matrix. For the nuclear ITS1 dataset, the SYM model was selected, characterized by equal base frequencies and a symmetric substitution matrix. Maximum likelihood (ML) trees were inferred in PAUP* using the best-fit models per partition, and node support was assessed with 1,000 bootstrap pseudoreplicates.

Bayesian inference (BI) analyses were performed in MrBayes v3.2.6 using the same partitioning scheme and substitution models obtained from PAUP*. Each analysis consisted of four Markov chains (three heated, one cold) under the default heating temperature (temp = 0.2) and was run for 10,000,000 generations, sampling every 1,000 generations. Convergence was evaluated by monitoring the stabilization of log-likelihood values and by examining standard diagnostics in MrBayes (e.g. PSRF ~1.0 and satisfactory ESS values for each parameter). The first

1,000 trees were discarded as burn-in, and posterior probabilities were calculated from the majority-rule consensus of the remaining trees.

To assess genealogical relationships and haplotype frequencies, haplotype data files were generated in NEXUS format using DnaSP v.6 (Rozas et al. 2017). For the mitochondrial markers, sequences were set as haploid and mitochondrial, with the *COI* gene defined as a protein-coding region using the *Drosophila* mitochondrial genetic code and *16S* treated as non-coding; sites with gaps were excluded, and invariant sites were included for both mitochondrial markers to estimate mutational distances correctly. Conversely, for the nuclear ITS1 region (set as diploid and autosomal), invariant sites were removed to reduce noise and complexity, and gaps were not considered. Finally, a network of genetic relationships and the number of mutational steps separating the sequences was constructed with Network 10 (fluxus-engineering.com).

Sequences from the giant oyster *Hyotissa hyotis* (*16S*: AY548883; *COI*: GQ166583; ITS1: KM460848) and the deep-sea oyster *Neopycnodonte cochlear* (*16S*: AY376600; *COI*: AB076939; ITS1: AB377696) (family Gryphaeidae) were used as outgroups for all analyses.

RESULTS

No successful spat settlement was detected on the spat collectors during the study period, as only six organisms were collected and allowed to fatten. Still, all died after three months without reaching an adequate size for identification. However, four oyster specimens settled on the rope holding collector number 2, reaching between 6 and 8 cm in length, and were later identified as the flat oyster *O. angelica*.

Four species of oysters were identified in the study area: *S. palmula*, *S. prismatica*, *Crassostrea corteziensis,* and *O. angelica*. The *16S* sequences unambiguously identified these species by BLAST analysis (Table 2).

From the first visit to the lagoon, the mangrove oyster *S. palmula* was identified as the most conspicuous species that was widely present on mangrove roots as well as on three reefs on rocky areas that were exposed to low tides (Fig. 2). For this species, 10 specimens were molecularly analyzed for *16S*, obtaining three haplotypes but with an unexpected result in haplotype 3 present in five specimens which showed 100% identity with several records reported for the Pacific oyster *M. gigas*. However, their morphological traits coincided with those of *S. palmula* (Fig. 3).

The same 10 specimens were analyzed for *COI*, yielding five haplotypes. Four haplotypes had >99% identity with *S. palmula*. Still, haplotype 5 had 100% identity with *M. gigas* and corresponded to the same five specimens whose *16S* sequences were also identical to *M. gigas*, corroborating the presence of the mitochondrial genome of *M. gigas* in *S. palmula* specimens. To confirm this result, *COI* sequences were also obtained from five *M. gigas* specimens from a commercial culture in the La Cruz coastal lagoon (located 128 km to the north), yielding the same haplotype.

Considering the above-described results, the ITS1 nuclear region of the five *S. palmula* specimens that exhibited mitochondrial sequences from *M. gigas* was analyzed. Three haplotypes were obtained, where two specimens showed >99% identity with sequences reported for *S. palmula*. The three remaining specimens shared the third haplotype and showed >99% identity with sequences reported for *M. gigas*.

Phylogenetic analysis was conducted after editing and identifying the sequences in conjunction with those obtained from GenBank. All phylogenetic trees obtained using PAUP* and MrBayes had the same topology. The *16S* sequences obtained allowed us to validate the morphological identification of the four ostreid species found in this study (Fig. 4). For this gene, *S. palmula* haplotype 3 was combined with *M. gigas* sequences. The phylogenetic tree with the *COI* of *S. palmula* showed that haplotype 5 (same specimens as *16S* haplotype 3) was integrated with *M. gigas* sequences (Fig. 5). The phylogenetic tree with the ITS1 nuclear region of *S. palmula* with the mitochondrial genes of *M. gigas* showed that two haplotypes were grouped with sequences reported for *S. palmula*. In contrast, the third haplotype (shared by three specimens) was integrated with sequences from *M. gigas* (Fig. 6).

The genetic network analysis, carried out with the same sequences used in the phylogenetic analysis, provided a more detailed picture of genealogical relationships and confirmed the introgression event in specimens morphologically similar to *S. palmula*. For the *16S* gene, the network showed segregated groups corresponding to each species, in which sequence OQ551179 from the five specimens morphologically identified as *S. palmula* clustered with *M. gigas* (Fig. 7).

Consistently, the *COI* network showed a significant divergence between *S. palmula* and *M. gigas*, with 148 mutational steps separating them. Here, sequence OQ709476 from the same five specimens as sequence OQ551179 clustered with several *M. gigas* sequences

Table 2. Ostreid species collected in the El Soldado coastal lagoon and the *16S*, *COI*, and ITS1 sequences obtained from a subsample of individuals, as well as their accession number in GenBank and the identity obtained with BLAST analysis.

Morphological identification	Collected individuals	Haplotypes by molecular marker	Number of individuals analyzed molecularly	GenBank accession number	Fragment sizes (pb)	BLAST identity to the closest species
Saccostrea palmula	67	*16S*				
		H1	4	OQ551177	530	100% *Saccostrea palmula*
		H2	1	OQ551178	530	100% *Saccostrea palmula*
		H3	5	OQ551179	530	100% *Crassostrea gigas*
		COI				
		H1	1	OQ709472	700	>99% *Saccostrea palmula*
		H2	1	OQ709473	700	>99% *Saccostrea palmula*
		H3	1	OQ709474	700	>99% *Saccostrea palmula*
		H4	2	OQ709475	700	>99% *Saccostrea palmula*
		H5	5	OQ709476	700	100% *Crassostrea gigas*
		ITS1				
		H1	1	OR750382	512	>99% *Saccostrea palmula*
		H2	1	OR750383	512	>99% *Saccostrea palmula*
		H3	3	OR750384	438	>99% *Crassostrea gigas*
Crassostrea corteziensis	2	*16S*				
		H1	2	OQ551175	532	100% *Crassostrea corteziensis*
Striostrea prismatica	11	*16S*				
		H1	2	OQ551176	567	>99% *Striostrea prismatica*
Ostrea angelica	25	*16S*				
		H1	4	OQ551180	530	>99% *Ostrea angelica*

Figure 2. *Saccostrea palmula* reef area within Estero El Soldado, Sonora, Mexico.

(Fig. 8). In contrast, the ITS1 nuclear network showed complete segregation between species, with the two specimens identified as mitochondrial hybrids (OR750382 and OR750383) grouping cohesively within the *S. palmula* cluster. At the same time, three (OR750384) were separated by 231 mutational steps (Fig. 9). This clear cytonuclear discordance -exotic mitochondria with native nuclear alleles- strongly supports the hypothesis of historical hybridization followed by backcrossing.

To obtain a more robust result, a concatenated phylogenetic analysis (*16S+COI*) was conducted by

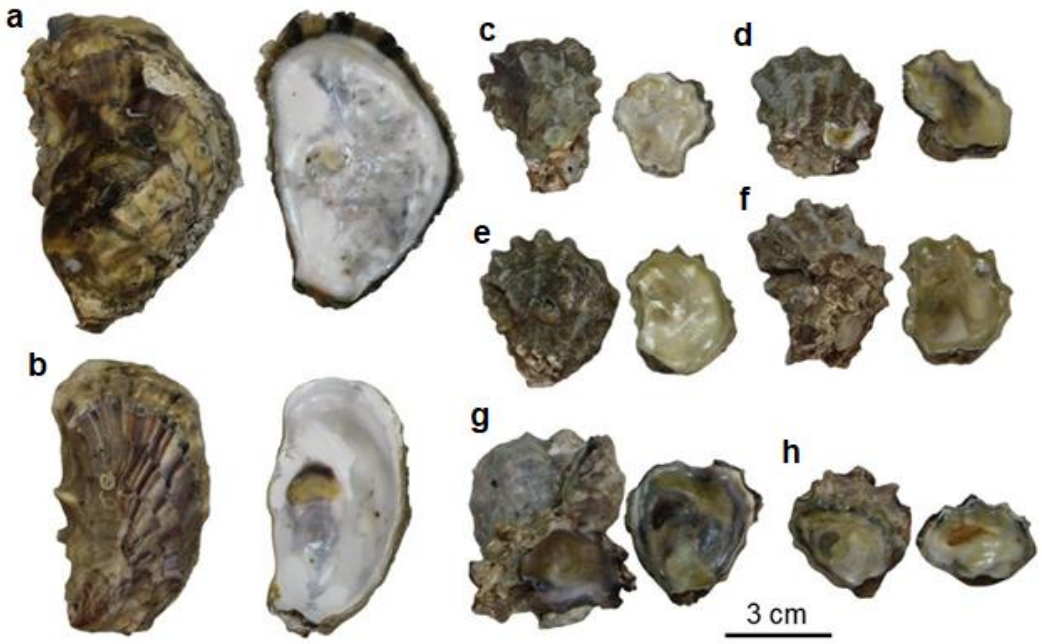

Figure 3. Shell morphology of oysters. a-b) *Magallana gigas* from an oyster farm located at Estero La Cruz, Sonora, c) pure specimen of *Saccostrea palmula*, d-f) specimens morphologically identified as *S. palmula* but with the mitochondrial genome and nuclear ITS1 region of *M. gigas*, g-h) specimens morphologically identified as *S. palmula* but with the mitochondrial genome of *M. gigas* and the nuclear ITS1 region of *S. palmula*.

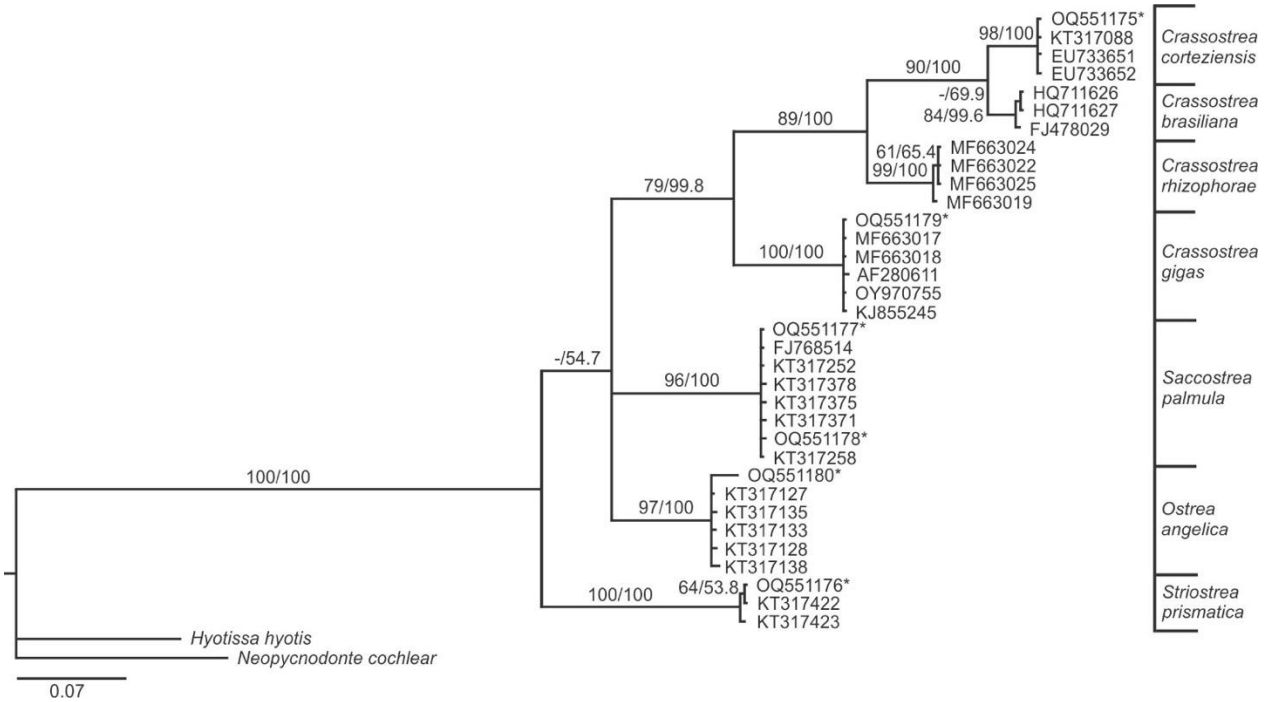

Figure 4. Maximum likelihood phylogenetic tree of mitochondrial *16S* rRNA sequences from ostreids collected in the El Soldado coastal lagoon (*) and sequences of other ostreids reported in GenBank. The OQ551179 sequence corresponds to haplotype 3 of the organisms morphologically identified as *Saccostrea palmula*. *Hyotissa hyotis* and *Neopycnodonte cochlear* were used as outgroups. Numbers on the arm: ML bootstrap values/BI posterior probabilities.

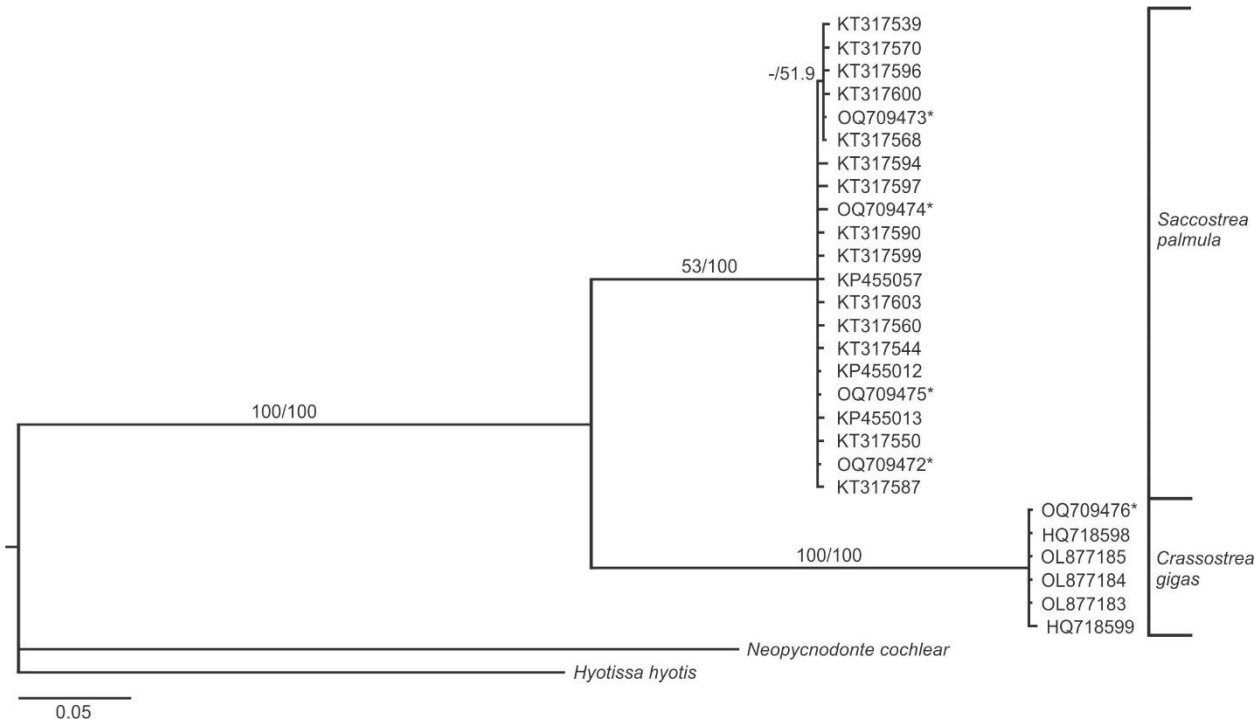

Figure 5. Maximum likelihood phylogenetic tree of mitochondrial *COI* sequences from *Saccostrea palmula* collected in the El Soldado coastal lagoon (*) and sequences of *Crassostrea gigas* reported in GenBank. The OQ709476 sequence corresponds to haplotype 5 of the organisms morphologically identified as *Saccostrea palmula*. *Hyotissa hyotis* and *Neopycnodonte cochlear* were used as outgroups. Numbers on the arm: ML bootstrap values/BI posterior probabilities.

joining all sequences derived from the mitochondrial DNA of the five specimens with evidence of introgression, together with the equivalent sequences of the species reported in GenBank. In this analysis, we confirmed that three morphologically identified specimens of *S. palmula* are closely related to *M. gigas* (Fig. 10).

DISCUSSION

To our knowledge, this study is the first to report the presence of *C. corteziensis* and *S. prismatica* in the El Soldado coastal lagoon, increasing the number of ostreid species recorded there from 2 to 4 since Martínez-Córdova (1996). Furthermore, this study showed evidence of introgression between *S. palmula* and *M. gigas*. The most conspicuous species was *S. palmula*, followed to a lesser extent by *S. prismatica*, *C. corteziensis*, and *O. angelica*.

The lack of success in spat collection can be explained by considering the environmental conditions present in Estero El Soldado during the period when the collectors were deployed. Although *M. gigas* is highly tolerant to wide ranges of salinity (10-50) and temperature (-1.8 to 35°C), reproduction and larval development occur only within a more restricted thermal range (Chávez-Villalba et al. 2003). During the time the collectors remained in the estuary (January 2020 to October 2022), water temperature followed a seasonal pattern, ranging from approximately 18°C in winter to 32°C in summer, with an average close to 25°C, while salinity remained relatively stable between 35 and 38 (Salas-Mejía et al. 2024). Although these conditions fall within the species' tolerance limits, studies in Mexico have shown that *M. gigas* can produce viable gametes and spawn without resulting in successful spat settlement in the natural environment (Chávez-Villalba 2014). In this context, Matsubara et al. (2023) indicate that larval settlement in this species depends on interactions among environmental conditions, biological cues, and the type of substrate available, suggesting that small variations in these factors may affect settlement success. In addition, reproductive activity in Estero El Soldado has been reported mainly from spring to late summer, with evidence of oocyte resorption toward the end of the

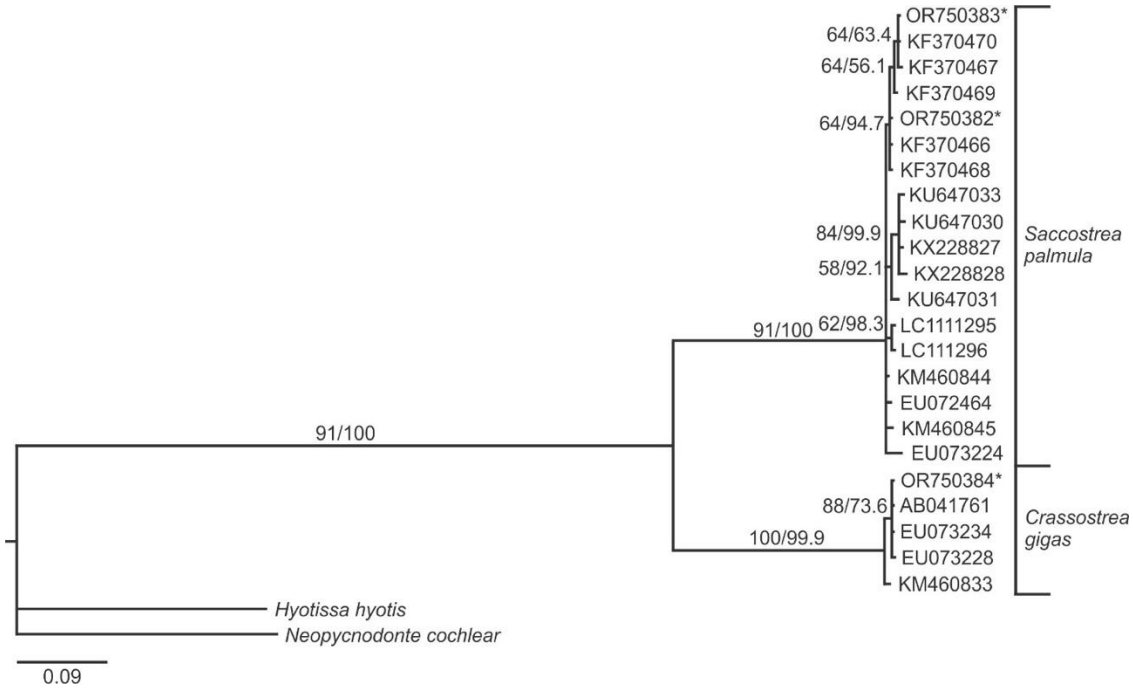

Figure 6. Maximum likelihood phylogenetic tree of sequences from the nuclear ITS1 region of ostreids with the morphology of *Saccostrea palmula* but with the mitochondrial genes of *M. gigas*, collected in the El Soldado coastal lagoon (*) and in conjunction with the sequences of *M. gigas* reported in GenBank. *Hyotissa hyotis* and *Neopycnodonte cochlear* were used as outgroups. Number on the arms: ML bootstrap values/BI posterior probabilities.

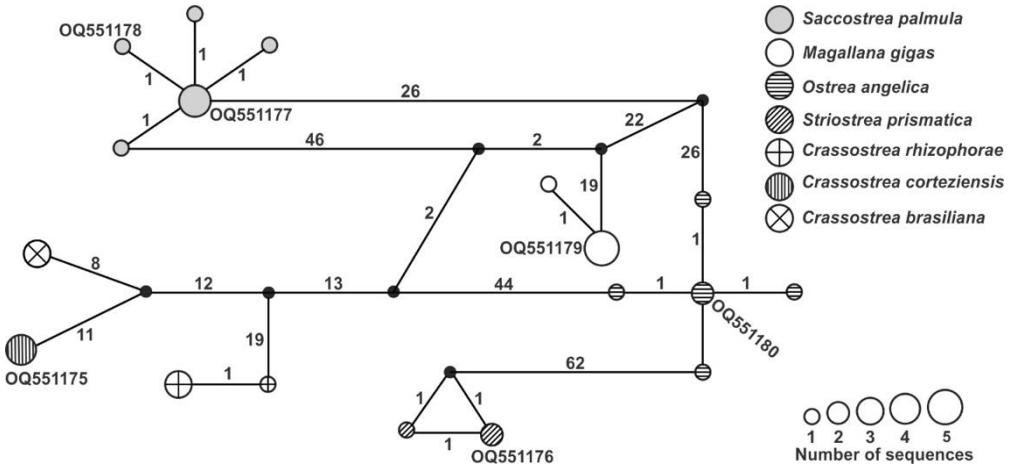

Figure 7. Genotype median joining network of mitochondrial *16S* rRNA sequences from ostreids collected in the El Soldado coastal lagoon (identified with GenBank accession numbers) and sequences of other ostreids reported in GenBank. Circle size is proportional to the number of individuals having the genotype. Number on the lines: the number of mutational steps between genotypes.

reproductive season (Chávez-Villalba et al. 2007). Therefore, the absence of spat settlement observed in this study is likely due to a combination of less favorable environmental conditions during key periods of the reproductive cycle and the lack of appropriate cues for larval settlement, rather than the absence of spawning itself.

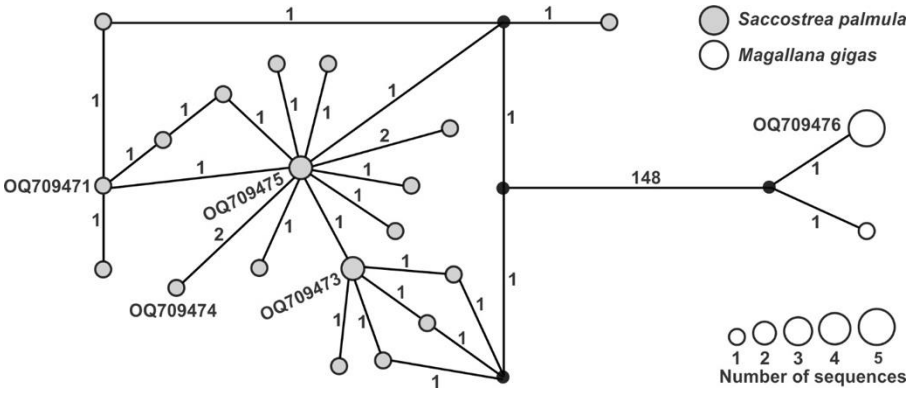

Figure 8. Genotype median joining network of mitochondrial *COI* sequences from *Saccostrea palmula* collected in the El Soldado coastal lagoon (identified with GenBank accession numbers) and sequences of *Magallana gigas* reported in GenBank. The OQ709476 sequence corresponds to specimens morphologically identified as *S. palmula*. Circle size is proportional to the number of individuals having the genotype. Number on the lines: the number of mutational steps between genotypes.

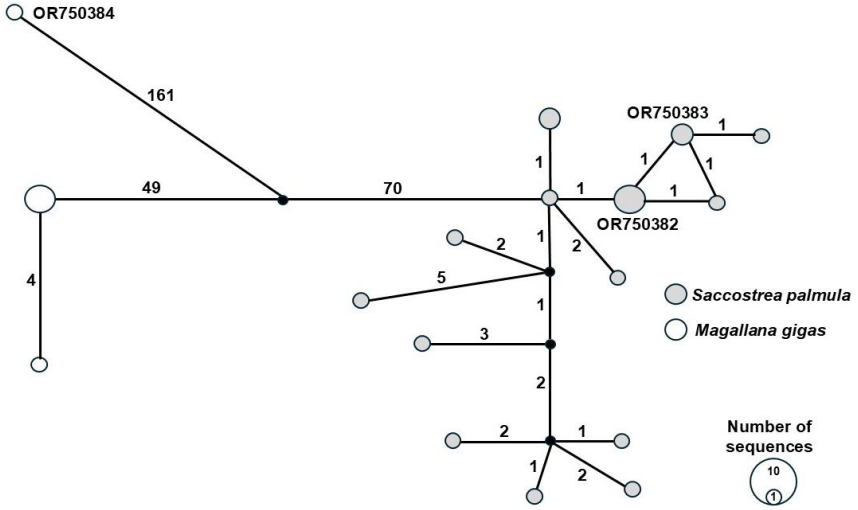

Figure 9. Genotype median joining network of sequences from the nuclear ITS1 region from *Saccostrea palmula* collected in the El Soldado coastal lagoon (identified with GenBank accession numbers) and sequences of *Magallana gigas* reported in GenBank. The OQ750384 sequence corresponds to specimens morphologically identified as *S. palmula*. Circle size is proportional to the number of individuals having the genotype. Number on the lines: the number of mutational steps between genotypes.

Molecular tools based on the sequences of *16S*, *28S*, *COI*, ITS1, and ITS2 have been widely used to identify species of the Ostreidae family and resolve the phylogeny between these species and the subfamilies that constitute Ostreidae, although with certain controversies in some cases (Jozefowicz & Foighil 1998, Hedgecock et al. 1999, Liu et al. 2011, Salvi et al. 2014, Hsiao et al. 2016, Hamaguchi et al. 2017, Salvi & Mariottini 2017, 2020, Guo et al. 2018). Thus, the inherent difficulties of phenotypic plasticity in identifying ostreid species can be overcome using molecular tools (Liu et al. 2011), allowing the discovery of introgression events that might otherwise go unnoticed, as in the case of *M. gigas* and *S. palmula* from the El Soldado coastal lagoon.

This study demonstrated that the *S. palmula* population at El Soldado is subject to introgression, likely due to 30 years of prior commercial culture of *M. gigas*, which ended just 20 years ago. The evidence presented here indicates that crosses were made between

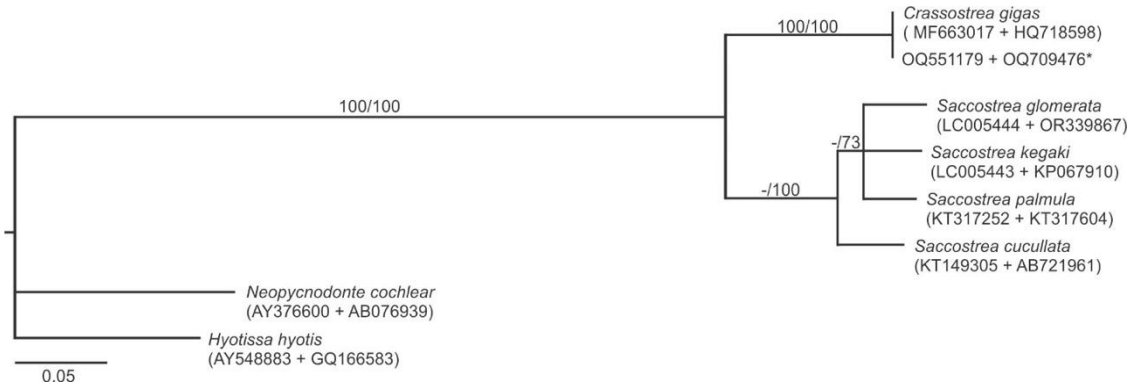

Figure 10. Maximum likelihood phylogenetic tree of concatenated *16S+COI* sequences from five organisms with *Saccostrea palmula* morphology but with the introgressed genes of *Crassostrea gigas* (*), collected in the El Soldado coastal lagoon and in conjunction with sequences of *C. gigas* and other *Saccostrea* species reported in GenBank. *Hyotissa hyotis* and *Neopycnodonte cochlear* were used as outgroups. Number on the arms: ML bootstrap values/BI posterior probabilities.

M. gigas females and *S. palmula* males. However, there are no elements to ensure that crosses were also made between *M. gigas* males and *S. palmula* females. The unequivocal placement of sequences from specimens morphologically identified as *S. palmula* into the *M. gigas* mitochondrial clades confirms the persistence of the exotic maternal lineage.

The most plausible explanation for the three *S. palmula* specimens that retain *S. palmula* morphology but carry *M. gigas* mitochondrial haplotypes (*16S* and *COI*) together with admixed ITS1 genotypes is introgressive hybridization, which may be progressing toward a hybrid swarm (Allendorf et al. 2013). It is further evidenced by the cytonuclear discordance observed in the genetic networks: while mitochondrial markers place these individuals within *M. gigas*, the nuclear ITS1 network groups them within the *S. palmula* lineage, suggesting that repeated backcrossing with native males has "diluted" the exotic nuclear genome while retaining the invasive mitochondrial capture. In a hybrid swarm, repeated hybridization, backcrossing with one or both parental species, and interbreeding among hybrids over multiple generations produce a population-wide genetic mosaic (Allendorf et al. 2013). This scenario (if widespread) could ultimately pose a conservation risk to the genetic integrity of *S. palmula*. We note, however, that our inference is based on a single nuclear locus (ITS1), which has well-known limitations, including potential biases associated with concerted evolution and locus-specific selection. Single-locus nuclear markers may not fully capture the heterogeneity of introgression across the genome and can either underestimate or overestimate the true extent of hybrid ancestry. For these reasons, the present results should be viewed as preliminary evidence of introgression consistent with hybridization, pending confirmation with higher-resolution, genome-wide SNP data, which should be addressed soon.

Although based on a reduced molecular subsample, the detection of *M. gigas* haplotypes in 50% of the sequenced individuals suggests that introgression into the *S. palmula* population at El Soldado may be substantial, as haplotypes occurring at moderate to high frequencies are likely to be detected even with limited sampling. Nevertheless, this pattern should be confirmed in future studies that incorporate a significantly larger number of sequenced individuals. If introgression is indeed high, a process of "genomic extinction" could occur, in which repeated hybridization and backcrossing erode the evolutionary legacy of the native species. Under this scenario, the genome-wide combination of alleles and genotypes that characterizes *S. palmula*, shaped over evolutionary time, could be progressively lost due to introgression from *M. gigas*, even if hybrid individuals exhibit reduced fitness (Allendorf et al. 2013).

Previous studies have demonstrated that *M. gigas* did not complete its sexual maturity cycle in the Gulf of California (Chávez-Villalba et al. 2007) or that the spawned gametes died (Chávez-Villalba 2014). The evidence of *M. gigas* genes in *S. palmula* shows that there was an exceptional window of environmental opportunity in El Soldado that favored full-term maturity and subsequent spawning of cultured *M. gigas*, coinciding with the reproductive season of *S.*

palmula. However, even more surprising is the fertilization of *M. gigas* gametes with an ostreid of a different genus. Gaffney & Allen (1993) noted that reports of interspecific hybridization in *Crassostrea* should be treated with skepticism; however, over time, it was shown that such hybridization is possible. Su et al. (2016) reported postzygotic barriers that prevent the development of the larval phase in embryos produced by crossing *Magallana angulata* (Crassostreinae) and *Saccostrea cucullata* (Saccostreinae), both sympatric in China. Similarly, the fact that *M. angulata* and *S. cucullata* cannot produce viable progeny does not imply that hybridization cannot occur between species of those genera. The molecular sequences reported in the present study demonstrate that hybridization is possible and has serious consequences for the native species. Hence, this discovery is extremely important, as it indicates a potential threat to the conservation of other native ostreid species in the Gulf of California.

The establishment of invasive species can be avoided through three important actions: eradication, control, and prevention (Allendorf et al. 2013). Eradication can only be conducted at an early stage of introduction or at the beginning of the invasion. In the case of the El Soldado coastal lagoon, the invasive species no longer exists, and eradicating this genetic contamination is impossible. Given the large number of commercial Pacific oyster farms in the coastal lagoons of the Gulf of California, the potential for introgression with native ostreids is high, and a detailed investigation is required.

To our knowledge, this study is the first to report that *S. palmula* populations at El Soldado have experienced a potential "hybrid swarm" process. This finding is significant because it emphasizes the ability of *M. gigas* to reproduce in an environment previously considered unsuitable for its maturation. Therefore, there is a need for more detailed investigations into the reproductive biology and genetics of these species to fully understand the possibilities and limitations of hybridization, as well as the specific genetic factors that influence it.

Our results should be verified in a more comprehensive study that quantifies the extent of introgression from *M. gigas* into *S. palmula*, using techniques such as RADseq to generate SNP datasets. These datasets could then be used in species assignment analyses to assess the degree of genomic similarity between species. Typically, genetic introgression is identified when a percentage of one species' genome is found in another, with the majority still corresponding to the original species. This knowledge is vital for managing feral populations of *M. gigas* and for developing sustainable aquaculture practices. Regarding El Soldado's biodiversity, this study expanded the site's oyster species catalog from 2 to 4. *S. palmula* was the most conspicuous species, followed to a lesser extent by *S. prismatica*, *C. corteziensis*, and *O. angelica*.

From a management perspective, our findings provide actionable steps to mitigate introgression risks for native ostreids in coastal lagoons of the Gulf of California. Although aquaculture of *M. gigas* has been prohibited in El Soldado since its designation as a Natural Protected Area, the presence of introgressed individuals highlights the need for regional surveillance. A practical approach for other lagoons where feral populations of *M. gigas* persist, and aquaculture is still active, would be to implement eDNA or larval qPCR screening during the reproductive season to detect *M. gigas* gametes and larvae, even at low densities. To quantify and monitor hybridization dynamics, we recommend developing a minimal RADseq SNP panel that distinguishes pure and admixed individuals of both species. Establishing a biannual monitoring schedule that coincides with the onset and peak of spawning would facilitate early detection of introgression events and support timely management actions. Close coordination between protected-area managers and regional aquaculture authorities will be essential to ensure that conservation strategies for *S. palmula* are aligned with ongoing farming practices and regional biosecurity needs.

Credit the author's contribution

J.M. Grijalva-Chon: conceptualization, funding acquisition, methodology, validation, supervision, writing and original draft; J.E Chávez-Villalba: methodology, supervision, review and editing; M.F. Martínez-García: methodology, formal analysis, writing and editing; J.A. Arreola-Lizárraga & C. Lodeiros-Seijo: supervision, review and editing; A. Varela-Romero: methodology, data curation, review and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interest

The authors declare they have no conflict of interest.

Data availability

The data for this study are available from the corresponding author upon reasonable request.

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