Phylogeny and Species Diversity of Gulf of California Oysters (Ostreidae) Inferred from Mitochondrial DNA*

Authors: Raith, Meredith, Zacherl, Danielle C., Pilgrim, Erik M., and Eernisse, Douglas J.

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Phylogeny and species diversity of Gulf of California oysters (Ostreidae) inferred from mitochondrial DNA*

Meredith Raith[1], Danielle C. Zacherl[1], Erik M. Pilgrim[2], and Douglas J. Eernisse[1]

[1]Department of Biological Science, California State University Fullerton, Fullerton, California 92834-6850 U.S.A.

[2]U.S. Environmental Protection Agency, Ecological Exposure Research Division, National Exposure Research Laboratory, Cincinnati, Ohio 45268 U.S.A.

Correspondence, Douglas J. Eernisse: deernisse@fullerton.edu

Abstract. The Olympia oyster, *Ostrea lurida* Carpenter, 1864, is the only native oyster on the west coast of temperate western North America and a conservation target for native species restoration throughout much of its known range, from British Columbia, Canada to Baja California, Mexico. This species was recently demonstrated to be genetically distinct from its southern congener, *O. conchaphila* Carpenter, 1857, but our new sampling, combined with previously published data, supports a tentative allopatric pattern, with the southern and northern species restricted to either side of Punta Eugenia in Baja California, Mexico, a known biogeographic boundary. We collected *O. conchaphila* and multiple co-occurring oyster species at 11 sites along the Pacific coast of Baja California Sur or within the Gulf of California, Mexico. Oyster surveys revealed at least six other co-occurring species, including two exotics, of Ostreidae Rafinesque, 1815 that were identified by sequencing 16S ribosomal DNA (16S) and cytochrome oxidase subunit I (COI) mitochondrial markers. In addition to our newly collected material, our phylogenetic analyses included Ostreidae from worldwide localities available in GenBank.

Phylogenetic estimates, using maximum likelihood, supported the sister species relationship between *Ostrea lurida* Carpenter, 1864 and *O. conchaphila* Carpenter, 1857. Together, they group as the sister lineage of *Myrakeena angelica* (Rochebrune, 1895), nested within a grouping of species currently assigned to *Ostrea* Linnaeus, 1758. Thus, we have revived the original name *Ostrea angelica* Rochebrune, 1895 and consider the monotypic genus, *Myrakeena* Harry, 1985 a junior synonym of *Ostrea*. We also collected *O. equestris* Say, 1834, native to the Caribbean and not previously reported in the Eastern Pacific. Our results are consistent with the recognition of only four subfamilies within Ostreidae: Ostreinae Rafinesque, 1815, Crassostreinae Scarlato and Starobogatov, 1979, Saccostreinae Salvi *et al*., 2014, and Striostreinae new subfamily. Another subfamily, Lophinae Vialov, 1936, is best synonymized with Ostreinae because it would otherwise be paraphyletic to that taxon. Sequences of *Saccostrea palmula* (Carpenter, 1857) revealed a striking lack of genetic variation that contrasted with their substantial phenotypic plasticity. Surprisingly, the morphologically distinctive species, *Ostrea tubulifera* Dall, 1914, was revealed as an ecotype of *S. palmula*, and so is herein considered a junior synonym of the latter species.

Key words: systematics, *Ostrea*, *Crassostrea*, *Saccostrea*, phylogeography

Oysters are economically important worldwide as fisheries species and ecologically important because they form dense beds, providing extensive habitat for a variety of organisms. Ecosystems with declining oyster populations may experience negative effects such as lower water quality and reduced biodiversity (Quayle 1988, Dumbauld *et al.* 2011). Because of their dual importance, oyster biology and genetics are studied extensively to inform restoration decisions, protect wild populations, and maintain healthy fishery populations.

The status of the Olympia oyster, *Ostrea lurida* Carpenter, 1864, is of particular interest to western North American researchers and shellfish farmers. It is the only oyster native to the western United States, and despite the historic collapse of its fisheries about a century ago, it has renewed economic value as a specialty food item. When Carpenter (1864) first described *O. lurida*, he reported its distribution to extend from what is presently British Columbia, Canada to the northern part of the Pacific coast of the Baja California peninsula (Polson *et al.* 2009; Fig. 1). Other authors have reported a broader range from Sitka, Alaska, U.S.A. to Cabo San Lucas, Baja California Sur, Mexico (Dall 1914, Hertlein 1959, Baker 1995), but these broader ranges were not confirmed by Polson and Zacherl (2009). By the late 1800s to early 1900s, the large-scale

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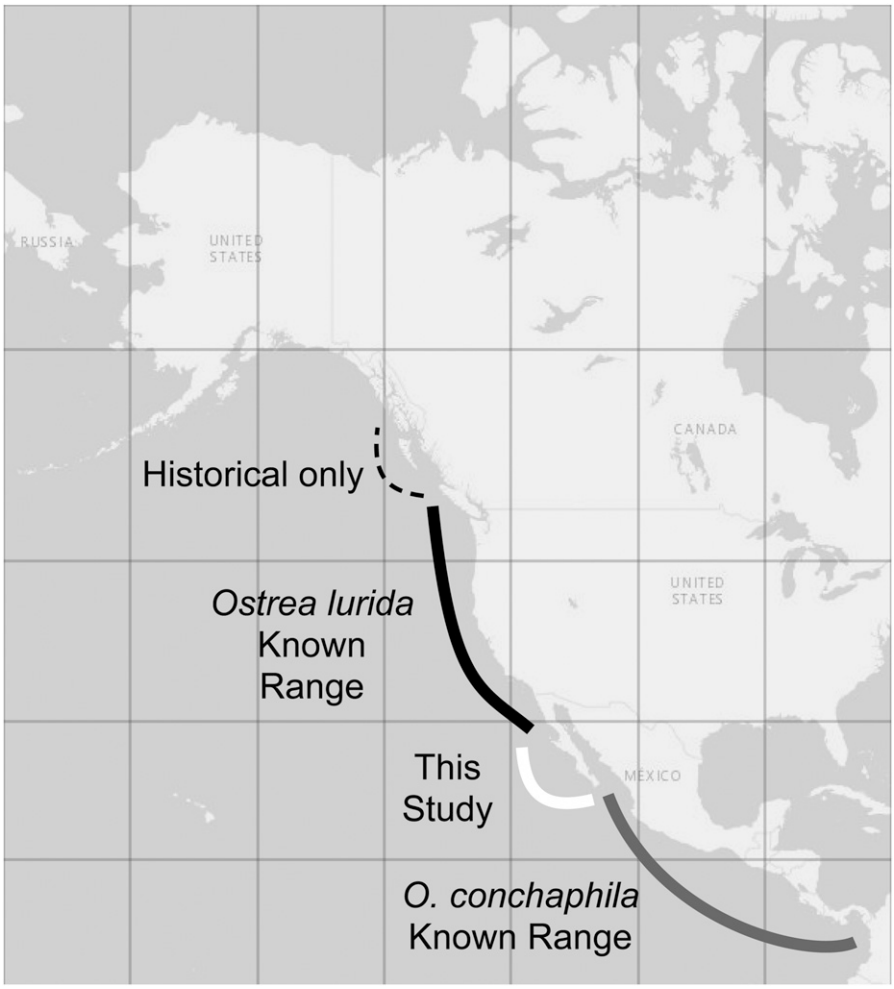

Figure 1. Previously reported range for *Ostrea conchaphila* and *O. lurida* after Polson *et al.* (2009). These had long been regarded as separate species but Harry (1985) proposed instead that they were a single widespread species, *O. conchaphila*. Bars are only approximate and northern limits of *O. lurida* and southern limits of *O. conchaphila* are based on literature reports and have not been confirmed in this study. Base map based on National Geographic *et al.* (2012).

commercial fishery of wild-caught Olympia oyster had collapsed due to over-harvesting and was replaced by short-lived oyster farming efforts that failed by the 1930s (reviewed in Baker 1995). In response to these declines, oyster farmers intentionally introduced barge loads of the much larger non-native Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), from Japan (Steele 1957). The Pacific oyster fishery grew rapidly and continues to dominate oyster farming on the West Coast (Barrett 1963, Conte 1996). Meanwhile, *O. lurida* has remained rare in western North America areas where its beds once flourished (Baker 1995, Conte 1996, Zu Ermgassen *et al.* 2012).

There are ongoing efforts to restore native Olympia oyster populations (*e.g.*, White *et al.* 2009, Wasson *et al.* 2014), and yet their phylogeographic structure is poorly known and their phylogeny is the subject of ongoing and recent debate, especially relative to its southern congener, *Ostrea conchaphila* Carpenter, 1857. About a century after Carpenter (1857) described *O. conchaphila* as occurring in Mazatlan, Mexico, to Panama and *O. lurida* as ranging from British Columbia, Canada to Cabo San Lucas, Baja California Sur, Mexico (Fig. 1), Hertlein (1959) speculated that there was a likely zone of overlap and possible hybridization occurring between southern California and Cabo San Lucas. Harry (1985) later proposed that the two nominal species were a single species, with *O. lurida* (type locality, Willapa Bay, Washington) as a junior synonym of its southern congener, *O. conchaphila* (type locality, Mazatlan, Mexico). Mixed acceptance of his synonymy led to some confusion in the literature because people used different names for the native oysters north of Mexico.

The controversy arising from the synonymizing of the two nominal species motivated Polson *et al.* (2009) to survey oysters from western North America using mitochondrial 16S ribosomal DNA (16S) and cytochrome oxidase subunit III (COIII) markers. They found only *Ostrea lurida* through Hertlein's proposed area of sympatry at least as far south as San Quintin, Baja California, Mexico. Additionally, haplotype analysis showed little structure among populations of *O. lurida* between San Quintin, Mexico and Willapa Bay, Washington, but did find an interesting phylogeographic break in the COIII data between Willapa Bay and the southern portion of Vancouver Island, British Columbia, Canada. Eernisse and coworkers (manuscript in prep.) have added cytochrome oxidase subunit I (COI) to this comparison and have found that the northern haplotype dominates not only the Vancouver Island populations sampled by Polson *et al.* (2009) but also extends to within Puget Sound, Washington. The lack of mitochondrial variation in southern localities for *O. lurida* contrasted with the substantial distinction that Polson *et al.* (2009) found when these were compared with *O. conchaphila* from near Mazatlan. Polson *et al.* (2009) concluded that the species as described by Carpenter (1864) were separate species.

The analysis by Polson *et al.* (2009) of their new sequences as supplemented by corresponding Ostreidae sequences from

GenBank produced a result with *Ostrea conchaphila* and *O. lurida* as reciprocally monophyletic sister species. However, this conclusion was tentative not only because samples between San Quintin and Mazatlan were missing but also because their analysis did not include multiple oyster species from the Gulf of California and the Pacific coast of Baja California whose morphological and phylogenetic affinities are poorly documented. Likewise, worldwide Ostreidae phylogenetic relationships have remained unresolved at multiple taxonomic levels. Unraveling the relationships is complicated by a number of factors, including the historic reliance on shell-based morphological characters (*e.g.*, shell color, sculpturing, dentition, or shape) in a group with notoriously plastic shell attributes. This "morpho-species" concept is potentially confounded by convergent similarities due to similar microhabitat and rampant phenotypic plasticity within species. There have been only a few attempts to apply other species concepts, such as the biological (Mayr 1942, Mishler and Donoghue 1982) or phylogenetic (Cracraft 1983, Avise 2004) species concepts, and only to particular taxa. Lam (2003) was among the first to test the effectiveness of conventional morphology-based oyster classifications with molecular data. The Polson *et al.* (2009) study helps to underscore how use of molecular tools can reveal potentially discrete species despite otherwise indistinguishable morphological characteristics.

Application of any species concept, however, can be confounded by spatial and taxonomic gaps in sampling. For example, most DNA-based phylogenetic analyses of Ostreidae have emphasized various subgroups in isolation or have a restricted geographic focus (Jozefowicz and Ó Foighil 1998, Wang *et al.* 2004, Lam and Morton 2006, Varela *et al.* 2007, Reece *et al.* 2008, Lazoski *et al.* 2011, Sekino and Yamashita 2013). As a case in point, Polson *et al.*'s (2009) phylogenetic estimate refined previous phylogenetic relationships but could still be affected by known taxonomic and geographic gaps in sampling. The previous phylogenetic estimates were laid out by Jozefowicz and Ó Foighil (1998), who placed *Ostrea conchaphila* (based on samples that are today considered *O. lurida*) as sister to *O. denselamellosa* Lischke, 1869 from South Korea, using molecular characters. Polson *et al.* (2009) refined their estimate with greater geographic sampling in multiple habitats along their described ranges, and resolved the revived nominal species *O. lurida* and a geographically restricted *O. conchaphila* as reciprocally monophyletic sister species. However, the gaps in geographic sampling that yet remain could profoundly revise the current phylogenetic hypothesis proposed by Polson *et al.* (2009). The ranges of *O. lurida* and *O. conchaphila* were previously only documented as effectively north and west or south and east of the Gulf of California, respectively, with neither species thought to be present within the Gulf. Hence, Polson *et al.* (2009) did not sample within the Gulf (Fig. 1), where either species might have occurred and where some of the multiple other unsampled nominal oyster species (Table 1), including *Myrakeena angelica* (Rochebrune, 1895),

Table 1. Selected previously-described oyster species in the Gulf of California. All species are reported with names as referenced in this manuscript with the inclusion of authority and date, additional nominal combinations for genera only, geographic range, and previous molecular analysis for any of the following mitochondrial gene regions: 16S, COI, COIII.

Species as Reported Here	Authority, Date	Other Genera	Geographic Range	Previous Molecular Analysis
Ostrea angelica	Rochebrune, 1895	*Myrakeena*	Gulf of California to Ecuador	No
Ostrea conchaphila	Carpenter, 1857	*Ostreola*	Gulf of California to Panama	Yes
Ostrea lurida	Carpenter, 1864	*Monoeciostrea* Orton, 1928	Northern Pacific to Gulf of California	Yes
Ostrea tubulifera	Dall, 1914	*Saccostrea*	Gulf of California	No
Crassostrea columbiensis	(Hanley, 1846)	*Ostrea*	Gulf of California, Ecuador	None from near type locality
Crassostrea corteziensis	(Hertlein, 1951)	*Ostrea*	Gulf of California to Panama	Yes
Crassostrea gigas	(Thunberg, 1793)	*Ostrea, Dioeciostrea* Orton, 1928, *Lopha* Röding, 1798	Introduced	Yes
Saccostrea palmula	(Carpenter, 1857)	*Dendostrea* Swainson, 1835, *Ostrea*	Gulf of California to Ecuador	Yes
Hyotissa hyotis	(Linnaeus, 1758)	*Ostrea, Mytilus* Linnaeus, 1758, *Pycnodonte* Fischer von Waldheim, 1835	Gulf of California to Ecuador	Yes
Striostrea prismatica	(Gray, 1825)	*Ostrea*	Gulf of California to Peru	No
Undulostrea megodon	(Hanley, 1846)	*Ostrea, Lopha*	Gulf of California to Peru	No

Ostrea tubulifera Dall, 1914, *Striostrea prismatica* (Gray, 1825) and *Undolostrea megadon* (Hanley, 1846), might even influence the putative sister species status of *O. lurida* and *O. conchaphila*.

The relationship of these purported sister species to other Ostreidae species is complicated by the unresolved phylogenetic relationships among Ostreidae genera and the lack of evidence for monophyly for the assemblage of species assigned to the oldest genus name, *Ostrea* Linnaeus, 1758 (*i.e.*, WoRMS recognizes 15 other Ostreidae extant genera as valid, Bouchet 2014). Many authors had recognized the importance of broad taxonomic and geographic sampling to discern relationships among oyster species (Ó Foighil and Taylor 2000, Terry *et al.* 2000, Leitão *et al.* 2004, Lapègue *et al.* 2006, Lam and Morton 2009), and multiple studies are focused on determining ostreid relationships, among species, among genera (*e.g.*, Lawrence 1995, Lam and Morton 2009), and across the family (Ó Foighil and Taylor 2000, Kirkendale *et al.* 2004).

One goal of this study, therefore, was to assemble morphological and DNA sequence characters for as many oyster (Ostreidae) species as possible from the Gulf of California, south to Mazatlan, and from the Pacific coast of Baja California Sur, using previous descriptions and reported occurrences to guide this effort (Table 1). A second goal was to combine our sequence data with other corresponding Ostreidae sequences in GenBank to help clarify phylogenetic relationships and test the earlier hypothesized sister species relationship between *Ostrea lurida* and *O. conchaphila* (Polson *et al.* 2009) using more extensive taxon and geographic sampling.

Thus, this study presents sequence comparisons of two mitochondrial gene regions, 16S and COI, from specimens collected from 11 sites in the Gulf of California and along the Pacific coast of Baja California Sur, with efforts made to represent a full range of morphotypic diversity found, to answer three questions:

1. What species of oysters are present in the vicinity of the Gulf of California?
2. Where does the north/south transition between *Ostrea lurida* and *O. conchaphila* occur and is there a region of range overlap?
3. What are the phylogenetic relationships among these species, including other available data for worldwide oysters for these gene regions, and are *O. lurida* and *O. conchaphila* still supported as sister species once additional taxa are added?

MATERIALS AND METHODS

Specimen acquisition

Voucher specimens at the Santa Barbara Museum of Natural History were examined and compared to published descriptions to become familiar with the species likely to be encountered at sampling sites. After a thorough investigation of morphological characters, we used pictures and descriptions of all the possible oyster species present in the Gulf of California while on sampling trips to aid with identification. Descriptions and analyses of morphological features in the field, and later in the laboratory, are detailed in Raith (2013). This study focuses only on the molecular results.

To sample broadly across the ranges of O*strea lurida* and *O. conchaphila*, as well as to collect and characterize other co-occurring oyster species, eight sites in the Gulf of California and three sites on the Pacific side of Baja California Sur were each surveyed on the lowest low tide during several spring tide series (see Table 2 for reported tidal height on day of collection) from June 2009 to January 2010 (Fig. 2). These sites contain estuaries or bays that have been previously known to harbor oyster species of interest and/or suitable habitat. Each site was searched for suitable habitat and for the presence of oysters for the duration of the tide or until at least 10 specimens of each morphotype were collected, when feasible. In some cases, multiple habitat types were sampled at different locations within a site. Sampling was conducted only in areas that were accessible, and so subtidal populations were not taken into consideration. Each oyster was opened in the field for tentative identification using morphological features. After provisional field identification (Raith 2013), oysters were placed in individual Whirl-Paks® (Nasco; Modesto, CA) partially filled with 95% ethanol until further analysis. Our phylogenetic analyses include new sequences for 435 total specimens, including 351 new specimens collected for this study and added COI sequences for 84 specimens whose 16S or COIII sequences were previously reported by Polson *et al.* (2009). Most new vouchers have been deposited in the Santa Barbara Museum of Natural History (SBMNH), with voucher numbers specifi ed with each sequence in GenBank and in Appendix A [10.4003/006.033.0206.s1].

DNA extraction and PCR amplification

Approximately 25 mg of the adductor muscle tissue per specimen was taken for DNA extraction. After additional refined identification in the laboratory, using morphological features such as chomata, plicae, and shell color (Raith 2013), the rest of the specimen was stored in 95–100% ethanol. Muscle tissue was digested using Proteinase K and digestion buffer solution while holding the tissue in a water bath at 57 °C overnight. Subsequent steps for DNA extraction were carried out using the DNeasy Blood and Tissue kit from Qiagen (Carlsbad, CA) following the manufacturer's recommendations for extraction.

The 16S mtDNA gene region was amplified using 30 μL reactions with 2 mM $MgCl_2$, 200pM dNTPs, 0.75 Units

Table 2. Sampling localities for sequenced oysters collected for this study from Mexican localities, including sample date, sample time, tidal height, habitat type, approximate latitude and longitude and the number of samples sequenced for each gene. Identifications to species are as determined in the sequence analysis portion of this study. Sequence tallies also include specimens collected for Polson *et al.* (2009) from the vicinity of Mazatlán and sequenced for COI here. Appendix A has more details on individual vouchers in our analyses from both of our studies, including those newly sequenced from north of Mexico.

Species	Site	Date	Sampling Time	Tidal Height (m)	Habitat	Latitude	Longitude	16S seq	COI seq
Ostrea conchaphila	Mazatlán	6/19/2009	1225	-0.41	Rocky shore	23° 10.446N	106° 23.773W	1	1
Crassostrea sp. A	Mazatlán	6/19/2009	1225	-0.41	Mangrove	23° 09.181N	106° 19.699W	10	0
Saccostrea palmula	Mazatlán	6/19/2009	1225	-0.41	Intertidal	23° 10.331N	106° 21.273W	54	25
O. conchaphila	Topolobampo	6/21/2009	1530	-0.69	Oyster farm	25° 36.163N	109° 02.317W	6	3
C. corteziensis	Topolobampo	6/21/2009	1530	-0.69	Mudflat	25° 35.143N	109° 07.074W	1	0
C. sp. A	Topolobampo	6/21/2009	1530	-0.69	Mudflat	25° 35.143N	109° 07.074W	5	1
Saccostrea palmula	Topolobampo	6/21/2009	1530	-0.69	Mudflat	25° 34.940N	109° 06.915W	35	13
O. angelica	Guaymas	6/23/2009	545	-3.5	Mudflat	27° 57.804N	110° 58.251W	6	4
O. conchaphila	Guaymas	6/23/2009	545	-3.5	Oyster farm	27° 54.593N	110° 57.207W	2	1
C. sp. A	Guaymas	6/23/2009	545	-3.5	Oyster farm	27° 54.593N	110° 57.207W	4	1
Saccostrea palmula	Guaymas	6/23/2009	545	-3.5	Oyster farm	27° 54.593N	110° 57.207W	39	10
O. angelica	Puerto Peñasco	6/24/2009	940	-1.11	Oyster farm	31° 17.640N	113° 26.395W	8	6
O. conchaphila	Puerto Peñasco	6/24/2009	940	-1.11	Rocky shore	31° 20.686N	113° 38.078W	7	5
C. gigas	Puerto Peñasco	6/24/2009	940	-1.11	Oyster farm	31° 17.640N	113° 26.395W	5	0
Saccostrea palmula	Puerto Peñasco	6/24/2009	940	-1.11	Rocky Shore	31° 20.686N	113° 38.078W	30	5
O. conchaphila	Bahía Concepcion	1/13/2010	1812	-3.5	Rocky shore	26° 45.788N	111° 53.488W	9	9
Saccostrea palmula	Bahía Concepcion	1/13/2010	1812	-3.5	Rocky shore	26° 45.788N	111° 53.488W	9	4
Saccostrea palmula	Loreto	1/14/2010	1520	-2.6	Rock/pilings	26° 01.001N	111° 20.527W	5	4
O. conchaphila	La Paz	1/17/2010	1630	-1.2	Rocky shore	24° 07.404N	110° 26.265W	3	3
Saccostrea palmula	La Paz	1/17/2010	1630	-1.2	Rocky shore	24° 07.404N	110° 26.265W	19	13
O. equestris	Los Cabos	1/16/2010	1530	-2.1	Rocky shore	23° 03.586N	109° 40.234W	7	7
Saccostrea palmula	Los Cabos	1/16/2010	1530	-2.1	Rocky shore	23° 03.586N	109° 40.234W	2	1
Striostrea prismatica	Los Cabos	1/16/2010	1530	-2.1	Rocky shore	23° 03.586N	109° 40.234W	1	1
O. conchaphila	Bahía Magdalena	1/15/2010	1548	-3	Rocky shore	24° 48.047N	112° 05.737W	5	5
O. equestris	Bahía Magdalena	1/15/2010	1548	-3	Rocky shore	24° 48.047N	112° 05.737W	1	1
C. sp. A	Bahía Magdalena	1/15/2010	1548	-3	Rocky shore	24° 48.047N	112° 05.737W	2	0
Saccostrea palmula	Bahía Magdalena	1/15/2010	1548	-3	Rocky shore	24° 48.047N	112° 05.737W	1	0
Striostrea prismatica	Bahía Magdalena	1/15/2010	1548	-3	Rocky shore	24° 48.047N	112° 05.737W	1	1
O. conchaphila	Laguna San Ignacio	1/13/2010	1630	-2.7	Oyster farm	26° 47.562N	113° 09.119W	15	15
C. sp. A	Laguna San Ignacio	1/13/2010	1630	-2.7	Oyster farm	26° 47.562N	113° 09.119W	1	0
Saccostrea palmula	Laguna San Ignacio	1/13/2010	1630	-2.7	Oyster farm	26° 47.562N	113° 09.119W	3	0
O. lurida	Guerrero Negro	1/12/2010	1600	-2.5	Rocky shore	28° 02.143N	114° 07.151W	4	4
C. sp. A	Guerrero Negro	1/12/2010	1600	-2.5	Rocky shore	28° 01.153N	114° 06.256W	1	0
Saccostrea palmula	Guerrero Negro	1/12/2010	1600	-2.5	Rocky shore	28° 02.143N	114° 07.151W	2	0

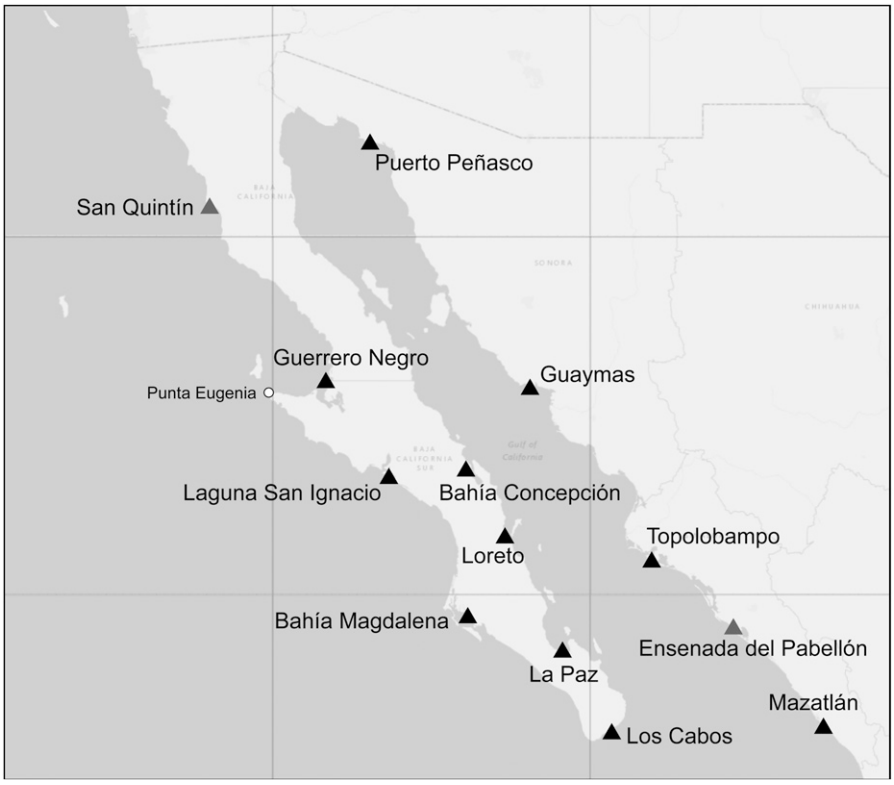

Figure 2. The 11 sampling locations for this study from Baja California Sur, Sonora, and Sinaloa, Mexico, represented by black triangles. The two previously sampled localities from Polson *et al.* (2009) are represented by gray triangles. The recognized biogeographic breakpoint of Punta Eugenia (at ~28°N) is denoted with a white circle. Base map as source as in Fig. 1.

HotStarTaq polymerase (Qiagen, Valencia, CA, USA), 1 µM forward and reverse primers, Qiagen 10X buffer (with $MgCl_2$), and 1 µL of our genomic DNA prep described above. Primers 16Sar (5′- CGC CTG TTT ATC AAA AAC AT – 3′) and 16Sbr (5′ - GCC GGT CTG AAC TCA GAT CAC GT – 3′) were used (Palumbi 1996). Thermal cycling conditions started with denaturation at 94 °C for 15 min (this extended time was needed to activate the version of HotStarTaq we used), then continuing at 94 °C for 30 sec, annealing as described next for 1 minute, and 72 °C for 1 min for 35 cycles. Final extension was 72 °C for 10 min. The annealing temperature varied according to a "touchdown" protocol described by Polson *et al.* (2009). Our first PCR cycle had an annealing temperature of 53 °C and this was decreased by 1 °C for each of next seven cycles, then another 25 cycles each had an annealing temperature of 48 °C. Later, we had similar high success of amplification with the annealing temperature remaining at 52 °C for each cycle. Our 16S PCR products were cleaned and sequenced commercially at the Duke University Institute for Genome Sciences and Policy (IGSP) core sequencing facility. Analysis of each pair of forward and reverse 16S sequences included the construction of sequence contigs using CodonCode Aligner v. 4.1.1 (http://www.codoncode.com).

The COI mitochondrial gene region was amplified using 20 µL reactions with 3 mM $MgCl_2$, 200 pM dNTPs, 1 Unit Taq polymerase (Qiagen, Valencia, CA, USA), 1 µM forward and reverse primers, Qiagen 10X buffer (with $MgCl_2$) and 20 ng of template. Primers LCOI490 (5′ - GGT CAA CAA ATC ATA AAG ATA TTG G - 3′) and HCO2198 (5′ - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3′) were used (Folmer *et al.* 1994). In cases where amplification was not successful, we were sometimes able to amplify COI by pairing HCO2198 with a different primer, veneroid-LCO (5′- YAG NAC YAA TCA TAA AGA TAT TGG - 3′; E. M. Pilgrim, unpubl.). Thermal cycling conditions started with denaturation at 94 °C for 2.5 min, continuing at 94 °C for 30 sec, 46 °C for 30 sec and 72 °C for 1 min for 35 cycles. Final extension was 72 °C for 10 min. PCR products were cleaned with Qiaquick PCR kit and sequenced using ABI Big Dye 3.1 (Carlsbad, CA, USA). Sequenced products were purified with DyeEx 96 Kits from Qiagen and run on ABI Prism 3730xl DNA Analyzer; DNA reads were assembled in Sequencher v. 4.8 (Gene Codes, Ann Arbor, MI, USA).

Relevant collection data for all vouchers for which we obtained sequences is detailed with the sequence records submitted to GenBank (accession numbers KT317088 – KT317610) and these are briefl y summarized by locality in Appendix A [10.4003/006.033.0206.s1]. This appendix also includes a listing of whether we obtained 16S or COI sequences for each of our vouchers, and also lists every 16S or COI sequence included in our analyses that was downloaded from GenBank.

DNA alignment and phylogenetic analysis

Alignment of sequences was carried out using MAFFT v. 7 (Katoh and Standley 2013) with the FFT-NS-I, E-INS-I, or Q-INS-I (slow, very slow, or extremely slow methods), depending on the alignment size, using the most refined (slowest) method that was feasible for a given set of sequences. Phylogenetic analysis was then performed on the aligned 16S

or COI sequences, or a combined gene data set, with the combined data set limited to those taxa (new or from GenBank) that had both genes available. The phylogenetic analyses employed the maximum likelihood criterion as implemented in RAxML (Stamatakis *et al.* 2008). The option of gamma model of rate heterogeneity was selected, and the best maximum likelihood tree was searched for, along with a bootstrap analysis (100 bootstrap replicates). Tree figures were produced using PAUP* v. 4.0a126 (Swofford 2002). All matrices and phylogenetic trees are deposited in Treebase (Sanderson *et al.* 1994) under Study ID 17984 and URL http//:purl.org/phylo/treebase/phylows/study/TB2:S17984.

RESULTS

Oyster species present in the Gulf of California

By sequencing the 16S ribosomal DNA (16S) and cytochrome oxidase subunit I (COI) mitochondrial markers of our collected oysters, we confirmed the presence in the Gulf of California of *Ostrea conchaphila* and at least five other co-occurring, recognized oyster species that were already reported to be present in the Gulf of California, all of family Ostreidae Rafinesque, 1815 (Tables 1 and 2). *Ostrea conchaphila* was collected at 5 sites within the Gulf of California located north and/or west of the previously documented northern range limit for *O. conchaphila* at Ensenada del Pabellon (cf. Polson *et al.* 2009) and the historic range limit at Mazatlan, Mexico. It was also found at two locations on the west coast of Baja California Sur, at Bahia Magdalena and Laguna San Ignacio (Fig. 2). Of other oyster species previously described as residing with the Gulf (Table 1), we observed the presence of *Saccostrea palmula* (Carpenter, 1857), "*Myrakeena*" *angelica*, *Striostrea prismatica*, and *Crassostrea corteziensis* (Hertlein, 1951), each at multiple sites (see Table 2), as well as an unidentified *Crassostrea* Sacco, 1897 species (*C.* species A, see discussion below). We also confirmed the known presence of the long-established exotic species, *C. gigas,* and another exotic species, for the first time in the Pacific Ocean, *O. equestris* (see discussion below). Lastly, we collected morphotypes consistent with *O. tubulifera* Dall, 1914 that grouped with *S. palmula* in our phylogenetic analyses (see discussion below).

North/south transition between *Ostrea lurida* and *O. conchaphila* and range overlap

Ostrea lurida was collected at only one site, Guerrero Negro on west coast of Baja California Sur (Fig. 2), just north and east of Punta Eugenia. In contrast, *O. conchaphila* was found at a total of 7 of the 11 field sites in this study, but not at Guerrero Negro. On the west coast of Baja California Sur, we collected *O. conchaphila* as far north as Laguna San Ignacio, just south and east of Punta Eugenia.

Phylogenetic relationships among Ostreidae species, including sister relationships

Maximum likelihood (RAxML) trees for the combined and separate 16S and COI datasets, including a broad assortment of oyster sequences from GenBank, resulted in overall similar groupings albeit with somewhat different taxa represented (Figs. 3, 4A–D, 5A–D). Because they were represented in GenBank especially by Tëmkin (2010), we were able to include a greater diversity of outgroups for our 16S data set, including more representatives of Gryphaeidae, the only other extant family besides Ostreidae within Ostreoidea.

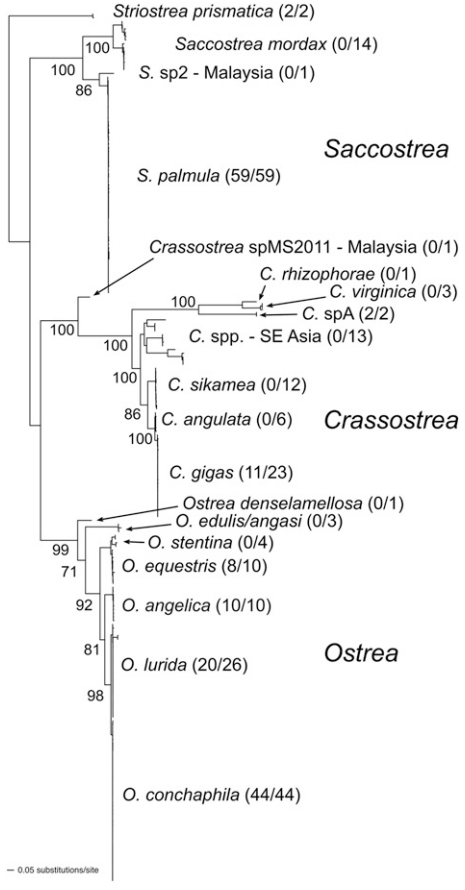

Figure 3. Best maximum likelihood tree from combined 16S and COI dataset, rooted with *Striostrea prismatica* as outgroup. For simplicity, bootstrap values are shown for selected internal nodes only. Vouchers from our study or from GenBank with both 16S and COI were included. Clear distinctions are evident between *Saccostrea*, *Crassostrea* and *Ostrea*. Numbers in parentheses indicate individuals from this study / all oysters in analysis for the corresponding taxon, including those from GenBank.

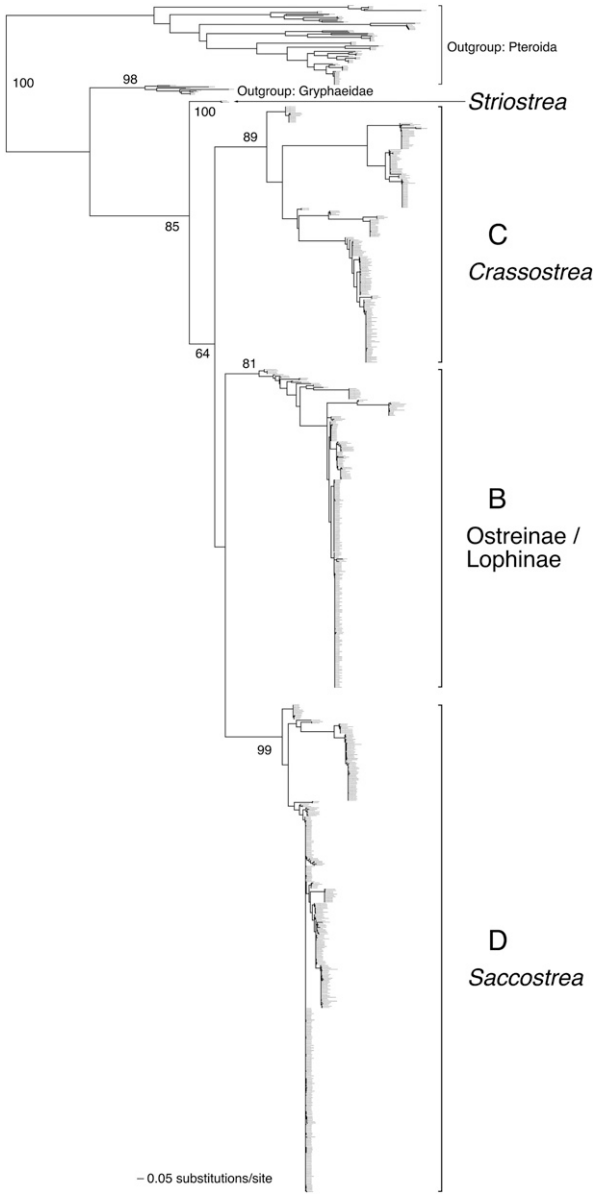

Figure 4A. Best maximum likelihood tree found for 16S only data set including all 16S sequences for this study and most of those available from GenBank, with available Gryphaeidae and selected pteroid 16S sequences employed as outgroups. As in Figure 3, the bootstrap values are shown only for selected basal internal nodes. Clear divisions with high bootstrap support are seen between *Crassostrea*, Ostreinae + Lophinae, and *Saccostrea*. See Figs. 4B-D for a more detailed presentation of selected portions of these results.

A combined data set of only individuals represented by both 16S and COI data, and using *Striostrea* Vialov, 1936 as outgroup, showed high bootstrap support (BSS) (98%) for *Ostrea lurida* and *O. conchaphila* as sister taxa (Fig. 3). "*Myrakeena*" *angelica*, here sequenced for the first time, grouped as sister to the *O. conchaphila*/*O. lurida* clade (BSS 81%). Some of our oysters that had proven difficult to identify in the field or lab grouped unambiguously with *O. equestris* downloaded from GenBank, a species not normally associated with the Gulf of California (Fig. 3 and see results below). *Saccostrea* Dollfus and Dautzenberg, 1920 and *Crassostrea* grouped independently of one other and also separately from Ostreinae + Lophinae. Notably, individuals denoted as *C.* sp. A here grouped separately from all other *Crassostrea* species (BSS 100%); it is unclear if the sequences labeled as *C.* sp. A are actually undescribed or possibly *C. columbiensis* (Hanley, 1846) (or *C. corteziensis*, see discussion below) as this species is reported as present in this region but samples from the proximity of the type locality in Ecuador were not available for inclusion in our molecular analyses.

Saccostrea palmula is the only member of its genus within our study area, but also groups separately from its worldwide congeners. Individuals initially identified as *Ostrea tubulifera* all group with *S. palmula*. Although we performed a separate analysis with the single representative of Gryphaeidae that had both 16S and COI available, we noticed that this single outgroup was highly divergent from our ingroup and its use as an outgroup and its inclusion for our 16S + COI data set tended to result in the unstable rooting of our ingroup. Instead, here we present (Fig. 3) our combined data set results with only *Striostrea prismatica* as outgroup, and this choice is justified by our 16S-only results (Fig. 4A) that had better representation of Gryphaeidae and other selected pteriomorph bivalves as outgroup sequences.

For our 16S-only results, summarized in Figure 4A, there was a clear distinction between four lineages: *Ostrea* + associated genera, *Crassostrea*, *Saccostrea*, and *Striostrea*. The first three of these are presented in more detail in Figures 4B–D. Figure 4B outlines *Ostrea* + associated genera in more detail based on 16S, again showing support for *O. conchaphila* and *O. lurida* as sister species. As in our combined gene result, together these two species were sister to "*Myrakeena*" *angelica.* Some of our other oyster sequences from the vicinity of the Gulf of California grouped together with available *O. equestris* sequences from the Western Atlantic or Caribbean where it normally occurs (Mikkelsen and Bieler 2007), and we have thus confirmed a new record for the Pacific Ocean. Collectively, all these New World *O. equestris* 16S sequences group together with (BSS 86%) Eastern Atlantic/Mediterranean oysters identified as *O. stentina* Payraudeau, 1826 (an older name than *O. equestris*) or *O. spreta* d'Orbigny, 1846, and these three nominal species are not separated when only this gene is considered, in contrast to the 16S + COI or COI-only results (see discussion).

Figure 4C outlines the 16S-only results for the *Crassostrea* grouping more closely. One individual in this study does group with 16S sequences identified as *C. corteziensis* in GenBank,

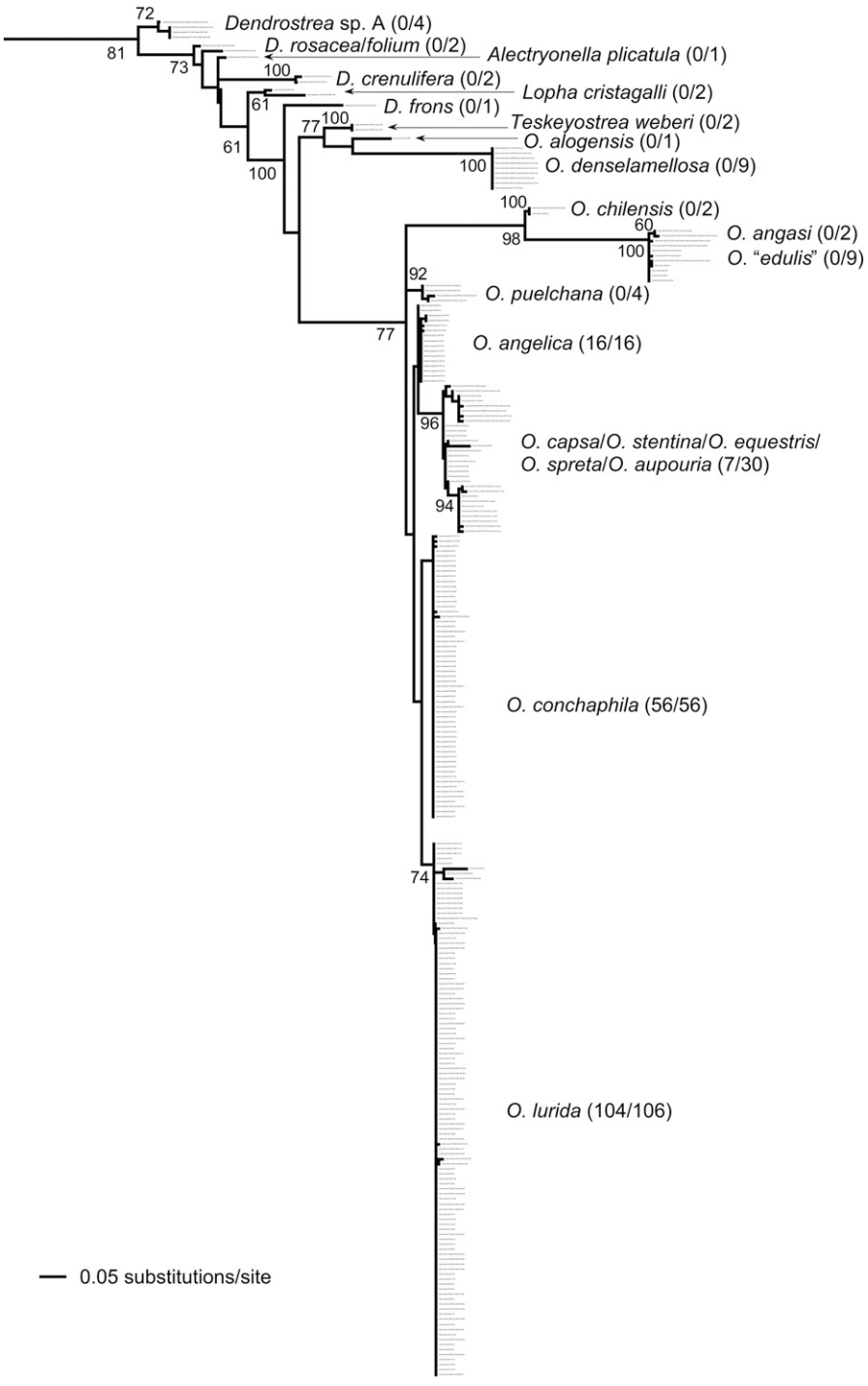

Figure 4B. Best 16S maximum likelihood tree as in Fig. 4A but showing detail of this tree for the Ostreinae + Lophinae portion. Bootstrap values are indicated for interspecific nodes only that are supported in > 50% of the 100 bootstrap replicates. Numbers in parentheses represent individuals from this study / all individuals used for the corresponding taxon. *O. conchaphila* and *O. lurida* are supported as sister taxa but with < 50% bootstrap support. *O. angelica* is supported as sister to these with 61% bootstrap support. *O. equestris* collected from the Gulf of California group with sequences collected from the Gulf of Mexico.

which groups weakly (BSS 52%) as sister lineage of the *C. gasar* Dautzenberg, 1891 group. A third clade that groups (BSS 84%) with *C. corteziensis* + *C. gasar* is itself strongly supported (BSS 99%) and referred to here as *Crassostrea* sp. A. As already suggested, the possibility remains that either this or the *C.* "*corteziensis*" clade might be *C. columbiensis* (see discussion). Note that these Gulf of California *C.* sp. A did not group as sister with *C. rhyzophorae* (Guilding, 1834) + *C. virginica* (Gmelin, 1791), as in the combined COI and 16S tree (Fig. 3), most likely because there were no COI sequences available for *C. corteziensis* or *C. gasar*.

Figure 4D outlines the 16S-only results for *Saccostrea* grouping more closely. All individuals collected in this study that group here have been identified as *S. palmula*, including morphologically diverse oysters as first noted by Polson *et al.* (2009). The combined *S. palmula* sequences from both studies had relatively little sequence variation across the Gulf of California, despite their apparent morphological plasticity. The *S. palmula* sequences were distinct from other *Saccostrea* but not supported as monophyletic with respect to at least some of the southeastern Asian *Saccostrea* spp. sequences from Lam and Morton (2006) and other studies. Instead, the Gulf of California *S. palmula* sequences were nested within a broader monophyletic grouping of *Saccostrea* species (Fig. 4D).

A maximum likelihood estimate based on all new COI data combined with most relevant GenBank COI sequences (not including some redundant sequences) is summarized in Figure 5A. A clear distinction between *Ostrea* (and associated genera), *Crassostrea*, *Saccostrea* and *Striostrea* was again evident using other available outgroups for this analysis. Figure 5B outlines the *Ostrea* clade showing support for *O. conchaphila* and *O. lurida* and as sister species (BSS 85%). Together they were again sister to "*Myrakeena*" *angelica*. There was modest bootstrap support for the *O. equestris*

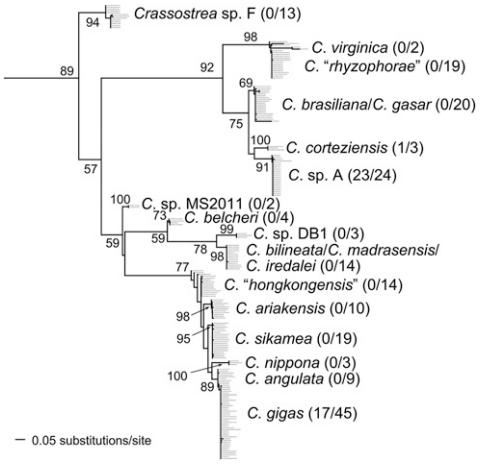

Figure 4C. Best 16S maximum likelihood tree as in Fig. 4A but with emphasis on the *Crassostrea* spp. portion. Bootstrap values are indicated for interspecific nodes supported in > 50% of the 100 bootstrap replicates. Numbers in parentheses represent individuals from this study / all individuals used, for each corresponding taxon. As has been addressed in other studies, *C. gigas* is closely associated with *C. angulata* + *C. nippona* (Seki, 1934) and some other worldwide vouchers that might represent additional species, but all of our Gulf of California vouchers for this species complex matched *C. gigas* most closely. Also note that we were able to obtain 23 sequences for *C.* sp. A but only one for *C. corteziensis*, although the species clearly group independently of each other.

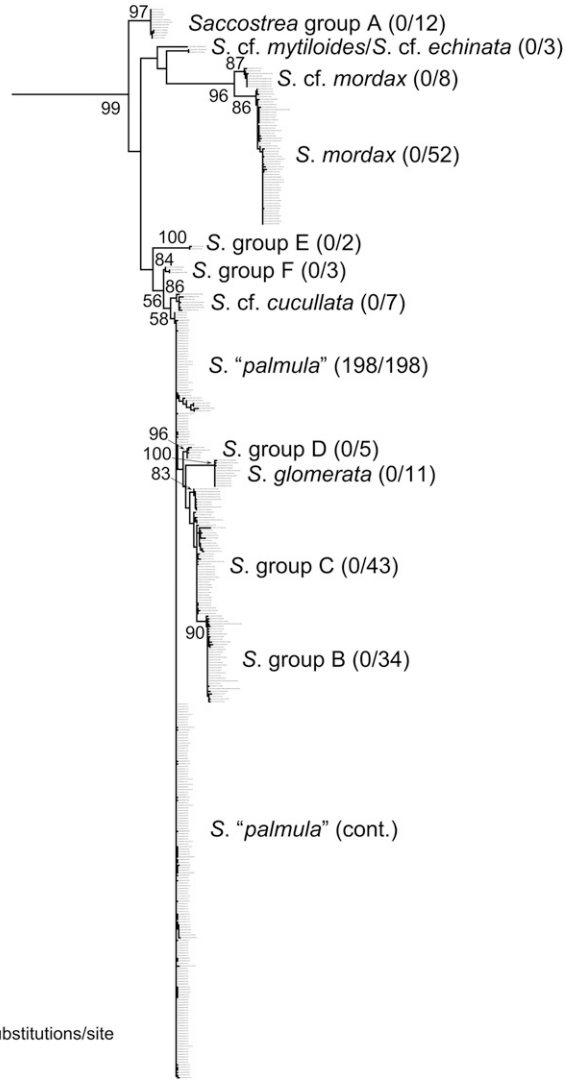

Figure 4D. Best 16S maximum likelihood tree as in Fig. 4A with emphasis on the *Saccostrea* spp. portion. Bootstrap values > 50% are shown for selected internal nodes. Numbers in parentheses represent individuals from this study / all individuals used in the tree, for each corresponding taxon. *Saccostrea palmula* exhibited relatively little haplotype diversity, including those specimens identified based on morphology as *Ostrea tubulifera* (see text). In this result, *S. palmula* is paraphyletic relative to certain individuals from the *S. cucullata* species group.

clade, including the previously discussed *O. equestris* individuals from this study.

Figure 5D outlines the *Crassostrea* group more closely showing *C. gigas* grouping together (BSS 99%) with sequences identified as *C. angulata* Lamarck, 1819 or *C.* "sp. KL2003." *Crassostrea* sp. A groups as sister to *C. gasar* (BSS 70%) but, as noted above, there are no COI sequences yet for other *Crassostrea* spp. in this group.

Figure 5C outlines the *Saccostrea* group showing *S. palmula* grouping sister to an unidentified Taiwanese species (BSS 76%). Still missing are sequences from more southern localities reported for *S. palmula*, as far south as Ecuador (Félix Pico 2007). Sequences whose GenBank identification is *S. cucullata* (Born, 1778) are unlikely to have come from a single species, with multiple haplotypes grouping independent of each other throughout the tree.

DISCUSSION

Overall, our analyses recovered *Ostrea conchaphila* and *O. lurida* as distinct sister species, as previously noted by Polson *et al.* (2009). We have newly resolved the grouping of these species together as sister lineage to a third Gulf of California species, prior to this study known as *Myrakeena angelica* (Rochebrune, 1895). *Myrakeena* Harry, 1985 has been considered the sole member of the nominal tribe, Myrakeenini Harry, 1985 (*e.g.*, Coan and Valentich-Scott 2012), but *M. angelica* has been controversial in its subfamily placement (Huber 2010). Based on the phylogenetic proximity of this species to *O. conchaphila* and *O. lurida*, we reinterpret the features

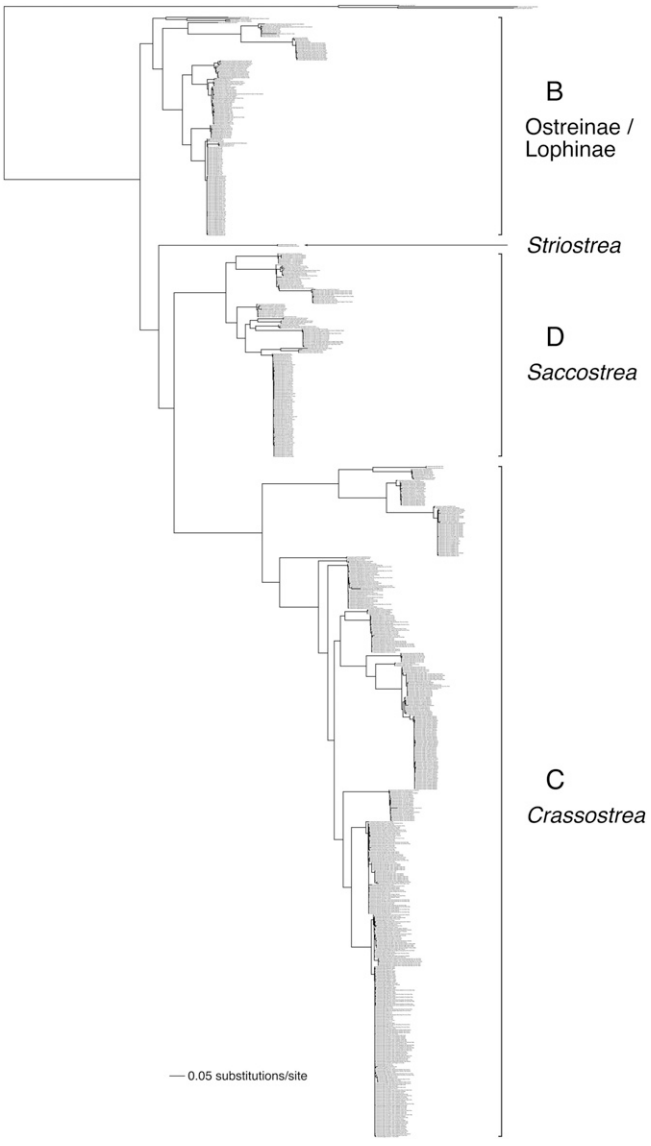

Figure 5A. Best maximum likelihood tree for COI data set only including all individuals from this study and selected COI sequences from GenBank, avoiding redundancy. The bootstrap values supporting selected basal nodes are shown. More distal outgroups used in this analysis are not shown, and the basal branch that would connect them has been shortened in length, as indicated by the paired diagonal lines. See Figs. 5B–D for a more detailed presentation of selected portions of these results. Additionally, a complete majority rule consensus tree of the 100 bootstrap replicates is presented in Appendix B [10.4003/006.033.0206.s1]. Clear divisions with high bootstrap support are seen between Ostreinae + Lophinae, *Crassostrea*, and *Saccostrea*, but these COI results did not resolve deep nodes in general, and the rooting of the ingroup differs from what was observed in the 16S results (Fig. 4A), which also had better representation of outgroup sequences.

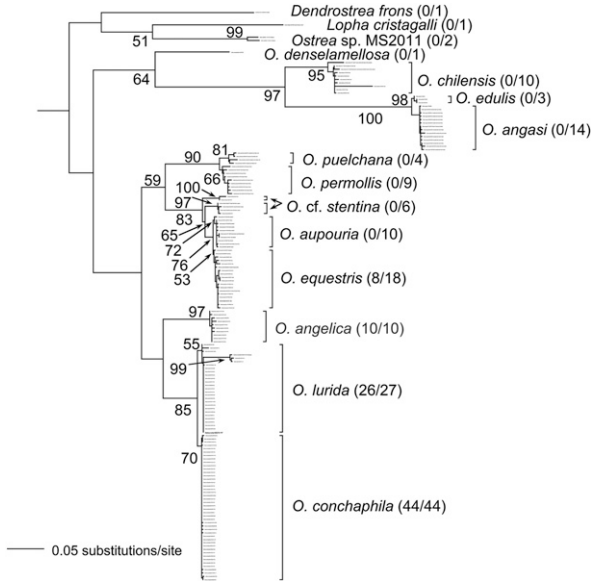

Figure 5B. Best COI maximum likelihood tree as in Figure 5A but with emphasis on Ostreinae + Lophinae portion. Bootstrap values > 50% are shown for interspecific nodes only based on 100 bootstrap replicates. Numbers in parentheses represent individuals from this study / all individuals used in the tree, for each corresponding taxon. *Ostrea conchaphila* and *O. lurida* are supported as sister taxa in 85% of the bootstrap replicates. *Ostrea angelica* is supported as sister to these in the best tree found, but with < 50% bootstrap support. Within *O. lurida*, a subclade indicated with 99% bootstrap support corresponds to our most northern (Vancouver Island) samples.

emphasized by Harry (see below) as derived for this species and here considered *Myrakeena* as a junior synonym of *Ostrea* and the species been reassigned as *Ostrea angelica*.

Ostrea conchaphila and *O. lurida* have sometimes been assigned to a separate genus or subgenus, *Ostreola* Monterosato, 1884, whose type species is *Ostrea stentina*. As reviewed by Huber (2010), the uses of *Ostreola* by Stenzel (1971) and Harry (1985) have not been accompanied by a clarified diagnosis. Coan *et al.* (2000) questioned its validity and others (Lapègue *et al.* 2006, Shilts *et al.* 2007) have considered it to be a junior synonym of *Ostrea*. Likewise, our analysis does not support any clear grouping that would warrant its further use.

Previously, it was unknown whether *Ostrea conchaphila* and *O. lurida* were sympatric between San Quintin, Baja California Sur, and Mazatlan, Mexico. We found an interesting distributional break at Punta Eugenia between these species, with no evidence of sympatry; *O. conchaphila* maintains its presence south of Punta Eugenia but extends further north into the Gulf of California than the previous northern range limit of Mazatlan, Mexico. Extending the conclusions of Polson *et al.* (2009), we found only *O. lurida* as far south as

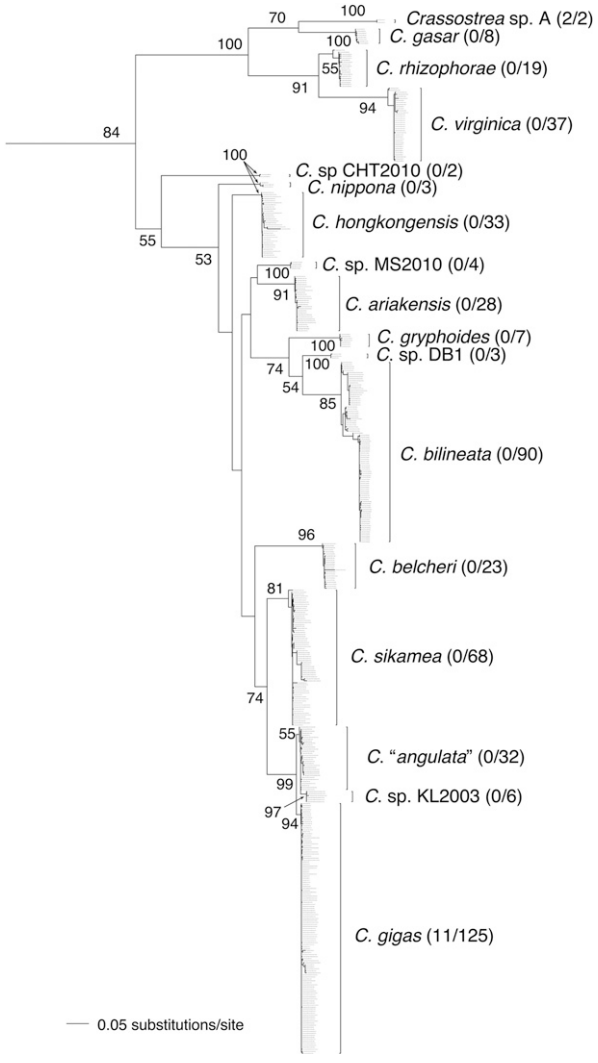

Figure 5C. Best COI maximum likelihood tree as in Figure 5A but showing detail of this tree for the *Crassostrea* spp. portion. Bootstrap values > 50% are shown for only interspecific nodes, based on 100 bootstrap replicates. Numbers in parentheses represent individuals from this study / all individuals used in the tree, for the corresponding taxon. As has been addressed in other studies, *C. gigas* is closely associated with *C. angulata* and some other worldwide vouchers that might represent additional species, but all of our Gulf of California vouchers for this species complex matched *C. gigas* most closely. Also note that we were able to obtain only two sequences for *C.* sp. A and none for *C. corteziensis*, in contrast to our 16S results (Fig. 4C).

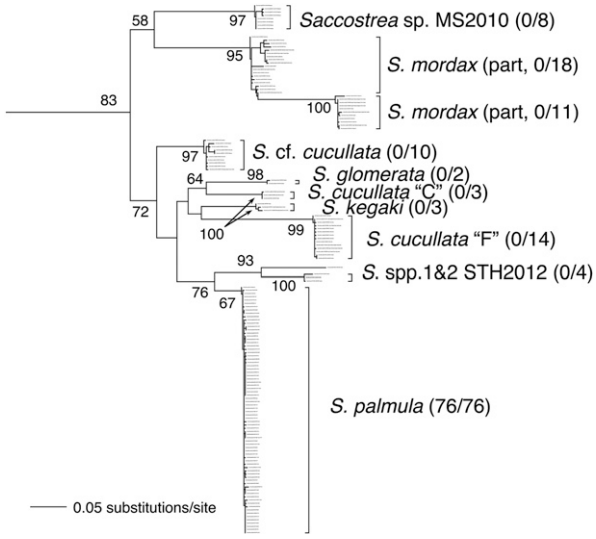

Figure 5D. Best COI maximum likelihood tree as in Figure 5A but showing detail of this tree for the *Saccostrea* spp. portion. Bootstrap values > 50% are shown for only interspecific nodes. Numbers in parentheses represent individuals from this study / all individuals used in the tree, for each corresponding taxon. *S. palmula* exhibited low haplotype diversity, including specimens that were first identified based on morphology as *Ostrea tubulifera* (see text).

Guerrero Negro, contradicting previously published southern range limits (Hertlein 1959, Harry 1985).

In addition to *Ostrea conchaphila* and *O. angelica*, we confirmed the presence of multiple other oyster species previously described from the Gulf of California, including *Saccostrea palmula*, *Striostrea prismatica*, and *Crassostrea corteziensis*, as well as an unidentified *Crassostrea* sp. A, and confirmed the known presence of the exotic species, *C. gigas* and, for the first time in the Pacific Ocean, the exotic species *O. equestris.* Lastly, we collected morphotypes consistent with *O. tubulifera* that grouped with *S. palmula*.

The Ostreidae species collected during our study, combined with those accessed from GenBank, consistently resolved into four subgroups; these correspond to the genera *Striostrea*, *Ostrea* (plus associated genera belonging to either Ostreinae or Lophinae), *Crassostrea*, and *Saccostrea*. This tentative conclusion is complicated by differences in how our ingroup, Ostreidae, was rooted by available outgroup sequences in our different analyses (see "Resolving Deep Nodes within Ostreidae" below for further details). Considering that mitochondrial sequences are not well suited to resolving such ancient basal relationships, and no relevant study employing multiple nuclear markers has yet been undertaken, we will organize this discussion to emphasize relationships within each of these Ostreidae subgroups.

Ostreinae + Lophinae

Our results are consistent with an Eastern Pacific clade consisting of three *Ostrea* species that we refer to as the *O. conchaphila* species group: *O. conchaphila*, *O. lurida*, and

O. angelica. The distributional break between the *O. lurida* and *O. conchaphila* at Punta Eugenia, with no evidence of sympatry, is interesting because past researchers have postulated differing scenarios explaining how Punta Eugenia might act as a biogeographic breakpoint. Hewitt (1981) discusses near-continuous eddies that are present north and south of Punta Eugenia, effectively separating the bodies of water on either side of the break. Gonzalez-Rodriguez *et al.* (2012) estimated that temperatures north and south of Punta Eugenia vary by an average of approximately 4 °C. Both currents and temperature could affect recruitment and retention of larvae (*i.e.*, for fishes and mollusks), subsequently influencing the makeup of adult populations. Some fishes (Bernardi *et al.* 2003, Dawson *et al.* 2006), bryozoans (Soule 1960), mollusks (Hall 1964) and other invertebrate species exhibit population breaks at Punta Eugenia. Isopods in the genus *Ligia* (Hurtado *et al.* 2010) have been shown to exhibit a northern/southern disjunction on either side of Punta Eugenia but they lack a planktonic larval stage, unlike oysters and most other marine animal groups.

Ostrea lurida and *O. conchaphila* exhibit divergence that could mirror similar divergence observed for other taxa, but further research is required to test whether their contemporary distributional break correspond to a specific vicariant event that disrupted gene flow or, alternatively, might reflect ongoing selection for alternative temperature regimes. One relevant observation is that the outer coast of the Baja California Peninsula, termed the Surian province by Valentine (1966), is characterized by an overlap of Californian and Panamic (or Panamanian) province species with the Californian species dominating in exposed rocky habitats and the Panamic species dominating estuarine and bay habitats (Valentine 1966, Kennedy 2000). Given the typical protected habitat of these oysters, our observation that the southern *O. conchaphila* is most common south of Punta Eugenia agrees with Valentine's (1966) general pattern. Because we did not sample extensively in the transition zone of Punta Eugenia, we recommend finer-scaled sampling throughout this area and the use of nuclear markers to address species presence/absence, possible hybridization, introgression and rooting patterns.

Our inclusion of COI sequences sheds further insight into phylogeographic patterns much further north as first discussed by Polson *et al.* (2009). They found that southern Vancouver Island, Canada, vouchers were distinct from all outer Pacific coast localities of *Ostrea lurida* to the south, especially for mitochondrial COIII but also a single base pair distinction in 16S. We added COI sequences for some of the same vouchers they previously studied, and found that these same Vancouver Island vouchers differed at COI sites. This is evident (Fig. 5B) from the 99% BSS for a COI subclade of *O. lurida*, which corresponds to these Vancouver Island vouchers. As in Polson *et al.* (2009), we consider this genetic distinction to be a mere phylogeographic break, not corresponding to separate species, because the Vancouver Island population was found to be nested inside *O. lurida* group, not as a reciprocally monophyletic sister lineage, and because 16S exhibits so little difference. Eernisse and coworkers (manuscript in prep.) have extended this study by also sampling Puget Sound populations and by further analysis of the extent of differentiation for COI and 16S.

Ostrea angelica is newly sequenced here and is supported as the sister lineage to *O. lurida* and *O. conchaphila*. Harry (1985) proposed *Myrakeena* as a monotypic genus for it, and Powell (2008) transferred it (as *M. angelica*) to Lophinae based on morphology. Most notably, it resembles members of Lophinae in having convex valves with deep radial plications and a long and narrow resilifer. This distinctive morphology was argued to separate it from members of *Ostrea*, which have flatter and smoother valves with short, broad resilifers (Coan and Valentich-Scott 2012). However, Inaba *et al.* (2004) and Huber (2010) have suggested *O. angelica* is nested within the subfamily, Ostreinae, whose type species is *O. edulis* Linnaeus, 1758. With some regret because of the distinguished malacologist who is commemorated by *Myrakeena*, our results suggest it is most appropriate as a junior synonym of *Ostrea* because its use would otherwise make *Ostrea* paraphyletic. Better phylogenetic resolution has revealed that the distinctive morphology of its type species is recently derived, not reflective of an ancient divergence.

Ostrea equestris, with a described range along the Atlantic coast and Caribbean (Shilts *et al.* 2007), is present in the Gulf of California. Our data show strong bootstrap support for the clade consisting of European/Mediterranean *O. stentina* and western Atlantic *O. equestris*, with similar relationships to those previously reported (Lapègue *et al.* 2006, Shilts *et al.* 2007). Kirkendale *et al.* (2004) examined whether populations in the Gulf of Mexico differed from populations on the Atlantic coast, as the species was ranging further northward than previously described. However, both regions shared similar haplotypes suggesting regular gene flow between areas and perhaps natural range expansion (Kirkendale *et al.* 2004). Eight individuals were found in this study at Cabo San Lucas or Bahia Magdalena, although not in high densities. Further sampling of this species would be useful in determining its full distribution around the Gulf of California. The sequences obtained in this study group with sequences of individuals collected from the Atlantic coast, but haplotype analyses should be carried out across its range to address competing hypotheses about its introduction into the Gulf of California, ranging from possible allopatric speciation and natural range expansion to anthropogenic introduction from either the intentional transport of oyster or unintentional transport (*e.g.*, via ballast water or ship fouling). Additional sampling

along the coast of Central America or the Panama Canal, coupled with the above-suggested haplotype analyses may distinguish among competing hypotheses about its introduction.

We found evidence that Lophinae is likely paraphyletic to Ostreinae, even with multiple unresolved relationships within this collective grouping. Despite the complications this will pose for its present morphological diagnosis, we recommend expanding Ostreinae to include Lophinae at least until better phylogenetic resolution would suggest otherwise. The Lophini tribe *sensu* Coan and Valentich-Scott (2012) have more pronounced chomata, hyote, and clasper spines than in other Ostreidae. However, when these morphological differences are considered in light of our 16S phylogenetic trees, pronounced plications and spines also occur in the *Ostrea stentina* group and in *O. angelica*.

Striostrea

Only two individuals of *Striostrea prismatica* were collected for this study, one from Bahia Magdalena and the other from Los Cabos, both in Baja California Sur, Mexico, but they group tightly in the combined and individual marker analyses with high bootstrap support suggesting that this species is distinct from others shown here. After the completion of our analyses, another *S. prismatica* was purchased from a beach vendor at Playa Cerritos, north of Mazatlan, and its identification confirmed by later sequencing (DJE, personal observation). This oyster was apparently collected on the same beach by fishermen using snorkels so this implies *S. prismatica* could be quite common in the shallow subtidal. Because the only previously available sequence for this genus is one 28S ribosomal DNA sequence for the senior synonym of its type species, *S. margaritacea* (Lamarck, 1819) (Ó Foighil and Taylor 2000), there has been little molecular data that might have resolved the uncertainty about the family or subfamily status of this genus, and no data available to test whether the four recognized species constitute a monophyletic group. For example, Harry (1985) erected the tribe Striostreini for this genus and together with *Saccostrea*. Both of these genera have prominent chomata (Coan and Valentich-Scott 2012). However, our results do not support a phylogenetic basis for such a grouping. All of our analyses have revealed that *S. prismatica* is very divergent from all other sampled Ostreidae. We propose Striostreinae new subfamily to include the single genus, *Striostrea*. Although *S. prismatica*, itself, has gone through multiple name changes and inclusions in different genera, its relationship to the type species of *Striostrea* is currently uncontroversial and it is clearly separate from the three other genera in analyses presented here, although a weakness of our proposal is the lack of 16S or COI for the type species of *Striostrea*. Our results also suggest that there are still unresolved basal relationships within Ostreidae that need to be addressed that we cannot address here, lacking data from more slowly evolving nuclear markers that would be more appropriate for resolving such ancient branching patterns.

Crassostrea

Within *Crassostrea*, specifically within a group of several nominal species (see Reece *et al.* 2008) associated with available sequences for the familiar Pacific oyster, *C. gigas*, this species lacks bootstrap support in our combined or 16S-only analyses, but has 94% BSS for its monophyly in our COI-only analysis (Fig. 5C). These results are relevant for putting our mitochondrial sequence results into perspective, because Banks *et al.* (1994) previously documented gametic incompatibility and allozyme-estimated genetic divergence between *C. gigas* and the very similar species, *C. sikamea* (Amemiya, 1928), one of the species in this group. Some sequences show long branches, suggesting that a few individuals vary greatly from others within *Crassostrea* but for unknown reasons. However, population structure was minimal even though *C. gigas* spans a wide range of localities, either native or introduced (due to its use as a fishery species). Based on sites sampled in the present study, *C. gigas* was generally uncommon except in aquaculture facilities where this species provided habitat for other oyster species (MR, personal observation).

Of note, we found two oyster species to be present on mangroves. Hertlein (1951) was apparently unaware of the nominal species, *Ostrea columbiensis* Hanley, 1846 (type locality in Santa Elena, Ecuador), when he proposed *O. corteziensis* (type locality at Bahía Kino, Gulf of California). Harry (1985) considered *C. corteziensis* to be a junior synonym of *C. columbiensis*, whereas Olsson (1961), Keen (1971), and Coan and Valentich-Scott (2012) all considered each as valid species, and they consistently listed both as present in the Gulf of California based on earlier reports. Coan and Valentich-Scott (2012) selected a neotype of *C. columbiensis* from historic material from the Ecuadorian type locality, but no sequences are available from anywhere south of the Gulf of California. Like Pérez-Enríque *et al.* (2008), we found distinct 16S clades. Our results imply additionally that they co-occur and that these likely separate species are not even sister species within *Crassostrea*. We have tentatively followed Pérez-Enríque *et al.* (2008) in our assignment of names, based solely on how our 16S sequences matched theirs, downloaded from GenBank. They did not mention the possibility that their "*Crassostrea* sp." could be *C. columbiensis* from the Gulf of California, and used Hertlein's *C. corteziensis* for oysters from localities where they have been cultured for over 35 years (Stuardo and Martínez 1975). Likewise, Torres-Rojas *et al.* (2014) referred to only "*C. corteziensis*" for oysters for a stable isotope study sampled from oyster farms throughout Sinaloa, and the presence of another

mangrove species could help clarify results in such comparative studies. We sequenced 16S as did Pérez-Enríque *et al.* (2008), and one of our vouchers (Fig. 4C) matches two of their "*C. corteziensis*" 16S sequences. In contrast, 23 of our vouchers, from multiple localities, matched one of their "*C.* sp." vouchers that we refer to as *C.* sp. A. We were only successful in obtaining two COI sequences (Fig. 5C) from any of these 24 total vouchers, both *C.* sp. based on their 16S sequence. The issue of appropriate names for distinct co-occurring apparent species within the Gulf of California must await sampling of *C. columbiensis* from near its type locality in Ecuador and other Panamic localities. Obtaining additional sequence data from COI or other gene regions could aid in resolving relationships within this relatively poorly studied portion of *Crassostrea*.

The need for multi-gene studies at more basal levels within *Crassostrea* is also apparent from the alternative rootings of Ostreidae that we observed but have not shown here, with some resolutions supporting *Crassostrea* as paraphyletic to other Ostreidae. Our results in general suggest a deep biphyletic division within *Crassostrea*, one that corresponds to two distinct mitochondrial gene orders noted by Wu *et al.* (2010). The order of genes on the mitochondrial genome of the *Crassostrea* type species, *C. virginica* (Gmelin, 1791), here along with other species on one side of the biphyletc division (Fig. 4C) informally denoted as *Crassostrea* subgroup "A," is distinct from an "unusually conserved" (Ren *et al.* 2010) gene order for *C. gigas* and five other nominal species, all representing our other *Crassostrea* subgroup "B" (Fig. 4C). Of these, *C. bilineata* (Röding, 1798) as *C. iredalei* (Faustino, 1932) and C. "sp. DB" (Wu *et al.* 2012) are most divergent from *C. gigas* within *Crassostrea* subgroup "B" (Fig. 4C) but still have a nearly identical gene orders. Likewise, we predict that *C. corteziensis* and *C.* sp. A will have a similar or even an identical mitochondrial gene order compared with *C. virginica*, because all three are members of subgroup "A." It is further possible that the two *Crassostrea* subgroups have diagnostic gene order differences, and these could also coincide with morphological distinctions noted by Harry (1985) in contrasting *C. virginica* and *C. gigas*. Salvi *et al.* (2014) have recently come to similar conclusions and have introduced a new genus, *Magallana* Salvi, Macali, and Mariottini, 2014, with type species *C. gigas*, and this corresponds well to our *Crassostrea* subgroup "B."

Previously, *Crassostrea* sp. A had been collected at Topolobampo (Perez-Enriquez *et al.* 2008), but in the present study the species was collected from Mazatlan, Topolobampo, Guaymas, Bahia Magdalena and Guerrero Negro, suggesting a wide range that spans the biogeographic breakpoint at Punta Eugenia. Much like the widespread *C. corteziensis*, *C.* sp. A also seems present in many areas (perhaps more common on rocky shores rather than in mangroves) and shows similar morphological features to its counterpart.

Saccostrea

The *Saccostrea* collected in this study consisted of only one species, *S. palmula*. For all of our analyses, this and other recognized congeners grouped as a monophyletic group that was distinctly separated from Ostreinae, *Crassostrea*, and *Striostrea.* Other studies have likewise shown multiple *Saccostrea* species as clearly distinct from *Crassostrea* (Brock 1990, Kirkendale *et al.* 2004, Lam and Morton 2006), contradicting the proposal by Lawrence (1995) to reassign *Saccostrea* species to *Crassostrea*. Our results reinforce the current consensus that such reassignment is inappropriate. Further, as already discussed above, the monophyletic grouping of *Saccostrea* species is clearly distinct from *Striostrea* in all analyses, suggesting that the inclusion of *Saccostrea* in the aforementioned Striostreinae is also not applicable. We, therefore, agree with the recent proposal by Salvi *et al.* (2014) that Saccostreinae Salvi, Macali, and Mariottini, 2014 deserves subfamily status.

Within *Saccostrea*, *S. palmula* was somewhat less separated from other included worldwide species of *Saccostrea*. This species is part of a group that should be referred to as the *Saccostrea cucullata* (Born, 1778) species group, referring to the oldest nominal species in this group, except that we found confusion in GenBank regarding the identity of sequences referred to *S. cucullata* by different authors. *Saccostrea palmula* is likely distinct from these other species, based on especially COI sequence divergence (Fig. 5D). Within *S. palmula* there is relatively little sequence divergence (Figs. 4D, 5D) across the multiple localities sampled here. While COI sequences are lacking for the above-mentioned *Saccostrea* spp. from southeastern Asia, it is interesting how much 16S sequence variation there is for those compared with the only slight (< 1%) 16S or COI sequence variation we found within *S. palmula* (Figs. 4D, 5D, calculations not shown). This disparity could imply that *S. palmula* has relatively recently colonized the Gulf of California (but see its archaeological and fossil history below), or it could mean there has been a relatively recent event that has homogenized mitochondrial sequence variation within the species (*e.g.*, genetic bottleneck or a selective sweep).

The lack of genetic variability within *Saccostrea palmula* might imply the possibility of human introduction into the Gulf of California, but this seems unlikely to us. First, *S. palmula* sequences are distinctive compared with available 16S (Fig. 4D) or COI (Fig. 5D) sequences available in GenBank. Second, any hypothetical introduction of *S. palmula* to Mexico would have need to have occurred very early, well before specimens of this species were brought as shells to England and later described in 1857 by Carpenter. Most

recorded introductions occurred later than this, such as the intentional introduction of a closely-related congener to Oahu in the Hawaiian Islands; Galtsoff (1964) observed that introduced Australian commercial oysters, *Saccostrea glomerata* (Gould, 1850) [as *Crassostrea commercialis* (Iredale and Roughley, 1933)], one of the species along with *S. palmula* within the *S. cucullata* species group (Fig. 4D), were successfully reproducing near Pearl Harbor, Oahu but were later wiped out by dredging operations associated with World War II. Third, there are reported *S. palmula* from archaeological (Feldman 1969) and fossil (Grant and Gale 1931) deposits from California to Ecuador. Such reports are always subject to some uncertainty given the difficulty of identifying this species by its shell alone. For example, Hertlein (1934) figured specimens of this species (as *Ostrea palmula*) from Pleistocene deposits at Laguna San Ignacio but cautioned that he could not rule out that they were *Ostrea angelica* instead. Still, Hertlein and Grant (1972) considered one of the earliest fossils to be described from California, the Pleiocene *Ostrea vespertina* Conrad, 1854, to be nearly indistinguishable from *Ostrea palmula* (= *S. palmula*), so the balance of evidence appears to favor *S. palmula* as a native species with a documented fossil history.

Lam (2003) and Lam and Morton (2006) have described a wide range of morphological plasticity in the *Saccostrea cucullata* species group oysters from a sampling of localities across the Indo-Pacific, considered alongside DNA sequence data. We also observed a wide range of morphologies for *S. palmula* within the Gulf of California but, as noted above, we observed very little sequence divergence. Some of our results should reduce the number of recognized species from this region. For example, individuals thought to be *Ostrea tubulifera* were identified based on conspicuous projections extending from the top valve. However, all these individuals, collected only in Mazatlan, Mexico, grouped within the Baja California *S. palmula* clade even after additional confirmatory sequencing from fresh dissections of voucher tissues. This outcome suggests *O. tubulifera* is best considered as a junior synonym for *S. palmula*. Further, Huber (2010) provides a description of "*S. tubulifera*" in his Compendium of Bivalves, implying some persisting confusion on its generic assignment and its recognition as a separate species or mere morphotype. Because of its rarity, Bernard (1983) had previously questioned whether its association with the Eastern Pacific was even valid. We were able to collect multiple individuals of this morphotype but they are not common (found at only one site in protected rocky areas) and they vary greatly in appearance from other confirmed vouchers of *S. palmula*. Additional studies clearly defining this morphotype's range and hypotheses addressing why this unique morphotype exists (*i.e.*, habitat structure, defense mechanisms) would add to the knowledge of oyster ecology.

Previous studies investigating the multiple Asian species of *Saccostrea* have shown considerable haplotype diversity among them (Lam and Morton 2003, 2004, 2009). For example, Lam and Morton (2006) found seven distinctly different lineages within the Indo-West Pacific, referred to them as *S. cucullata* A-G. These lineages appear independent of each other but are intermingled with other species such as *S. glomerata* (Gould, 1850), *S. mordax* (Gould, 1850) (which also shows two distinct haplotypes), and *S. kegaki* Torigoe and Inaba, 1981 (Lam and Morton 2006). Sequences from other authors have added to this diversity, as is evident in our more inclusive analysis.

There are still phylogenetic relationships that need to be addressed within *Saccostrea*. For instance, the 16S dataset suggests *S. palmula* is paraphyletic to some of its Asian congeners. We recognize that this result combined with the considerable morphological plasticity found for *S. palmula* are potential confounding issues that could affect its current separate species status. Still, it does appear that the *S. palmula* sequences are at least distinctive in comparison to other closely allied Asian species in the *S. cucullata* species group. Despite the large number of sequences we have obtained for the Gulf of California, our data set is insufficient to address the phylogenetic relationships within this species group, and a few worldwide species of *Saccostrea* that are considered valid have not yet been sampled.

Within *Saccostrea* there do appear to be up to three separate lineages apart from the *S. cucullata* group. Most of these available sequences in GenBank have been identified by the submitting authors as *S. mordax*, even though recent authors have considered this to be a junior synonym of *S. scyphophilla* (Peron and Lesueur, 1807). Therefore, phylogenetic relationships within *Saccostrea* remain partly unresolved but the incorporation of additional species and markers is expected to aid in clarifying relationships within this genus.

Resolving deep nodes within Ostreidae

Depending upon which outgroups were used in reference to the Ostreidae ingroup, the results varied, especially for the combined 16S + COI and COI-only analyses. For instance, in the COI-only analysis, the Ostreinae + Lophinae group varied in placement within Ostreidae when the outgroup composition was changed in analyses; if Gryphaeidae (the only other available family in Ostreoida) plus Pteroidea (an arbitrarily selected more distal outgroup selected from the extremely diverse Pteriomorpha, to which Ostreoida belongs) were both used as outgroups, Ostreidae tended to root with Ostreinae + Lophinae as basal (Fig. 5A) or sometimes with a paraphyletic *Crassostrea* as basal, the latter result also observed in some of our 16S + COI results with only a single available Gryphaeidae sequence set. This lack of stability could suggest that the available outgroup COI sequences

for Ostreidae might be too divergent, so that they tend to be attracted to long branches within the ingroup. It likely also reflects that this particular rapidly evolving mitochondrial marker is better at resolving the tips of the tree rather than the basal nodes. In the analyses shown here, the outgroup Gryphaeidae is especially divergent in its COI sequences, and this appears to influence the stability of rooting for both the COI-only analysis and the combined 16S + COI analysis; the latter analysis was additionally confounded by having only three Gryphaeidae sequences for COI. The Gryphaeidae 16S sequences are less divergent from our ingroup than is the case for COI, judging from their shorter internal branch lengths connecting them to our ingroup, relative to those within our ingroup (Fig. 4A). For this reason, and because there are more taxa within Gryphaeidae that have been sequenced for 16S than for COI, the rooting observed for the 16S analysis was also used for our 16S + COI analysis.

It is important to note that although our COI results appear to conflict with the 16S results in the inferred basal relationships, the conflicting branching pattern is not strongly supported in our COI analysis. Basal nodes generally lacked support in our COI and combined 16S + COI analyses, with the latter likely influence by the inclusion of COI sequences. For example, in our COI-only analysis (Fig. 5A), all of the nodes connecting our four main ingroup groupings have < 50% bootstrap support, and the Ostreinae/Lophinae grouping likewise has < 50% bootstrap support. Such lack of bootstrap support for basal nodes is not surprising given the rapid pace of COI evolution. For this reason COI is more often employed for phylogeographic comparisons or DNA barcoding, not for resolving ancient phylogenies. Because of this outgroup divergence, and noting there is only a single Gryphaeidae voucher for both 16S and COI, rooting of the combined tree was based on the rooting observed in the 16S analysis. These different rooting patterns are interesting and important, but the rapidly evolving mitochondrial 16S and especially COI markers used here cannot address basal relationships. Using a broader set of nuclear genes on these species would produce useful data in addressing this issue.

Both Ó Foighil and Taylor (2000) and Kirkendale *et al.* (2004) have previously used a nuclear gene, 28S ribosomal DNA, along with at least 16S to infer deep relationships across Ostreidae. Salvi *et al.* (2014) used a selection of these sequences combined with new nuclear ITS2 sequences to come to similar conclusions. Our analyses have much denser taxon sampling but are still largely congruent with the results of these previous studies. As in these, we support a monophyletic grouping of Ostreinae and Lophinae and the separation of *Saccostrea* from *Crassostrea*. Ó Foighil and Taylor (2000) and Kirkendale *et al.* (2004) reported the *C. virginica* group apart from the *C. gigas* group but without strong bootstrap support. Salvi *et al.* (2014) reported strong support for both Crassostreinae and a deep split within the subfamily but included only 10 Crassostreinae taxa in their combined data set. We found only weak support for *Crassostrea* monophyly (Fig. 3, Fig. 4A), with low bootstrap values possibly reflecting our much more inclusive taxon sampling and exclusive use of mitochondrial data, but agreed in finding a deep divergence within Crassostreinae. Likewise, Ó Foighil and Taylor (2000) found a different species of *Striostrea* grouping weakly with *Saccostrea*, whereas *Striostrea prismatica* was basal in at least our 16S analysis (Fig. 4A) where we had the best representation of outgroups. These are fascinating alternative hypotheses that deserved a modern multi-locus and broad taxon sampling analysis of phylogeny.

CONCLUSION

Ostrea lurida and *O. conchaphila* were resolved as sister taxa as in the Polson *et al.* (2009) 16S phylogenetic estimate, even with use of more comprehensive datasets covering additional geographic areas and with the addition of COI sequences. The two species show no evidence of sympatry and, based on sequence comparisons, both species extend further than their previously published ranges (Hertlein 1959, Harry 1985) — *O. conchaphila* further north and *O. lurida* further south. Although many additional species from the Gulf of California were added to this phylogeny, extensive work remains to be completed in order to understand the full ranges of species that are described but have not been identified with molecular techniques. For example, *Undulostrea megodon* (Hanley, 1846) should be examined in field surveys and subsequently added to molecular phylogenies. Also, the confusion involving the distribution and identity of *Crassostrea corteziensis* and *C. columbiensis* needs to be addressed. Further, subtidal populations of oysters were not taken into consideration here, and the region spanning Punta Eugenia was not extensively sampled. Both avenues may lead to interesting findings concerning species composition and interactions. The discovery of the likely non-native *O. equestris* in the Gulf of California has important conservation implications, with its impact on native species still in need of study. Lastly, some progress has been made in identifying four separate lineages within Ostreidae that have been provisionally treated as separate subfamilies, setting the stage for future multi-locus nuclear gene analyses still needed to resolve basal relationships within Ostreidae.

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