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

Meredith Raith¹, Danielle C. Zacherl¹, Erik M. Pilgrim², and Douglas J. Eernisse¹

¹Department of Biological Science, California State University Fullerton, Fullerton, California 92834-6850 U.S.A.
²U.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 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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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 co-workers (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.

<table>
<thead>
<tr>
<th>Species as Reported Here</th>
<th>Authority, Date</th>
<th>Other Genera</th>
<th>Geographic Range</th>
<th>Previous Molecular Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ostrea angelica</em></td>
<td>Rochebrune, 1895</td>
<td>Myrakeena</td>
<td>Gulf of California to Ecuador</td>
<td>No</td>
</tr>
<tr>
<td><em>Ostrea conchaphila</em></td>
<td>Carpenter, 1857</td>
<td>Ostreola</td>
<td>Gulf of California to Panama</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Ostrea lurida</em></td>
<td>Carpenter, 1864</td>
<td><em>Monocostrea</em> Orton, 1928</td>
<td>Northern Pacific to Gulf of California</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Ostrea tubulifera</em></td>
<td>Dall, 1914 (Hanley, 1846)</td>
<td>Saccostrea</td>
<td>Gulf of California</td>
<td>No</td>
</tr>
<tr>
<td><em>Crassostrea columbiaenis</em></td>
<td></td>
<td>Ostrea</td>
<td>Gulf of California, Ecuador</td>
<td>None from near type locality</td>
</tr>
<tr>
<td><em>Crassostrea corteziensis</em></td>
<td>(Hertlein, 1951)</td>
<td><em>Ostrea</em></td>
<td>Gulf of California to Panama</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Crassostrea gigas</em></td>
<td>(Thunberg, 1793)</td>
<td><em>Dendostrea</em> Swainson, 1835, <em>Ostrea</em></td>
<td>Gulf of California to Ecuador</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Saccostrea palmula</em></td>
<td>(Carpenter, 1857)</td>
<td><em>Mytilus</em> Linnaeus, 1758, <em>Pycnodonte</em> Fischer von Waldheim, 1835</td>
<td>Gulf of California to Ecuador</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Hyotissa hyotis</em></td>
<td>(Linnaeus, 1758)</td>
<td><em>Ostrea</em></td>
<td>Gulf of California to Peru</td>
<td>No</td>
</tr>
<tr>
<td><em>Striostrea prismatica</em></td>
<td>(Gray, 1825)</td>
<td><em>Ostrea</em></td>
<td>Gulf of California to Peru</td>
<td>No</td>
</tr>
<tr>
<td><em>Undulostrea megodon</em></td>
<td>(Hanley, 1846)</td>
<td><em>Lopha</em></td>
<td>Gulf of California to Peru</td>
<td>No</td>
</tr>
</tbody>
</table>
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 Ostrea 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 COIIII 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 specified 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 MgCl₂, 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Site</th>
<th>Date</th>
<th>Sampling Time</th>
<th>Tidal Height (m)</th>
<th>Habitat</th>
<th>Latitude</th>
<th>Longitude</th>
<th>16S seq</th>
<th>COI seq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ostrea conchaphila</td>
<td>Mazatlán</td>
<td>6/19/2009</td>
<td>1225</td>
<td>-0.41</td>
<td>Rocky shore</td>
<td>23° 10.446N</td>
<td>106° 23.773W</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Crassostrea sp. A</td>
<td>Mazatlán</td>
<td>6/19/2009</td>
<td>1225</td>
<td>-0.41</td>
<td>Mangrove</td>
<td>23° 09.181N</td>
<td>106° 19.699W</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Saccostrea palusula</td>
<td>Mazatlán</td>
<td>6/19/2009</td>
<td>1225</td>
<td>-0.41</td>
<td>Intertidal</td>
<td>23° 10.331N</td>
<td>106° 21.273W</td>
<td>54</td>
<td>25</td>
</tr>
<tr>
<td>O. conchaphila</td>
<td>Topolobampo</td>
<td>6/21/2009</td>
<td>1530</td>
<td>-0.69</td>
<td>Oyster farm</td>
<td>25° 36.163N</td>
<td>109° 02.317W</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>C. cortesiensis</td>
<td>Topolobampo</td>
<td>6/21/2009</td>
<td>1530</td>
<td>-0.69</td>
<td>Mudflat</td>
<td>25° 35.143N</td>
<td>109° 07.074W</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C. sp. A</td>
<td>Topolobampo</td>
<td>6/21/2009</td>
<td>1530</td>
<td>-0.69</td>
<td>Mudflat</td>
<td>25° 35.143N</td>
<td>109° 07.074W</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Saccostrea palusula</td>
<td>Topolobampo</td>
<td>6/21/2009</td>
<td>1530</td>
<td>-0.69</td>
<td>Mudflat</td>
<td>25° 34.940N</td>
<td>109° 06.915W</td>
<td>35</td>
<td>13</td>
</tr>
<tr>
<td>O. angelica</td>
<td>Guaymas</td>
<td>6/23/2009</td>
<td>545</td>
<td>-3.5</td>
<td>Oyster farm</td>
<td>27° 54.593N</td>
<td>110° 57.207W</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>O. conchaphila</td>
<td>Guaymas</td>
<td>6/23/2009</td>
<td>545</td>
<td>-3.5</td>
<td>Oyster farm</td>
<td>27° 54.593N</td>
<td>110° 57.207W</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Saccostrea palusula</td>
<td>Guaymas</td>
<td>6/23/2009</td>
<td>545</td>
<td>-3.5</td>
<td>Oyster farm</td>
<td>27° 54.593N</td>
<td>110° 57.207W</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>O. conchaphila</td>
<td>Puerto Peñasco</td>
<td>6/24/2009</td>
<td>940</td>
<td>-1.11</td>
<td>Rocky shore</td>
<td>31° 20.686N</td>
<td>113° 38.078W</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>C. gigas</td>
<td>Puerto Peñasco</td>
<td>6/24/2009</td>
<td>940</td>
<td>-1.11</td>
<td>Oyster farm</td>
<td>31° 17.640N</td>
<td>113° 26.395W</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>O. conchaphila</td>
<td>Bahia Concepcion</td>
<td>1/13/2010</td>
<td>1812</td>
<td>-3.5</td>
<td>Rocky shore</td>
<td>26° 45.788N</td>
<td>111° 53.488W</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Saccostrea palusula</td>
<td>Bahia Concepcion</td>
<td>1/13/2010</td>
<td>1812</td>
<td>-3.5</td>
<td>Rocky shore</td>
<td>26° 45.788N</td>
<td>111° 53.488W</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Saccostrea palusula</td>
<td>Loreto</td>
<td>1/14/2010</td>
<td>1520</td>
<td>-2.6</td>
<td>Rock/pilings</td>
<td>26° 01.001N</td>
<td>111° 20.527W</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>O. conchaphila</td>
<td>La Paz</td>
<td>1/17/2010</td>
<td>1630</td>
<td>-1.2</td>
<td>Rocky shore</td>
<td>24° 07.404N</td>
<td>110° 26.265W</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Saccostrea palusula</td>
<td>La Paz</td>
<td>1/17/2010</td>
<td>1630</td>
<td>-1.2</td>
<td>Rocky shore</td>
<td>24° 07.404N</td>
<td>110° 26.265W</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>O. aequus</td>
<td>Los Cabos</td>
<td>1/16/2010</td>
<td>1530</td>
<td>-2.1</td>
<td>Rocky shore</td>
<td>23° 03.586N</td>
<td>109° 40.234W</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Saccostrea palusula</td>
<td>Los Cabos</td>
<td>1/16/2010</td>
<td>1530</td>
<td>-2.1</td>
<td>Rocky shore</td>
<td>23° 03.586N</td>
<td>109° 40.234W</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Striostrea prismatica</td>
<td>Los Cabos</td>
<td>1/16/2010</td>
<td>1530</td>
<td>-2.1</td>
<td>Rocky shore</td>
<td>23° 03.586N</td>
<td>109° 40.234W</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O. conchaphila</td>
<td>Bahia Magdalena</td>
<td>1/15/2010</td>
<td>1548</td>
<td>-3</td>
<td>Rocky shore</td>
<td>24° 48.047N</td>
<td>112° 05.737W</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>O. aequus</td>
<td>Bahia Magdalena</td>
<td>1/15/2010</td>
<td>1548</td>
<td>-3</td>
<td>Rocky shore</td>
<td>24° 48.047N</td>
<td>112° 05.737W</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. sp. A</td>
<td>Bahia Magdalena</td>
<td>1/15/2010</td>
<td>1548</td>
<td>-3</td>
<td>Rocky shore</td>
<td>24° 48.047N</td>
<td>112° 05.737W</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Saccostrea palusula</td>
<td>Bahia Magdalena</td>
<td>1/15/2010</td>
<td>1548</td>
<td>-3</td>
<td>Rocky shore</td>
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<td>112° 05.737W</td>
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<td>112° 05.737W</td>
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<td>1</td>
</tr>
<tr>
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<td>1/13/2010</td>
<td>1630</td>
<td>-2.7</td>
<td>Oyster farm</td>
<td>26° 47.562N</td>
<td>113° 09.119W</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>C. sp. A</td>
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<td>1630</td>
<td>-2.7</td>
<td>Oyster farm</td>
<td>26° 47.562N</td>
<td>113° 09.119W</td>
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<tr>
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<td>1630</td>
<td>-2.7</td>
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<tr>
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<td>1/12/2010</td>
<td>1600</td>
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<td>28° 02.143N</td>
<td>114° 07.151W</td>
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<tr>
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<td>28° 02.143N</td>
<td>114° 07.151W</td>
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</table>
HotStarTaq polymerase (Qiagen, Valencia, CA, USA), 1 μM forward and reverse primers, Qiagen 10X buffer (with MgCl₂), 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 MgCl₂, 200 pM dNTPs, 1 Unit Taq polymerase (Qiagen, Valencia, CA, USA), 1 μM forward and reverse primers, Qiagen 10X buffer (with MgCl₂) and 20 ng of template. Primers LCO1490 (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 Qiagen 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 briefly 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 MAFFFT 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 Bahía 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 Têmkin (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.
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,
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*

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**Figure 4B.** Best 16S maximum likelihood tree as in Fig. 4A but showing detail of this tree for the Ostreinae + Lophaeinae 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.
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...
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 Ostreaangelica. Ostrea conchaphila and O. lurida have sometimes been assigned to a separate genus or subgenus, Ostreola Monterosato, 1884, whose type species is Ostreastentina. 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 Ostreaconchaphila 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 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 [10.4003/006.033.020.pdf]. 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. Ostreaconchaphila and O. lurida are supported as sister taxa in 85% of the bootstrap replicates. Ostreaangelica 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.

Figure 5C. Phylogeny of Gulf of California oysters.
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 resilifier. This distinctive morphology was argued to separate it from members of Ostrea, which have flatter and smoother valves with short, broad resilifiers (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 prismatic* 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. prismatic* 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. prismatic* could be quite common in the shallow subtidal. Because the only previously available sequence for this genus is one 285 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. prismatic* is very divergent from all other sampled Ostreidae. We propose Striostreinae new subfamily to include the single genus, *Striostrea*. Although *S. prismatic*, 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 at Bahía Kino, Gulf of California). While 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-Enrique 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-Enrique 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-Enrique 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 biphyletic 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. iredadai (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-Enrique 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. palma. 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 Striostreae 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. palma 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 palma is likely distinct from these other species, based on especially COI sequence divergence (Fig. 5D). Within S. palma 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. palma (Figs. 4D, 5D, calculations not shown). This disparity could imply that S. palma 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 palma might imply the possibility of human introduction into the Gulf of California, but this seems unlikely to us. First, S. palma sequences are distinctive compared with available 16S (Fig. 4D) or COI (Fig. 5D) sequences available in GenBank. Second, any hypothetical introduction of S. palma to Mexico would have needed 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 anglica* instead. Still, Hertlein and Grant (1972) considered one of the earliest fossils to be described from California, the Pleocene *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 Pterioidea (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 
attacked 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 group-
ing likewise has < 50% bootstrap support. Such lack of boot-
strap 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 barcod-
ing, 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 rela-
tionships. Using a broader set of nuclear genes on these 
sepies 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 monophy-
letic 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 monophy-
ly (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 representa-
tion of outgroups. These are fascinating alternative hypothe-
ses 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 addi-
tional 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 identi-
fied 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. eques-
tris 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 
our separate lineages within Ostreidae that have been provi-
sionally 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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