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YOU SAY *CONCHAPHILA*, I SAY *LURIDA*: MOLECULAR EVIDENCE FOR RESTRICTING THE OLYMPIA OYSTER (*OSTREA LURIDA* CARPENTER 1864) TO TEMPERATE WESTERN NORTH AMERICA

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ABSTRACT The western North American bivalve mollusc known as the Olympia oyster, long known as *Ostrea lurida* Carpenter 1864†, is a historically exploited native species that has been largely displaced by larger nonnative oysters. There is much renewed interest in documenting and restoring its native populations and recent successful culturing has attracted a specialty market for these oysters. Yet its name was called into question when it was synonymized with *O. conchaphila* Carpenter 1857, an oyster whose type locality is Mazatlán, Sinaloa, Mexico. Others have considered it more plausible that the Olympia oyster is a more northern species, distinct from *O. conchaphila*, but morphological or molecular evidence either way has been lacking. Here we used a molecular approach to test the single *versus* two-species hypotheses with samples from Sinaloa, Mexico, near the type locality of *O. conchaphila* (Mazatlán, Mexico), and samples from Willapa Bay, WA, the type locality of *O. lurida*, as well as samples from intermediate locations. Based on our combined and separate analyses of two mitochondrial DNA (mtDNA) markers, 16S ribosomal RNA (16S) and cytochrome oxidase III (CO3), native *Ostrea* from Sinaloa, Mexico are reciprocally monophyletic with a clade from multiple other localities between Baja California, Mexico and British Columbia, Canada, including Willapa Bay, WA. Corrected pairwise sequence comparisons for 16S indicate these two groups last shared a common ancestor 1.5–3.9 mya (2.06% sequence divergence). Based on these results and assuming that the Sinaloa group represents the true *O. conchaphila,* molecular evidence supports *O. conchaphila* and *O. lurida* as separate species. Posthoc morphological comparisons uncovered no significant support for morphological distinction between the two taxa, underscoring the difficulty associated with using morphology alone to distinguish closely related oyster species. Despite the present lack of any morphological diagnostic differences for separating these nominal species, the molecular data are not consistent with the synonymy of the species and support the reinstatement of *O. lurida* from all the localities north of central Baja California.

KEY WORDS: Olympia oyster, *Ostrea conchaphila*, *Ostrea lurida*, Ostreidae, systematics, phylogeny, cryptic species

INTRODUCTION

Many marine taxa are known to exhibit a high degree of phenotypic plasticity that challenges systematic biologists and creates problems for conservation efforts. Knowlton (1993) described the abundance of cryptic marine species, which are characterized as being almost impossible to distinguish based on morphology alone. Species definitions have been the subject of many debates (Donoghue 1985) and, in recent decades, even the most popular species concepts have been criticized, such as the biological species concept (Mishler & Donoghue 1982, Donoghue 1985, Cracraft 1987, Kluge 1990) and the phylogenetic species concept (Avise 2004). Regardless of one's species definition, the use of molecular markers has enabled scientists to identify previously cryptic species and to resolve taxonomic inconsistencies (Knowlton 1993, Simison & Lindberg 1999, Dawson & Jacobs 2001, Crummett & Eernisse 2007, Shilts et al. 2007, Hewson 2008).

Recent efforts to restore populations of the Olympia oyster‡, *Ostrea conchaphila*, some involving transplants of oysters between estuaries separated by large distances, have been conducted despite lingering uncertainty about the oyster's true identity. The taxonomic problem is whether *Ostrea lurida* Carpenter 1864, the name once widely used for the Olympia oyster, should be considered a junior synonym of *O. conchaphila* Carpenter 1857. This synonym was proposed by Harry (1985) without explanation but is presumed to be based on morphological examination of museum specimens combined with the notion that the species was more widely ranging than previously thought. Prior to Harry's study, *O. lurida* and *O. conchaphila* were thought to be separate species, with the range of *O. conchaphila* from Mazatlán, Mexico to Panama (Dall 1914) and the range of *O. lurida* from Sitka, AK, USA to Cabo San Lucas, Baja California Sur, Mexico. Neither species was thought to exist in the contiguous coastline between the two species' ranges, including all of the Gulf of California, Mexico (Dall 1914). An exception (although not addressing the Gulf of California) was Hertlein's (1959) discussion of a possible transition zone between these species based on intermediate-appearing oysters from San Diego, CA and northern Baja California, Mexico, which would necessitate a considerable northern extension of the range of *O. conchaphila*. After Harry (1985) proposed this was all a single species, some (e.g., Baker 1995) have questioned the

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†The taxonomy of the Olympia oyster has been in dispute since Harry (1985) proposed synonymy of *Ostrea lurida* Carpenter 1864 and *Ostrea conchaphila* Carpenter 1857. Polson et al. 2009 provide molecular evidence that the Olympia oyster refers to the nominal species, *Ostrea lurida* Carpenter 1864. In view of their genetic data, and for consistency, the original taxon, *Ostrea lurida*, is used throughout this volume to refer to the Olympia oyster, which is distributed from approximately Baja California (Mexico) to southeast Alaska.

‡The common name as sanctioned by the American Fisheries Society is Olympia oyster, though other names are commonly used including, "native oyster," "California oyster," "Yaquina oyster."

synonymy whereas others have indiscriminately used one name or the other, mostly in reference to oysters from Washington or British Columbia.

A separate issue is Harry's (1985) revival of *Ostreola* Monterosato 1884 (type species *Ostrea stentina* [Payraudeau, 1826]), which he also extended to include *O. conchaphila*. Subsequent morphological (Coan et al. 2000) and molecular (Kirkendale et al. 2004, Lapègue et al. 2006, Shilts et al. 2007) studies have not supported *Ostreola* as distinct from *Ostrea*. In particular, Shilts et al. (2007) have called for the dissolution of *Ostreola* and reassignment to *Ostrea*.

Until the present study, no one has tested Hertlein's (1959) predicted zone of overlap in southern California and along the Baja California peninsula, and this information would be especially useful considering ongoing native oyster restoration efforts. For example, government agencies such as NOAA and nongovernmental organizations such as The Nature Conservancy are interested in expanding restoration projects to locations south of San Francisco Bay, CA (Dick Vander Schaaf, The Nature Conservancy, pers. comm.) to include Hertlein's (1959) zone of overlap.

Given the high phenotypic plasticity of these oysters, the use of molecular markers applied in a geographic context could potentially be useful for resolving whether the native oyster is one or more species. Parallel studies with oysters in different ocean basins (Lam & Morton 2006, Lapègue et al. 2006, Shilts et al. 2007, Wang & Guo 2008) have already demonstrated the usefulness of molecular markers. Here, for the first time, we have tested Harry's (1985) synonymy of the two species with DNA sequence comparisons, followed by morphological comparisons that were *posthoc* with respect to groupings identified by our molecular analyses. We analyzed two mitochondrial DNA (mtDNA) markers, 16S ribosomal DNA (16S) and cytochrome oxidase subunit III (CO3), for oysters collected at multiple localities between Willapa Bay, WA (the type locality of *O. lurida*) and Mazatlán, Mexico (the type locality for *O. conchaphila*). Our sampling sites also included seven localities between southern California, USA and the Pacific coast of Baja California, Mexico to test for the presence of Hertlein's (1959) predicted zone of overlap.

MATERIAL AND METHODS

Specimen Acquisition

All samples collected whose sequences were used in the analysis, along with localities and sample numbers, are listed in Table 1. Samples from Willapa Bay, WA were collected and identified as the Olympia oyster by Russell Rogers (WA Department of Fish and Wildlife) and were given to us as soft tissue only. One ingroup sequence *O.* "*conchaphila*" from Barkley Sound, B.C., Canada, and all outgroups used in the analyses were downloaded from GenBank (Table 2). Some of the names listed in Table 2 reflect recent taxonomic changes, such as favoring *Ostrea* over *Ostreola* for those species commonly assigned to the latter (see Introduction). When collected in the field, all oysters were opened and then immediately preserved in 95% ethanol. We randomly selected a subset of samples collected from each site for analysis, and these were each given unique voucher labels. Because samples from Willapa Bay, WA were soft tissue only, the entire sample was extracted.

DNA Extraction and "Touchdown" Polymerase Chain Reaction (PCR)

For the DNA extraction and purification, we used DNeasy tissue kits from Qiagen (Valencia, CA) and followed the manufacturers' recommendations. For the tissue, approximately 25 mg of the adductor muscle was dissected and digested in a Proteinase-K and lysis buffer bath overnight at 56°C. Double-stranded products of partial 16S were amplified *via* PCR with universal animal primers (Palumbi 1996): 16Sar: 5′—CGC CTG TTT ATC AAA AAC AT—3′, 16Sbr: 5′—GCC GGT CTG AAC TCA GAT CAC GT—3′. Double-stranded products of CO3 were amplified *via* PCR with primers modified for oysters (P. Baker unpublished): CO3F: 5′— AAA AGT TCA AAG CGG TCT TA —3′, CO3R: 5′— AGC TAA CAT ACG AAC AAG GC —3′.

A HotStarTaq kit from Qiagen was used to prepare 50 µL reactions for amplification of 16S and CO3. Each reaction included 4.0 µL of 25 mM $MgCl_2$ (CO3 only), 5 µL of 10 × buffer, 5 µL of dNTPs (10 mM), 2 µL of primer (1 µL forward/1 µL reverse, 10 µM), 1 µL genomic DNA template, 0.25 µL HotStarTaq polymerase, and deionized sterile water to bring the reaction volume to 50 µL per reaction. Reactions were carried out *via* a GeneAmp PCR System 2400 (Perkin-Elmer) thermocycler. HotStarTaq activation and initial denaturation temperatures were 95°C for 15 min (a step specific to the HotStarTaq) and then 94°C for 1 min thereafter (denaturation only). For 16S, initial annealing temperatures of 53°C for eight cycles of 1 min each (decreased by 1°C each cycle) were used; final annealing temperatures of 48°C for 25 cycles of 1 min each were used and an extension temperature of 72°C was used for 1 min per cycle and for the final extension period of 10 min, followed by a hold at 10°C until samples were removed to storage at 5°C. For CO3, initial/final annealing temperatures of 50/45°C for 30 cycles of 1 min each were used and an extension temperature of 72°C was used for 1 min per cycle and for the final extension period of 10 min. PCR product was visualized with agarose-gel electrophoresis (1.5%), ethidium bromide staining, and UV illumination to confirm successful amplification of the mtDNA region of interest.

DNA Sequencing Alignment and Analyses

PCR product cleanup and sequencing were performed commercially at the Duke University Institute for Genome Sciences and Policy DNA sequencing facility (Duke IGSP, Durham, NC). Each sequence contig was assembled from a pair of sequences from opposite strands using CodonCode Aligner software (CodonCode Corp, Dedham, MA). All new sequences were submitted to GenBank and have been assigned the accession numbers FJ768501-FJ768589 (16S) and FJ768590-FJ768673 (CO3).

All sequences were aligned by eye with the use of MANIA software (D. L. Swofford & D. J. Eernisse, unpublished). There was very little sequence length variation, and so alignment was generally not problematic. The DNA sequence alignments of 16S and CO3 were 502 and 417 sites, respectively. Heuristic searches were performed for the combined, and separate, data set, or sets, using maximum parsimony (MP) and maximum likelihood (ML) optimality criteria. Altogether, 161 specimens were in our combined data set, including 111 for 16S and 86 for CO3. 16S sampling emphasized the contrast between *Ostrea*

TABLE 1.

Sampling localities for sequenced western North American oysters with the number of samples sequenced for each gene. Names are as determined in this study. 16S sampling emphasized the contrast between *Ostrea conchaphila* and *O. lurida* and tested for Hertlein's (1959) proposed region of overlap whereas CO3 sequencing emphasized tests of phylogeographic structure within *O. lurida*.

Species	Locality (Code)	GPS Coordinates	16S Seqs	CO3 Seqs
Ostrea lurida	Ladysmith Harbor, Vancouver Id., B.C. (LH)	N 48°59.321′, W 123°48.241′	2	4
Ostrea lurida	Ahmah Island, Vancouver Id., B.C. (AI)	N 48°56.911′, W 125°5.239′	1	3
Ostrea lurida	Willapa Bay, WA (WB)	N 46°29.916′, W 124°01.766′	21	5
Ostrea lurida	Yaquina Bay, OR (YB)	N 44°36.807′, W 124°4.799′	0	5
Ostrea lurida	Coos Bay, OR (CB)	N 43°19.313′, W 124°23.156′	0	5
Ostrea lurida	Humboldt Bay, CA (HB)	N 40°45.225′, W 124°12.92′	0	5
Ostrea lurida	Tomales Bay West, CA (TB)	N 38°13.763′, W 122°58.556′	0	5
Ostrea lurida	Drakes Estero, CA (DE)	N 38°0.302′, W 123°0.307′	0	4
Ostrea lurida	Pt. San Quentin, San Francisco Bay, CA (SF)	N 37°56.508′, W 122°30.315′	0	4
Ostrea lurida	Elkhorn Slough, Monterey Co., CA (ES)	N 36°48.631′, W 121°46.996′	0	3
Ostrea lurida	Mugu Lagoon. CA (ML)	N 34°6.104′, W 119°5.882′	0	4
Ostrea lurida	Alamitos Bay, CA (AL)	N 33°44.264′, W 118°7.114′	5	5
Ostrea lurida	Newport Bay, CA (NB)	N 33°37.173′, W 117°53.542′	4	5
Ostrea lurida	Aqua Hedionda, CA (AH)	N 33°08.579′, W 117°19.370′	4	3
Ostrea lurida	Batiquitos Lagoon, CA (BL)	N 33°08.579′, W 117°19.370′	4	5
Ostrea lurida	Scripps Pier, La Jolla, CA (LJ)	N 32°52.087′, W 117°15.307′	5	1
Ostrea lurida	Mission Bay, CA (MB)	N 32°47.673′, W 117°13.468′	4	5
Ostrea lurida	San Diego Bay, CA (SD)	N 32°42.505′, W 117°10.260′	4	3
Ostrea lurida	Bahía San Quintin, Baja California (SQ)	N 30°27.858′, W 115°57.834′	4	3
Ostrea conchaphila	Ensenada del Pabellon (N), Sinaloa (EdPn)	N 24°30.063′, W 107°41.245′	2	2
Ostrea conchaphila	Ensenada del Pabellon (S), Sinaloa (EdPs)	N 24°28.843′, W 107°33.396′	7	5
Saccostrea spp.	Urias Lagoon, Mazatlán, Sinaloa (Maz)	N 23°12.33′, W 106°24.315′	24	0
Saccostrea sp.	Bahía de Kino, Sonora (BdK)	N 28°45.55′, W 111°54.8′	1	0
Crassostrea gigas	Bahía de Kino, Sonora (BdK)	N 28°45.55′, W 111°54.8′	1	0

conchaphila and *O. lurida* and tested for Hertlein's (1959) proposed region of overlap whereas CO3 sequencing emphasized tests of phylogeographic structure within *O. lurida*. The larger 16S data set included 22 sequences downloaded from GenBank, whereas only two of these (*Crassostrea virginica* and *C. gigas*) were available for inclusion in our CO3 data set. The other 84 CO3 sequences were either *Ostrea* "*lurida*" ($n = 24$ from 8 widely separated localities) or *O. conchaphila* ($n = 7$ from 2 localities in close proximity). To combine the two data sets, 50 ingroup specimens with CO3 sequences but without 16S sequences were deleted from the analysis (i.e., the combined analysis included the same 111 taxa as in the 16S-only analysis). This avoided confounding tree searching by having a "missing data" set of taxa from one data set that shared no characters with a similar set from the other.

For the MP criterion, we used the PAUP* 4.0b10 software package (Swofford 2003). All gaps were treated as missing data to avoid treatment of adjacent gaps as separate characters. The two-part search strategy used by Kelly and Eernisse (2008) was used to improve the efficiency of searching, specifically to avoid having searches become unnecessarily trapped in nonoptimal tree islands. The first part involved 1,000 replicate searches with only 10 trees held per replicate, keeping all minimum length trees found. Then the second part involved swapping on all minimum-length trees found with the trees-held restriction removed, starting with trees already in memory and with maxtrees set to 1,000. Branch robustness was estimated by 1,000 bootstrap replicate searches, each with 10 replicate random stepwise addition sequence heuristic searches.

For the ML criterion, we used genetic algorithm for rapid likelihood inference (GARLI) v. 0.951 (Zwickl 2006). Because this program searches for trees with a fast and accurate genetic algorithm whose speed is not substantially influenced by the number of taxa, it can handle much larger data sets than are conventionally analyzed with the ML criterion. The version of GARLI used has automatic model selection, which is optimized for the particular data set during analysis. (A newly released v. 0.96 of the program adds additional model assignment options that were not previously available.) Ten separate GARLI searches were performed and the tree selected was the one with the most optimal (closest to zero) final likelihood score. Branch robustness was estimated with 100 bootstrap replicate searches using GARLI. To check whether these results agreed with a more conventional ML search, we performed such a search with PAUP* with the number of taxa greatly reduced to 29 taxa to allow the feasible completion of searches. For this analysis, we used FINDMODEL (Tao et al. 2005) to identify an appropriate model for each data set. Based on this selection, we performed a heuristic search for the combined data set using the general time-reversible plus gamma (GTR + Γ) model with optimality criterion set to minimum evolution and tree-bisection-reconnection (TBR) branch swapping algorithm.

Corrected pairwise differences within and between species were calculated using Arlequin version 3.11 (Excoffier et al. 2005) with 1,000 permutations, transitions and transversions equally weighted, and acceptable level of missing data set to 5 percent. Corrected pairwise differences were used to estimate intra and interspecific percent sequence divergence by dividing

TABLE 2.

GenBank sequences used in analyses (16S except as noted).

Species	Locality in GenBank	Accession
*Ostrea lurida**	Barkley Sound, BC, Canada	AF052071
Outgroups		
Ostrea algoensis	Port Alfred, South Africa	AF052062
Ostrea angasi	St. Helen, Tasmania, Australia	AF052063
Ostrea auporia	Hauraki Gulf, New Zealand	AF052064
Ostrea chilensis	Hauraki Gulf, New Zealand	AF052065
Ostrea denselamellosa	South Korean hatchery stock	AF052067
Ostrea edulis	France	DQ280032
Ostrea equestris	Big Pine Key, FL, USA	AF052074
Ostrea puelchana	San Matias Gulf, Argentina	AF052073
Ostrea spreta	Argentina	DQ640402
Ostrea weberi	Florida, USA	AY376601
Alectryonella plicatula	Pohnpei, Micronesia	AF052072
Cryptostrea permollis	Florida panhandle, USA	AF052075
Dendostrea folium	Aitutaki, Cook Islands	AF052069
Dendostrea frons	Carrie Bow Cay, Belize	AF052070
Lopha cristagalli	Guam	AF052066
Saccostrea commercialis	Moreton Island, Queensland, Australia	AF353100
Saccostrea cuccullata	Australia	AF458901
Crassostrea ariakensis	China	AY160757
Crassostrea rhizophorae	Brazil	AJ312938
Crassostrea gigas	Bangor, U.K. (cultured)	AJ553903
Crassostrea gigas (CO3)	None listed (native to northwestern Pacific)	AF177226
Crassostrea virginica	None listed (native to northwestern Atlantic)	AF092285
Crassostrea virginica (CO3)	Delaware Bay, USA	AY905542

*This ingroup sequence from Genbank, *O. ''conchaphila,''* was reidentified in the current study as *O. lurida*.

the mean pairwise differences between species by the number of base pairs used in the pairwise analysis.

Morphological Description

To verify the identities of the samples collected from and around Mazatlán, several morphological characters previously identified as potentially diagnostic within the *Ostreidae* (Keen 1958, Torigoe 1981, Harry 1985, Castillo Rodriguez & García-Cubas 1986, Quayle 1988, Coan et al. 2000) were considered. The morphologies of the samples from Mexico were examined in a ''blind'' manner, with no *a priori* knowledge of the molecular outcomes. Characters were examined in selected specimens from mainland Mexico from which DNA was extracted (Table 3).

Post-hoc examinations of nine specimens (Table 3) from Hertlein's (1959) zone of overlap (Southern California, USA and Baja California, Mexico) were also completed and compared with the blind-examined specimens in an effort to validate findings from the molecular analyses. Also examined was the shell of a syntype specimen of *Ostrea lurida* from the Redpath Museum at McGill University, Montreal, Canada. The specimen, RMM 125, is presumed to be part of the type lot described by Philip Carpenter. The only reason that we have avoided designating this specimen the lectotype for *O. lurida* is our lack of knowledge of other syntype specimens. The syntypes of *Ostrea conchaphila* are housed at The Natural History Museum in London, England. Images of one of these specimens selected by Kathie Way, as a candidate for lectotype designation, were obtained, but too late to include in this manuscript.

Shell and soft-tissue characters used in the morphological examinations are described individually later. For illustrations of some of these characters see Harry (1985).

Chomata Sweep and Chomata Shape

Chomata are small (often under 1 mm), repeating, tubercule or denticle-like shell features on the internal shell margin on either side of the hinge. Pits (catachomata) lie in the left, or attached valve, and matching denticles (anachomata) lie in the right valve (Torigoe 1981). Chomata shape may be rounded (less than twice as long as wide) or elongate (more than twice as long as wide).

Chomata extend (sweep) in a series from the hinge area around the margin towards the ventral side. Chomata can be a useful character to distinguish the genus *Crassostrea* from *Ostrea*, as *Crassostrea* are believed to lack this character (Harry 1985). In some taxa (e.g., *Saccostrea*) the chomata extend all the way to the ventral margin (100% sweep), but, in other taxa, the chomata extend only a fraction of that distance.

Plicae

Plicae (plications) are matching folds in both shell valves that are expressed in a zigzag manner, particularly near the ventral shell margin, so that the valves close precisely. Individual plica-like folds can be mimicked by the shell conforming to substratum topology.

Shell Color

Shell color, both internal and external, is sometimes listed as a character for oysters, especially for *Crassostrea* (e.g., Keen 1958, Quayle 1988, Coan et al. 2000), where the color is typically described as chalky white, but these same sources admit its high variability. Furthermore, shell color can be altered or eliminated by weathering and preservation techniques (Patrick Baker, pers. obs.). Descriptions of *Ostrea conchaphila* (and *lurida*) often note an olive-green interior color (e.g., Keen & McLean 1971, Hertlein 1959).

Other Shell Characters

External shell sculpture, in the form of foliations or spines, has been described for some taxa (Torigoe 1981). Secondary mantle retractors occur in the genus *Saccostrea* and appear on the shell as a series of small oval muscle scars paralleling the pallial line (Torigoe 1981).

TABLE 3.

Morphological characters examined among a subset of samples used in the molecular analyses (see Table 1 and Figure 1 for locality codes in the first column).

Specimen	ID Field*	ID Laboratory**	ID Molecular§	Chomata Sweep	Chomata Shape	Plicae	External Color	Mantle Tentacles	Anal Papilla
BdK 0701	*O. conchaphila*	*Crassostrea* sp.[a]	*Crassostrea* sp.	None	Absent	No	No	Monomorphic	NA
BdK 0702	*O. conchaphila*	*Crassostrea* sp.[a]	*Saccostrea* sp.	<50%	Round	Yes	No	Polymorphic	Internal
Maz 0503	*O. conchaphila*	*Saccostrea* sp.[a]	*Saccostrea* sp.	100%	Elongate	Yes	Yes	Monomorphic	None
Maz 0510	*O. conchaphila*	Unresolved[a]	*Saccostrea* sp.	<50%	Round	Yes	No	Monomorphic	None
Maz 0512	*O. conchaphila*	Unresolved[a]	*Saccostrea* sp.	<50%	Round	Yes	No	Polymorphic	Simple
EdPn 0702	*O. conchaphila*	*O. conchaphila*[a]	*O. conchaphila*	<50%	Round-elongate	Yes	Yes	Polymorphic	Simple
EdPs 0701	*O. conchaphila*	*O. conchaphila*[a]	*O. conchaphila*	<50%	Round-elongate	Yes	No	Monomorphic	None
EdPs 0703	*O. conchaphila*	*O. conchaphila*[a]	*O. conchaphila*	<50%	Round-elongate	Yes	Yes	Polymorphic	Simple
EdPs 0704	*O. conchaphila*	*O. conchaphila*[a]	*O. conchaphila*	<50%	Round-elongate	Yes	Yes	Polymorphic	Simple
SD 0601	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round	Yes	Yes	Polymorphic	NA
SD 0603	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round	Yes	Yes	Polymorphic	Simple
AH 0505	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round	Yes	Yes	Polymorphic	NA
NB 0601	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round	No	No	Polymorphic	Simple
NB 0602	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round	No	No	Polymorphic	Simple
SQ 0504	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round	Yes	Yes	Polymorphic	NA
SQ 0505	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round	Yes	Yes	Polymorphic	Simple
LJ 0503	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round	No	No	monomorphic	NA
LJ 0505	*O. conchaphila*	*O. conchaphila*[b]	*O. lurida*	<50%	Round-elongate	No	No	polymorphic	NA

* Taxon ID based upon field identification using external morphological characters.
** Taxon ID based upon both ([a]) pre and ([b]) posthoc laboratory examination of internal and external morphological characters.
§ Taxon ID after molecular results; *Saccostrea* sp. is likely *S. palmula*, the only Panamic member of this genus.

Soft Tissue Characters

Torigoe (1981) and Harry (1985) used mantle tentacles and the morphology of anal papillae as diagnostic characters although Coan et al. (2000) questioned their value and, likewise, Baker (pers. obs.) observed that preservation techniques might change their appearance. Therefore, characters were reduced to polymorphism of tentacles on the inner two mantle folds (those with the most distinct tentacles), and the presence or absence of an external anal papilla.

RESULTS

Molecular Analyses

Trees that were optimal for the maximum parsimony (MP) and maximum likelihood (ML) optimality criteria, for the combined and separate 16S and CO3 datasets, all resulted in similar topologies: a northern group (*O. ''lurida''* from British Columbia to Baja California) and a southern group (*O. ''conchaphila''* from EdPn and EdPs in Sinaloa, combined as EdP hereafter) are reciprocally monophyletic with respect to all other included taxa. Variation among the first 1,000 MP trees found is adequately represented by one of these and was selected arbitrarily (Fig. 1). The sampling scheme for 16S ($n = 111$) and CO3 ($n = 84$) data sets differed considerably in the localities sampled (see Methods and Table 1). Only 36 of the 86 taxa with CO3 data were included in our combined analysis of 111 total taxa (Fig. 1 and Fig. 2). Analysis of the separate CO3 data set with either only these 36 included taxa or else with the complete 86-taxon CO3 data set produced results entirely consistent with analysis of the 16S data set and the combined set. For example, for our MP analysis, the nodes for each focal species or for both sister species together in the CO3-only data set are supported with high (>95%) bootstrap support. These results agree with the 16S-only and combined sets, though the latter produced somewhat lower bootstrap support (e.g., Fig. 1). The MP and ML bootstrap support values mostly agreed with each other, but with somewhat lower support for nodes with ML. Results of a PAUP* search using the ML criterion, with a necessarily reduced set of taxa, were consistent with those from the more extensive GARLI analysis (Fig. 2). This implies that the particular topological results emphasized here were independent of the optimality criterion (MP or ML) or ML approach (PAUP* or GARLI) used. The best MP and ML trees did differ somewhat in the topology of the most proximal outgroups for *O. conchaphila* + *O. lurida* (Fig. 1 and Fig. 2, respectively). In our best ML tree, a greater proportion of the total branch length is found in the separation of our ingroup and the most proximal outgroups (Ostreinae + Lophinae) from our more distal outgroups from *Saccostrea* and *Crassostrea* (both in Crassostreinae). Although Ostreinae is paraphyletic to Lophinae in our best trees (as indicated by the quotation marks around Ostreinae in Fig. 2A), there is no strong bootstrap support for such nesting of Lophinae within Ostreinae, so it is possible that further resolution will support their reciprocal monophyly.

The bootstrap support supporting the monophyly of *O. lurida* and *O. conchaphila* are each high for both MP and ML (Figs. 1 and 2B). However, the joining of these as sister species is more equivocal. Our best trees for MP and ML resolves them as sister taxa but with low bootstrap support. The bootstrap proportions supporting the node joining *O. conchaphila* + *O. lurida* is higher for MP (69%) than for ML (43%). The only alternative grouping observed for MP and ML with >10% bootstrap support had *O. lurida* as sister taxon of all other Ostreinae + Lophinae species, including *O. conchaphila*. This

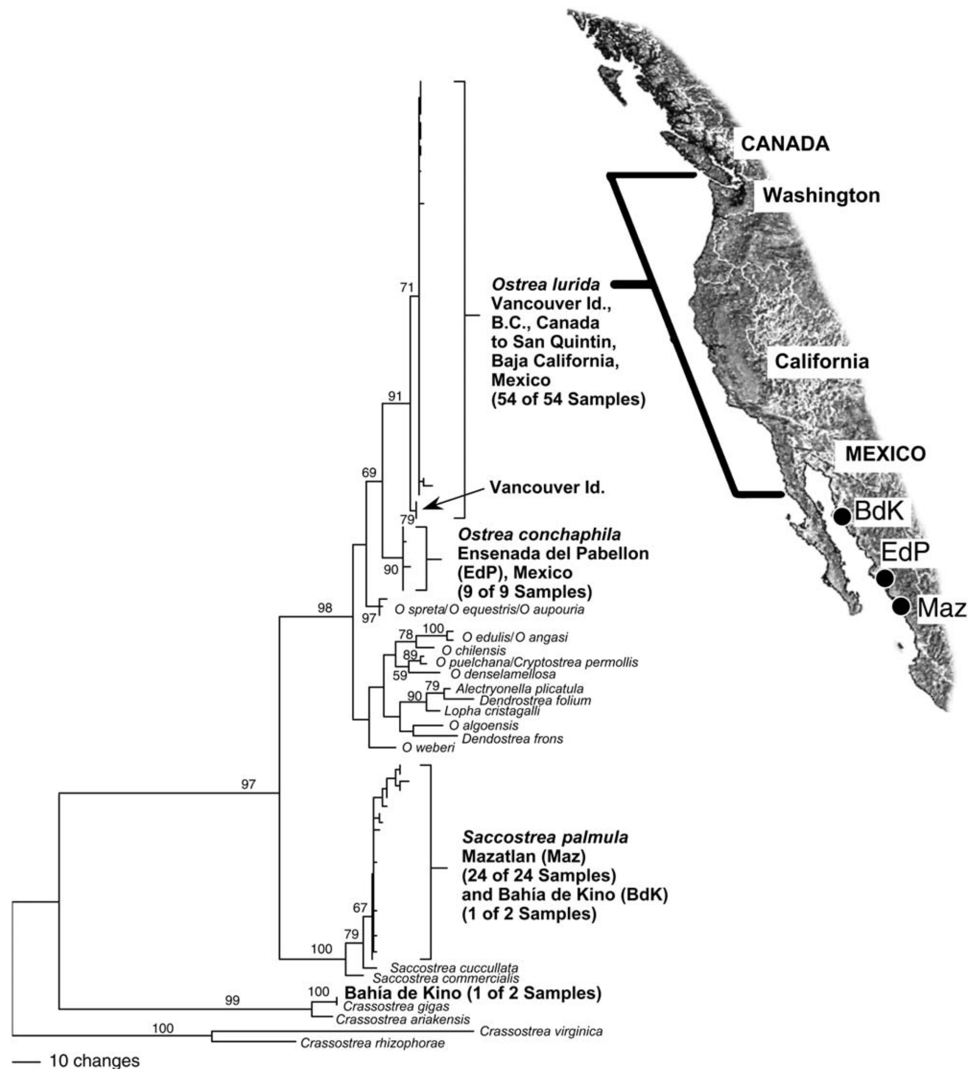

Figure 1. One of 1000 most parsimonious trees found based on the 111-taxon combined 16S ($n = 111$) and CO3 ($n = 36$) dataset. This was very similar to the ML (Fig. 2) and 16S MP results (not shown). Numbers above nodes represent MP bootstrap values. Bootstrap support values > 50% were generally rare for intraspecific nodes, and these have not been shown except for the case of *Ostrea lurida*, which is influenced by phylogeographic pattern in the CO3 data set (Figs. 3–4). Numbers in parentheses indicate the number of samples from a given locality. The map is modified from a NASA image.

arrangement was supported in 11% of the MP bootstrap replicates and 35% of the ML replicates.

The average percent sequence divergence between paired combinations of 16S sequences for *O. conchaphila versus O. lurida* was 2.06%. Samples with 16S only from Mazatlán (Maz) and Bahía de Kino (BdK) did not group with either *Ostrea* species. All Maz samples and one BdK sample grouped with *Saccostrea* spp., whereas the other BdK sample grouped with *Crassostrea* spp. (almost certainly *C. gigas*, assuming the sequence from GenBank is correctly identified as from this species). Independent morphological examinations only partially supported their assignment to these genera as described later. We used morphology to identify one of the specimens (see later) as *S. palmula* (Carpenter, 1857), and this was later resolved as *Saccostrea* with DNA. Because this is the only recognized species of *Saccostrea* from the Panamic region of the eastern Pacific (P. Valentich-Scott, pers. comm.), we have provisionally assigned all of the specimens resolved with DNA as *Saccostrea* to this species.

Separate analysis of the complete CO3 data set (as well as especially our MP combined data set analysis) supported a phylogeographic separation within *O. lurida*. Ignoring autapomorphic substitutions in six of the 84 total ingroup taxa, the entire CO3 data set reduces to the variable sites summarized in Figure 3 and the corresponding haplotype network in Figure 4. In particular, sites for either side of the southern end of Vancouver Island (B.C., Canada) were identical to each other but separated from Willapa Bay (southwestern Washington, USA). All other *O. lurida* sites from Oregon, CA, and Baja California, Mexico were identical to the Willapa Bay site, ignoring autapomorphies. The southern and northern haplotype groups within *O. lurida* are equally separated from the much more distant *O. conchaphila*, with each having an identical nucleotide state in common with *O. conchaphila*, relative to the other at two sites. Because two highly divergent CO3 sequences for *Crassostrea* spp. were the only available outgroups for our CO3 data set, these did not provide a useful rooting for inferring whether these alternative matching states

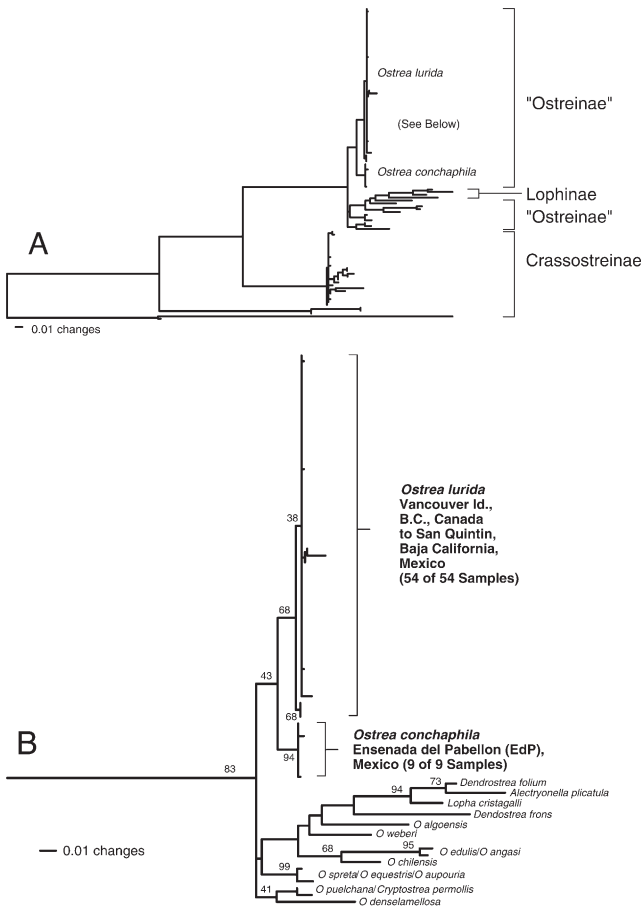

Figure 2. The tree with the best maximum likelihood (ML) score estimated by the program GARLI in 10 separate searches. A. Entire total evidence data set corresponding to the parsimony result in Figure 1. Note the proportionally greater internal branch length separating Crassostreinae from "Ostreinae" + Lophinae in this ML result, compared with one of the most parsimonious trees (Fig. 1). Results within *Saccostrea* and *Crassostrea* (Crassostreinae) were similar to those in Figure 1. B. Portion of the same ML result, emphasizing "Ostreinae" + Lophinae. Numbers above nodes are bootstrap values based on 100 replicate bootstrap searches in GARLI. Bootstrap values < 50% have been provided for selected nodes for comparison with the *parsimony* bootstrap results (Fig. 1).

are plesiomorphic or derived. Because of this lack of proximal outgroups and because *O. conchaphila* is relatively far from *O. lurida*, the rooting depicted in Figure 4 for *O. conchaphila* midway between the Vancouver Island and more southern haplotype group is arbitrary.

The phylogeographic contrast discovered for CO3 was not seen in the three 16S sequences obtained from the Vancouver Island populations *versus* the other 108 more southern sequences. This is probably best explained by the generally slower substitution rate of the 16S gene region.

Morphological Examinations

Independent Morphological Identification of Mainland Mexico Samples

The findings from the independent morphological examinations of samples from Mexico were in agreement with the molecular results in most cases (Table 3). All samples from two adjacent sites within Ensenada del Pabellon, Sinaloa, Mexico were tentatively identified as *Ostrea conchaphila* based on the presence of a rounded or slightly elongate chomata that extended <50% along the internal shell margin on either side of the hinge (Table 3); this was in agreement with the molecular results. In contrast, one sample from Mazatlán (Maz 0503) was identified as *Saccostrea palmula* based on the presence of chomata all the way around the shell, a purple interior margin and the absence of anal papilla and secondary mantle retractor scars. The identifications of the other two samples from Mazatlán could not be positively resolved using morphological characters. All three Mazatlán samples grouped with *Saccostrea* sp. in the molecular results. From Bahia de Kino, one specimen (BdK 0701) was identified as *Crassostrea* sp. based on the absence of chomata and the thick, chalky shell, and this finding was congruent with the molecular results. Despite the presence of chomata in the other BdK sample examined (BdK0702), this sample was also tentatively identified as *Crassostrea* sp. based on the thick, chalky shell (Table 3), however, this sample grouped with *Saccostrea* sp. in the molecular findings.

Post-Hoc Morphological Examination of Samples from Hertlein's Zone of Overlap

Our post-hoc morphological examinations were not able to resolve any consistent differences between the putative northern *O. lurida* and the southern *O. conchaphila* (Table 3). For each character there was at least one sample that differed from the mode (e.g., chomata shape, Table 3). Morphological distinctions were also not apparent from the examination of a syntype specimen of *Ostrea lurida*.

Specific observations from the independent and post-hoc examinations are reported later for each character.

Chomata Sweep and Chomata Shape

Chomata were present in all specimens except BdK 0701 (Table 3). In some specimens, this chomata shape was intermediate and/or

```
CO3Site        abcdefghijklmnop
Oco-Southern   TGTCACATGTTTGTAA
Olu-Northern   AACTGTGTAGTCACGC
Olu-AllOther   AACTGTACGGCCACGC
Pattern        1111112321311111
```

Figure 3. Summary of observed CO3 variation by three haplotype groups. Variation was consistent with dividing localities into southernmost, northernmost, and all other localities in between, as listed in the CO3 column of Table 1. Oco-Southern (S) includes both *Ostrea conchaphila* sites, EdPn and EdPs; Olu-Northern (N) includes both Vancouver Island sites for *O. lurida*, LH and AI. Olu-All Other (A) includes all other *O. lurida* localities. Sample sizes for three observed haplotypes: S = 7; N = 7; A = 70. This includes three individuals (two S and one A) that differed from the other normal haplotype patterns by only a single unique base pair difference, but none of this variation occurred at sites a-m. The similarity "Pattern" tallies to 12 for A + N ("1") and two each for A + S and N + S ("2" and "3"). No assumption was made to root the similarity matching patterns because the only available outgroups (*Crassostrea* spp.) were highly divergent from these three haplotypes. Alignment sites summarized above were as follows, relative to the complete mitochondrial genome for *Crassostrea gigas*, AF177226: a = 190 ; b = 210; c = 231; d = 258; e = 309; f = 366; g = 395; h = 399; i = 435; j = 453; k = 459; l = 495; m = 501; *n* = 519; o = 558; and *P* = 582. Of these, only site 'g' represents a nonsynonymous substitution.

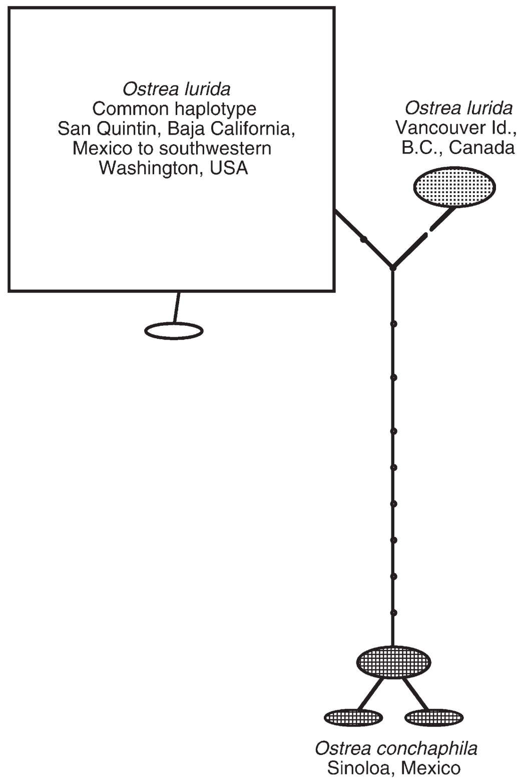

Figure 4. Haplotype network for *Ostrea lurida* (top) and *O. conchaphila* (bottom), with the node length of the branches separating different haplotypes corresponding to the nucleotide substitutions observed. Table 1 lists the sample sizes per locality in the CO3 column. As computed with TCS version 1.21 (Clement et al. 2000), the lowest available connection limit of 90% was the only setting in which the relatively distant *O. conchaphila* was observed to connect with the remaining network. To avoid terminal sites in the CO3 alignment with missing data for many of the sequences, these were trimmed before construction of the network. These trimmed sites included three variable sites (a, b, and p in Fig. 3) whose inclusion would have only increased the length between *O. conchaphila* and *O. lurida*. Note that the root connecting *O. conchaphila* to the haplotype network for *O. lurida* is arbitrarily placed at the midpoint.

variable, designated as round-elongate in Table 3. In none of the specimens examined, however, was the presence or absence of chomata ambiguous, at least under low-power magnification.

Plicae

Plicae were scored as absent if they did not appear to arise independently of underlying substratum topology. In some specimens, plicae were numerous and regular, but in others they were irregular or few in number (Table 3).

Shell Color

Internal shell color was ambiguous in all samples except for Maz 0503 in which it was deep purple; internal shell color is not included in Table 3. External shell color, where it differed unambiguously from white or off-white, was recorded in Table 3.

Other Shell Characters

External shell sculpture in the form of foliations or spines was not present in any of the specimens examined in this study. Secondary mantle retractor scars were present in specimen Maz 0503, which helped in diagnosing it as *Saccostrea*. Both samples from BdK had relatively thick shells throughout (>2 mm) and were chalky in texture, which is a common description to *Crassostrea* shells.

Soft Tissue Characters

For some of the samples, characters such as anal papillae and mantle tentacles could not be defined precisely because of tissue damage, poor preservation, and possible environmental plasticity. The presence/absence of an anal papilla and the relative size of mantle tentacles on the two inner folds were variable within suspect taxa.

DISCUSSION

The combined and separate analyses of the 16S and CO3 sequence data sets do not support Harry's synonymy of *Ostrea lurida* with *Ostrea conchaphila,* but instead support the original classifications by Carpenter (1857, 1864), who treated them as separate oyster species. Our evidence thus provisionally supports the reinstatement of *Ostrea lurida* as the Olympia oyster. From the analysis of the 16S data set, samples from the type locality of *O. lurida*, Willapa Bay, WA, along with seven other locations in Southern California and Baja California are unambiguously supported as a monophyletic grouping, within which we noted only minor variation. Likewise, 16S sequences of oysters from two adjacent locations within Ensenada del Pabellon (EdP in Fig. 1), Sinaloa, Mexico, near the type locality of *O. conchaphila*, group as a well-supported phylogenetic lineage. Relative to the most proximal available outgroups, specifically various *Ostrea* spp. listed in Table 2, *O. lurida* and *O. conchaphila* are weakly supported as sister species. However, Keen and McLean (1971) note the presence of at least 3 other putative *Ostrea* species in the Gulf of California, for which molecular data are unavailable, but who might serve as sister taxa to either focal species in this study.

Bootstrap support for the sister relationship between the focal species was higher for MP (68%) than for ML (43%). For either MP or ML, the highest and lowest bootstrap support proportions were observed for the CO3-only and 16S-only data sets, respectively, but even the latter was reasonably well supported in the case of MP. As detailed earlier in our results, alternative grouping with the next highest bootstrap proportion for both MP (11%) and ML (35%) placed *O. lurida* as basal within the "Ostreinae" + Lophinae monophyletic grouping. Still, the best trees for either optimality criterion places *O. conchaphila* and *O. lurida* as sister species. The lack of bootstrap support could be caused by the considerable branch length divergence of the involved nodes in their separation from the next available outgroups (*Saccostrea* and *Crassostrea* spp.), or the presently sparse intraspecific sampling for our other "Ostreinae" + Lophinae outgroups. Alternatively, the low bootstrap support might reflect the phylogenetic possibility that these native North American oyster species might not be sister species. For example, we have not included any extant or extinct *Ostrea* spp. from the northwestern Pacific and perhaps one or more of these could have a closer relationship to the similarly cooler water *O. lurida*.

Interesting phylogeographic structure was observed within *O. lurida* for the CO3 data set and this coincided with the geographic separation between Willapa Bay, WA and the

southern part of Vancouver Island, B.C., Canada. A break in this vicinity is unexpected. For marine organisms, most have found little evidence of a biogeographic break in this area (e.g., Kelly & Eernisse 2007), although some have found a break further north (e.g., Hart et al. 2005) or coinciding with the exposed coast *versus* inland passage discontinuity between British Columbia and Alaska (e.g., Lindstrom et al. 1997). It is possible that the Vancouver Island localities are populated with oysters that are relicts of Pleistocene glacial refugia, which have been inferred for areas north of these Vancouver Island localities. Specifically, glacial refugia during the late Wisconsin glaciation have been supported for the Queen Charlotte Islands, northern British Columbia (Hetherington et al. 2003) and in the Alexander Archipelago of southeastern Alaska (Carrara et al. 2007). At least one terrestrial species, the lodgepole pine, might have relicts in glacial refugia on the outer coast of Vancouver Island (Godbout et al. 2008). However, the present evidence for phylogeographic separation needs further study with more localities and more gene regions before such historical hypotheses can be accurately inferred.

Because of their extremely plastic morphologies, oysters present difficult challenges for identifications based solely on shell characteristics in the field and such identifications can be especially challenging or even impossible, even after careful examination of internal anatomy and shell structure in the laboratory. For example, the two samples from Bahia de Kino were identified as *Ostrea conchaphila* in the field, and were reidentified as *Crassostrea* sp. based on shell morphology in the laboratory. The subsequent molecular analyses grouped only one of the two samples as nearly identical to *C. gigas* with 100% MP bootstrap support, the other grouped with our other specimens presumed to be *Saccostrea palmula* (Fig. 1, Table 2). The need, in some cases, to rely on molecular analyses to positively identify specimens underscores the challenges associated with resolving the systematics of this troublesome group. Regarding the morphological characters examined, the presence/absence of chomata was the only unambiguously diagnostic character and it was only informative at a generic level, not between species of *Ostrea*.

Aside from the presence/absence of chomata, no other shell or soft tissue characters were 100% congruent with molecular data. Only one of the three *Saccostrea* samples examined had unambiguously elongate chomata all the way around the shell margin. Whereas all *Ostrea* samples examined had chomata, some were rounded whereas others were slightly elongate. Specimens identified as *O. lurida* from molecular study (Tables 1 & 3) were nearly as likely to have external shell color or shell plications as not. Soft tissue characters were more consistent, but for each character, at least one specimen, even in this small sample size, differed from the mode. The potential value of anal papillae was further limited by tissue damage. The variability we observed is consistent with comments by Keen (1958) and Coan et al. (2000) on *Ostreidae* morphology; Coan et al. (2000) emphasize the need for molecular data for more reliable identification. In all, there was considerable intraspecific and interpopulation variation that confounded any attempt to separate the genetically distinct lineages of *O. lurida* from *O. conchaphila* by morphology alone.

For the combined analyses of the 16S and CO3 datasets, *O. conchaphila* and *O. lurida* were well supported as distinct taxa (Figs. 1–2). Unfortunately, we were unable to find any examples of a calibrated molecular clock for either 16S or CO3 for other bivalves, but an approximate comparison is available for a molecular clock calibrated for 16S in Pacific porcelain crabs (Stillman & Reeb 2001), based on this, the range of estimated pairwise divergence times for *O. conchaphila versus O. lurida* is about 1.5–3.9 mya. The evidence for the reciprocal monophyly of *O. conchaphila* and *O. lurida* is indicative of a significant absence of gene flow between the two groups. However, again, we found no diagnostic morphological character corresponding to either the northern or southern groups (Table 3). Although we have contrasted oysters from near the type locality of *O. conchaphila* with oysters from the type locality of *O. lurida*, much further north in Washington, we have not carefully studied the range of morphological variation across our *O. lurida* localities with enough detail to determine whether there might be diagnostic shell or anatomical traits to separate *O. lurida* from *O. conchaphila*. Moreover, we have only collected *O. conchaphila* from one pair of nearby sites near Mazatlán. Clearly, further morphological study, including more intensive sampling throughout the range of *O. conchaphila*, is needed.

Based on our molecular evidence, we only found *O. lurida* within Hertlein's (1959) putative zone of overlap that is supposed to exist between these two species. Samples from the seven localities in southern California and northern Baja California (Table 1) all were effectively indistinguishable from *O. lurida* at the type locality, Willapa Bay, WA, and this was true for both mtDNA gene regions. In the future, sampling from other locations is warranted to determine the existence, if any, of a zone of overlap, and any such zone is predicted to occur further south than where we sampled, perhaps on the outer coast of Baja California Sur. A transition zone might also exist within the Gulf of California, however, previously published reports imply that neither species occurs within the Gulf of California (e.g., Keen & McLean 1971). Still, this could be because of lack of study, because molecular data on Gulf of California oyster species are mostly lacking. Thus, the only stretch of ostensibly inhabited coastline that was not sampled in this study extends from locations south of Bahia San Quintin, Baja California to the previously reported southern range endpoint for *Ostrea lurida* at Cabo San Lucas, Baja California Sur. Recent extensive intertidal searches in Cabo San Lucas failed to locate any oysters (Polson & Zacherl, this issue). The only mention of the existence of a zone of overlap was by Hertlein (1959), who also described three separate morphotypes found in southern California. Considering the high degree of phenotypic plasticity in oyster shell morphology, a zone of overlap more likely never was present in southern California and northern Baja California. Instead, Hertlein (1959) might have documented evidence of the high degree of phenotypic plasticity exhibited by *O. lurida*.

To bridge the 2 most popular species concepts, the biological species concept and the phylogenetic species concept, the concordance principle (CP) introduced by Avise & Ball (1990), recognizes a species based on the phylogenetic concordance of multiple independent molecular markers. Moreover, CP calls for reproductive isolation but is based on intrinsic rather than extrinsic (i.e., geographic) forces. With this study, we have provided molecular evidence across two nonindependent (i.e., tightly linked) mtDNA markers in a geographic context. Our phylogenetic estimates support *O. lurida* and *O. conchaphila* as two distinct taxa, with data still lacking for any transitional localities, should they exist. Finding a zone of overlap (sympatry)

within which there is little evidence for ongoing hybridization would have provided stronger support that *O. lurida* and *O. conchaphila* are separate species and not simply extremes of a latitudinal cline. Future studies would benefit from sampling variable nuclear markers to test for such hybridization. Recently Wang and Guo (2008) found ITS length variation across multiple species of *Crassostrea* but unfortunately no variation was detected across the *Ostrea* spp. compared.

The provisional decision to again regard these clades as separate species here, reviving Carpenter's *Ostrea lurida* and rejecting Harry's (1985) synonymy, is based on the evidence from this study for historical separation of the oysters from the Panamic *versus* the cooler northeastern Pacific. Whereas no one has yet demonstrated diagnostic shell or anatomical characters, this situation is in no small part caused by their notorious plasticity, and having the present phylogenetic estimate will help define where morphological contrasts with a genetic basis are likely to be found. With roughly 1.5–3.9 my separation, our mitochondrial markers were successful in distinguishing these species. The Olympia oyster becomes a sister species whose southern range limit has yet to be carefully explored along the Baja California Peninsula, and much less is yet known about *O. conchaphila*. In addition to molecular distance, this pair of oyster species is separated both by geography and contrasting climates. Although other *Ostrea* spp. and other locations in Mexico need to be sampled, evidence to date implies a sibling species pair with one in the subtropical Panamic marine biogeographical province and the other extending along the cooler Californian and Oregonian temperate provinces to the north. Our preliminary evidence for further phylogeographic structure within *O. lurida* is potential evidence that such historical processes might be ongoing. Thus, calling for the reinstatement of *Ostrea lurida* as it was originally described and once widely accepted will help focus research on the remaining mysteries of the Olympia oyster throughout its range.

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