Classification of Small Flat Oysters of Ostrea stentina Species Complex and a New Species Ostrea neostentina sp. nov. (Bivalvia: Ostreidae)

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CLASSIFICATION OF SMALL FLAT OYSTERS OF *OSTREA STENTINA* SPECIES COMPLEX AND A NEW SPECIES *OSTREA NEOSTENTINA* SP. NOV. (BIVALVIA: OSTREIDAE)

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ABSTRACT Small flat oysters of the genus *Ostrea* were collected from China, Japan, and the United States for identification and phylogenetic analysis. Fragments of mitochondrial cytochrome oxidase subunit I and 16S rRNA genes were sequenced in 57 specimens. Shell morphology and phylogenetic analyses identified four closely related groups in the *Ostrea stentina*/*equestris*/*aupouria* species complex, supporting recent and ongoing speciation. Groups 1 and 2 are both *O. equestris* (Say, 1834), representing two divergent populations from the Asian Pacific and the Americas, respectively. Groups 3 and 4 are two separate species independent of *O. equestris*. Group 4 is *O. stentina* occurring in northern Spain, Portugal, Morocco, and northern Tunisia. Group 3 is a new species *Ostrea neostentina* sp. nov. found in southeastern Spain, eastern Tunisia, Hong Kong, and Japan. The occurrence of *O. equestris* in both Atlantic and Pacific oceans, from Argentina, Columbia, the United States, Gulf of California, Japan, and China to New Zealand, is unusual and highlights wide connectivity of *Ostrea* species as part of the marine ecosystem. This study provides the first record of *O. equestris* and *O. neostentina* sp. nov. along the southern coast of China and adds to the understanding of the diversity, global distribution, and evolution of *Ostrea* species.

KEY WORDS: oyster classification, evolution, phylogenetics, COI, *Ostrea stentina*, *Ostrea equestris*, Ostreidae, Bivalvia, *Ostrea neostentina*

INTRODUCTION

Oysters are widely distributed in world oceans and estuaries. They are important fishery resources and important ecological service providers (Beck et al. 2011). Oysters support important aquaculture industries worldwide (Guo et al. 1999, Guo 2009). Despite their importance, the diversity and distribution of living oysters is not fully understood. Classification of oyster species has been problematic because of plasticity in shell morphology. Many oyster species have been misidentified, resulting in considerable confusion in classification (Harry 1985). Genetic studies over the past two decades have significantly improved oyster taxonomy, and about 100 species of living oysters are recognized (Bayne 2017, Guo et al. 2018). Still, many taxonomic uncertainties exist, and new species are to be discovered.

The ostreid genus *Ostrea* contains many fossils and living species. Harry (1985) revived the genus *Ostreola* (Monterosato, 1884), with the type species *Ostreola stentina* and member species *Ostreola equestris* and *Ostreola conchaphila*. Subsequently, *Ostreola* was reverted to *Ostrea* for lacking distinctive morphological characters (Harry 1985, Coan et al. 2000) and genetic support (Kirkendale et al. 2004, Lapègue et al. 2006, Shilts et al. 2007, Polson et al. 2009). Studies have indicated that some species of *Ostrea*/*Ostreola* are synonyms of *O. stentina* (Payraudeau, 1826). Ranson (1967) considered that subspecies *O. stentina var. isseli* (Bucquoy, Dautzenberg & Dollfus, 1887), *O. stentina var. prepratxi* (Bucquoy, Dautzenberg & Dollfus, 1887), and *O. stentina syrica* (Pallary, 1938), as well as *Ostreola parenzani* (Settepassi in Parenzan, 1974) and *Ostreola curvata* (Risso, 1826) were synonyms of *O. stentina*. Harry (1985) synonymized *Ostrea spreta* (d'Orbigny, 1846) from Southern Brazil and Caribbean Antillean islands with the northwest Atlantic/Caribbean *Ostrea equestris* (Say, 1834). Molecular study of *O. equestris* (Kirkendale et al. 2004) has revealed that the American *O. equestris* and New Zealand *Ostrea aupouria* (Dinamani & Beu 1981) are close sister taxa. Other studies (Shilts et al. 2007, Raith et al. 2015) included the Mediterranean/Eastern Atlantic–type species, *O. stentina*, and indicated that *O. equestris* and *O. aupouria* were synonyms of *O. stentina*. Salvi et al. (2014) also suggested that *O. aupouria*, *O. equestris*, *O. spreta*, and *O. stentina* may be one species. Hamaguchi et al. (2017) recently recorded *O. stentina* in Japan as part of the *O. stentina* complex that included *O. stentina*, *O. aupouria*, *O. equestris*, and *O. spreta*. It has been suggested that *O. equestris* is a separate species, which is different from two other species under the name *O. stentina* (Guo et al. 2018). Nevertheless, taxonomic status of the *O. stentina* complex is uncertain and requires further analysis, especially given its wide geographic distribution ranging from southern Mediterranean coasts, southwestern coast of the Iberian Peninsula, southern coast of Argentina, western and eastern Atlantic coasts, Gulf of California in eastern Pacific, and Asian Pacific. Taxonomic status of some other closely related *Ostrea* species pairs is also uncertain or controversial, including *Ostrea edulis* and *Ostrea angasi*, *Ostrea conchaphila* and *Ostrea lurida*, and *Ostrea permollis* and *Ostrea puelchana* (Coan et al. 2000, Kenchington et al. 2002, Hurwood et al. 2005, Shilts et al. 2007, Polson et al. 2009, Salvi et al. 2014, Raith et al. 2015, Guo et al. 2018).

More than 30 oyster species have been reported to occur along the coast of China, most of which belong to *Crassostrea* and *Saccostrea*, with only two species of *Ostrea*, *Ostrea denselamellosa* and *Ostrea circumpicta* (Xu & Huang 1993, Li & Qi 1994, Wang et al. 2004, Reece et al. 2008, Xu & Zhang 2008,

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Wang et al. 2008, 2010, 2013, Hamaguchi et al. 2014, Guo et al. 2018). The large, flat, and round shells with dense plates along the edge, can be easily identified as *Ostrea denselamellosa*. The occurrence of *O. circumpicta* in China has not been genetically confirmed.

To identify possible *Ostrea* species and clarify their taxonomic status, small flat oysters were collected from China, Japan, and the United States to identify possible *Ostrea* species by genus-specific PCR markers (Wang & Guo 2008) and sequenced for mitochondrial cytochrome oxidase subunit I (COI) and 16S rRNA genes. Phylogenetic and morphology analysis revealed that the *Ostrea stentina* complex consisted of three independent species: *Ostrea equestris* from eastern Atlantic, western and eastern Pacific; *O. stentina* from eastern Atlantic; and a new species *Ostrea neostentina* sp. nov. from Mediterranean to Asia. This study provides the first record of *O. equestris* and *O. neostentina* sp. nov. in China and adds to the understanding of the diversity, distribution, and evolution of *Ostrea* species.

MATERIALS AND METHODS

Samples and Identification by Multiplex Genus-Specific PCR

Oysters analyzed in this study were small flat oysters collected from five different locations along the southern coast of China, one location in Japan, and one in North Carolina (Fig. 1, Table 1). Oysters from China and Japan were collected and fixed in 95% ethanol and transported to Shellfish Research Laboratory, Institute of Oceanology, Chinese Academy of Sciences. Oysters from North Carolina were from the collection of Haskin Shellfish Research Laboratory, Rutgers University. Samples of *Ostrea edulis*, *Ostrea angasi*, and *Ostrea lurida* were sequenced at Haskin Shellfish Research Laboratory.

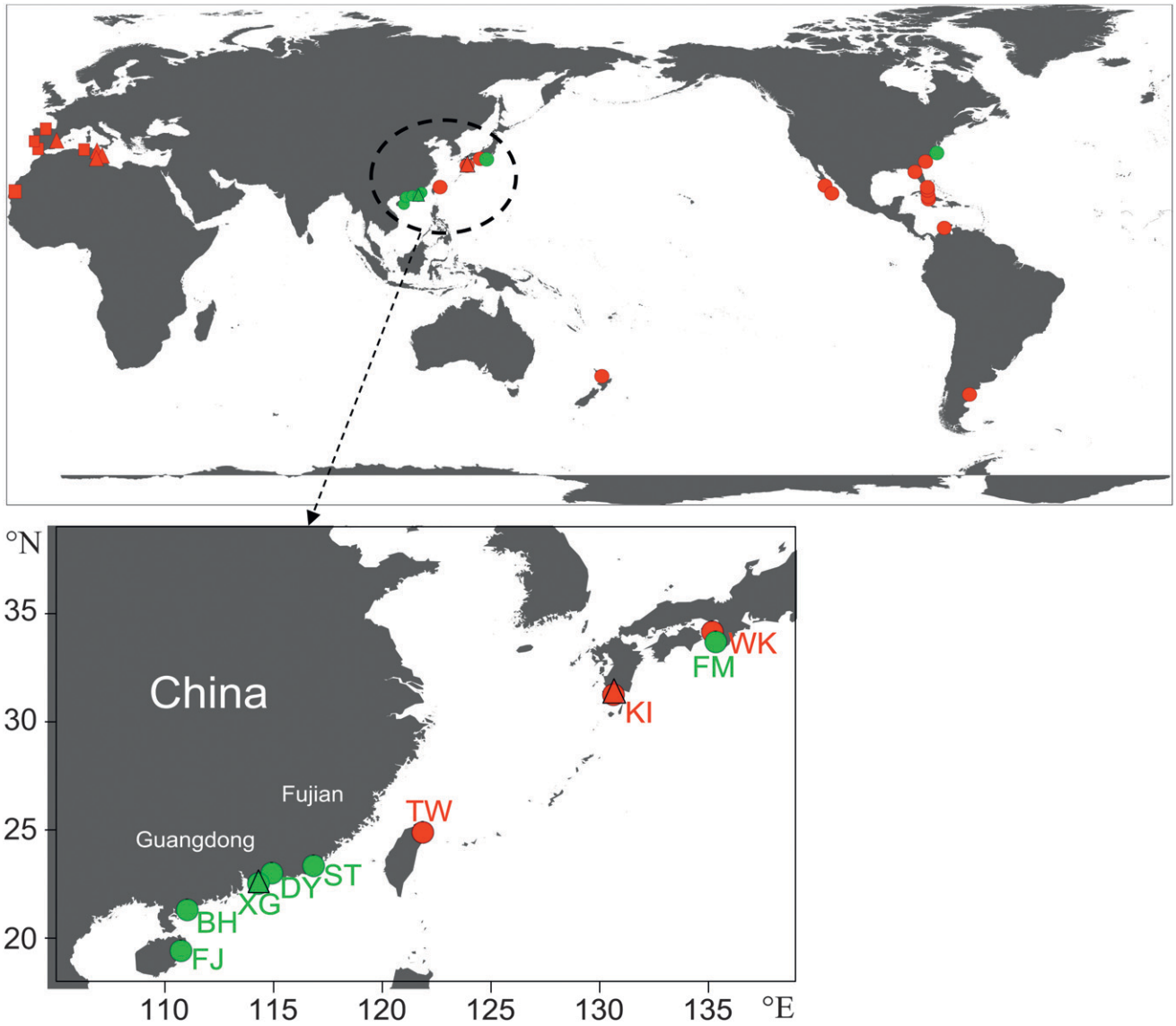

Figure 1. Reported distribution of *Ostrea stentina* complex species (marked as red) and sites sampled in this study (marked as green). Site information is provided in Tables 1 and 2. Circles represent *Ostrea equestris*, triangles represent *Ostrea neostentina*, and squares represent *O. stentina*.

TABLE 1.

Location, sample size, and sequence accession numbers for oysters collected in this study.

Abbreviation	Sampling site	GPS coordinates	Number of specimens	GenBank numbers of submitted sequences	
				COI	16S
1. FM	Full Moon Island, Japan	135.336° E, 33.69° N	2	1 (KY986322)	2 (KY986305,09)
2. ST	Shantou, China	116.84° E, 23.34° N	1	1 (KY986328)	1 (KY986306)
3. DY	Daya Bay, China	114.92° E, 23° N	25	24 (KY986326; MK370331–353)	25 (KY986307,10; MK370370–392)
4. XG	Hong Kong, China	113.968° E, 22.32° N	16	12 (MK370319–330)	16 (MK370354–369)
5. BH	Bohe, China	111.03° E, 21.3° N	3	2 (KY986323,27)	3 (KY986308,19–20)
6. FJ	Fengjiacun, China	110.74° E, 19.41° N	3	2 (KY986324–25)	3 (KY986311–12)
7. NC	North Carolina	75.64° W, 35.69° N	7	7 (KY986329–35)	7 (KY986313–18,21)

Multiplex genus-specific PCR of Wang and Guo (2008) was used to separate *Ostrea* species from *Crassostrea*, *Saccostrea*, and *Hyotissa* species. The *Saccostrea*/*Ostrea*–specific primer 28Soa166r was used to identify *Ostrea* and *Saccostrea* species, and the latter was easily identified by shell morphology.

Sequencing

DNA was extracted from the adductor muscle using the TIANamp Genomic DNA Kit (Tiangen Biology, Beijing, China). The COI fragment was amplified with universal primers: LCO1490, 5′–GGTCAACAAATCATAAAGATATTGG–3′ and HCO2198, 5′–TAAACTTCAGGGTGACCAAAAAATCA–3′ (Folmer et al. 1994). The 16S rRNA fragment was amplified with 16sar, 5′–CGCCTGTTTATCAAAAACAT–3′ and 16sbr, 5′–CCGGTCTGAACTCAGATCACGT–3′ (Palumbi et al. 1991).

PCR was performed in 25 μL of reaction mixture containing 2 μL 10× PCR buffer, 1.5 mM Mg^{2+}, 200 μM dNTPs, 1 U *Taq* DNA polymerase, and 0.4 μM forward and reverse primers, using the following parameters: an initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 sec, 48°C (16S) or 51°C (COI) for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min.

PCR products were verified on 1.5% agarose gels containing 0.2 μg/mL ethidium bromide, visualized on a UV transilluminator, and purified using DP214 Universal DNA Purification Kit (Tiangen Biotech). Sequencing was performed in both directions on an ABI 3730 Genetic Analyzer (Applied Biosystems). Sequences obtained in this study were submitted to NCBI (http://www.ncbi.nlm.nih.gov/) under gene accession numbers KY986305-KY986321, MK370354–392 (16S) and KY986322-KY986335, MK370319–353 (COI) (Table 1).

Phylogenetic Analysis

Reported sequences of *Ostrea stentina* complex species were downloaded from NCBI GenBank, and their location and accession numbers are provided in Table 2. Sequences (COI and 16S rRNA) from the following reference species were also included in the phylogenetic analysis: *Ostreola conchaphila* (KT317494.1 and FJ768528), *Ostrea lurida* (KT317504.1 and FJ768559), *Ostrea angelica* (KT317449.1 and KT317140), *Ostrea permollis* (DQ226526.1 and AY376606), *Ostrea puelchana* (DQ226521.1 and AF052073), *Ostrea edulis* (KJ818235.1 and AF540595), *Ostrea denselamellosa* (KP067907.1 and HQ660995), *Crassostrea gigas gigas* (JF700177.1 and JF808180), *Crassostrea gigas angulata* (KC170323.1 and KC170322), and *Hyotissa imbricata* (AB076917.1 and KC847136). Haplotypes for 16S rRNA and COI sequences were identified using DnaSP 5 software (Librado & Rozas 2009). Sequences used for phylogenetic analysis were aligned using CLUSTAL X (Thompson et al. 1994) and trimmed to the same length with manual adjustments.

Neighbor-joining (NJ) trees were constructed with MEGA 7 (Kumar et al. 2016) using Kimura's two-parameter (K2P) distances. Bayesian analysis was carried out using the program MrBayes V.3.2.6 (Ronquist et al. 2012). The best fitting models GTR + I + G and HKY + G were selected using Modeltest v3.7 (Posada & Crandall 1998) for 16S rRNA and COI, respectively, based on the Akaike information criteria before Bayesian inference (BI) analyses. Analysis for 20 million generations was run, and parameters and trees were sampled every 10,000 generations, discarding 25% of the total sampled trees from the beginning of the chain as burn-in. Bayesian posterior probabilities were obtained from a tree with clade credibility values. The *Hyotissa imbricata* species was chosen as the outgroup.

Genetic distances among the *Ostrea stentina* complex and other reference species were calculated using MEGA 7 with the K2P model.

RESULTS

Shell Morphology

Shells of *Ostrea stentina* complex species collected in this study are presented in Figure 2. All oysters collected, except one specimen from Hong Kong (A1 and A2), closely resemble *Ostrea aupouria* (=*virescens*) and *Ostrea equestris* in shell morphology as previously reported (Fig. 3). These oysters are generally small and oval or round in shape, with height greater than length in most specimens. The left valves, which have raised margins and shallow umbonal cavities, adhere to the substrate over a large area. The right valves are flatter than the left valves and have narrow, flat commissural shelves. There are a series of fine crenellates on both sides of the ligament along the edges of the right valves. Interior surfaces of the valves vary from dull-gray to green, depending on the environment. The adductor muscle scar is pointed and usually reniform and with greenish color around it. The umbo of the specimen collected from Hong Kong marked as A1 and A2 is closer to posterior,

TABLE 2.

Location and sequence accession numbers of *Ostrea stentina* complex species from GenBank.

Species	Abbreviation	Sites	Number of sequences (GenBank accession number)	
			COI	16S
Ostrea stentina	8. WK	Wakayama, Kemi, Japan	3 (LC051582–84)	3 (LC051572–74)
	9. KI	Kagoshima, Ibusuki, Japan	7 (LC051585–91)	7 (LC051575–81)
Ostrea sp.	10. TW	Taiwan, China	2 (JQ027291–92)	–
Ostrea aupouria	11. HG	Hauraki Gulf, New Zealand	8 (AF112288, DQ226533, AY376627–32)	1 (AF052064)
Ostrea equestris	12. BM	Bahía Magdalena, Mexico	1 (KT317503)	1 (KT317198)
	13. LC	Los Cabos, Mexico	7 (KT317496–502)	7 (KT317191–97)
	14 SR	Skidaway River, GA	6 (AY376614–19)	–
	15. CK	Cedar Key, FL	7 (AY376620–26)	–
	16. SB	Sprigger Bank, FL	1 (AY376613)	–
	17. BP	Big Pine Key, FL	1 (DQ226522)	1 (AF052074)
	18. WS	West Summerland Key, FL	5 (AY376607–11)	2 (AY376603–04)
	19. FK	Fiesta Key, FL	1 (AY376612)	–
	20. NB	Neguange Bay, Colombia	1 (DQ226523)	–
	21. SM	San Matias Gulf, Argentina	5 (DQ640078–82)	1 (DQ640402)
O. stentina	22. AS	Avilés, Spain	3 (KJ818237–39)	1 (KJ818210)
	23. MM	Mar Menor Lagoon, Spain	3 (DQ226515–17)	1 (DQ180744)
	24. KN	Kneiss Islands, Tunisia	1 (DQ313181)	1 (DQ313178)
	25. GH	Gannouche, Tunisia	1 (DQ313181)	1 (DQ313178)
	26. SA	Sado estuary, Portugal	1 (DQ313182)	1 (DQ313179)
	27. MI	Mira estuary, Portugal	1 (DQ313182)	1 (DQ313179)
	28. MO	Dakhla Bay, Morocco	1 (DQ313183)	1 (DQ313180)
	29. HM	Gulf of Hammamet, Tunisia	–	7 (EU409052–57,63)
	30. BI	Bizerte Lagoon, Tunisia	–	5 (EU409058–62)

DQ313182 is too short for phylogenic analysis and not used in the analysis.

and the adductor muscle scar is rounder than that in other specimens; the adductor muscle scar of A1 and A2 is similar to that of specimens from the Gulf of Hammamet (Dridi et al. 2008).

Genus Assignment

Multiplex genus-specific primers provided genus assignment of all oysters collected in this study. Amplified products showed that the collected oysters were either *Ostrea*, *Saccostrea*, or *Dendostrea* species (Fig. 4). Shell morphology of *Saccostrea* and *Dendostrea* species is distinctive: *Saccostrea* species have a deep umbonal cavity and internal chomata on both shells, and *Dendostrea* species have radiating ribs on the surface of the right valve. Thus, *Ostrea* species were identified with molecular markers and separated from *Saccostrea* and *Dendostrea* species based on shell morphology.

Phylogenetic Analyses

From 57 specimens of *Ostrea* species collected and identified in this study, 49 COI sequences and 57 16S sequences were obtained. After alignment and trimming, 429 bp of 16S and 603 bp of COI sequences were used for phylogenetic analysis. Among the 49 COI sequences, 25 haplotypes were identified (Table 3), and 13 haplotypes were identified among the 57 16S sequences (Table 4). Oysters from China did not share any haplotypes with oysters from North Carolina in either genes.

The NJ trees constructed based on COI and 16S are presented in Figures 5 and 6, respectively, which show the same topology. Haplotypes obtained in this study clustered together with sequences from the *Ostrea stentina* complex species, *Ostrea aupouria*, *Ostrea equestris*, and *O. stentina*. Within the large cluster, the sequences obtained in this study were closer to *O. aupouria* and *O. equestris* than to *O. stentina*.

According to the phylogenetic trees, the *Ostrea stentina* complex could be divided into four groups: group 1 includes *O. aupouria*/*stentina* sequences from China, Japan, and New Zealand; group 2 includes *Ostrea equestris* sequences from Gulf of California, Argentina, Florida, and North Carolina; group 3 includes *O. stentina* sequences from Mar Menor Lagoon in southeastern Spain, eastern Tunisia (Kneiss Islands—Gannouche, Gulf of Hammamet), one from Japan, and one from Hong Kong, China; and group 4 includes *O. stentina* sequences from Avilés in northern Spain; Portugal; Dakhla Bay, Morocco; and northern Tunisia (Bizerte Lagoon). Except for one specimen from Japan and one from Hong Kong, the groupings on the phylogenetic trees are mostly in accordance with geographical distribution in the Pacific, the Americas, and eastern Atlantic.

Genetic Distances

The K2P genetic distances between the obtained 16S haplotypes (except Hap 13) and *Ostrea aupouria*/*Ostrea equestris* were 0.2%–1.0%, smaller than that between the haplotypes and *Ostrea stentina* (1.0%–1.9%) (Table 5). The largest 16S distance within the *O. stentina* complex (1.9%) was larger than that between two subspecies *Crassostrea gigas gigas* and *Crassostrea gigas angulata* (1.05%–1.32%) (Wang et al. 2010), as well as

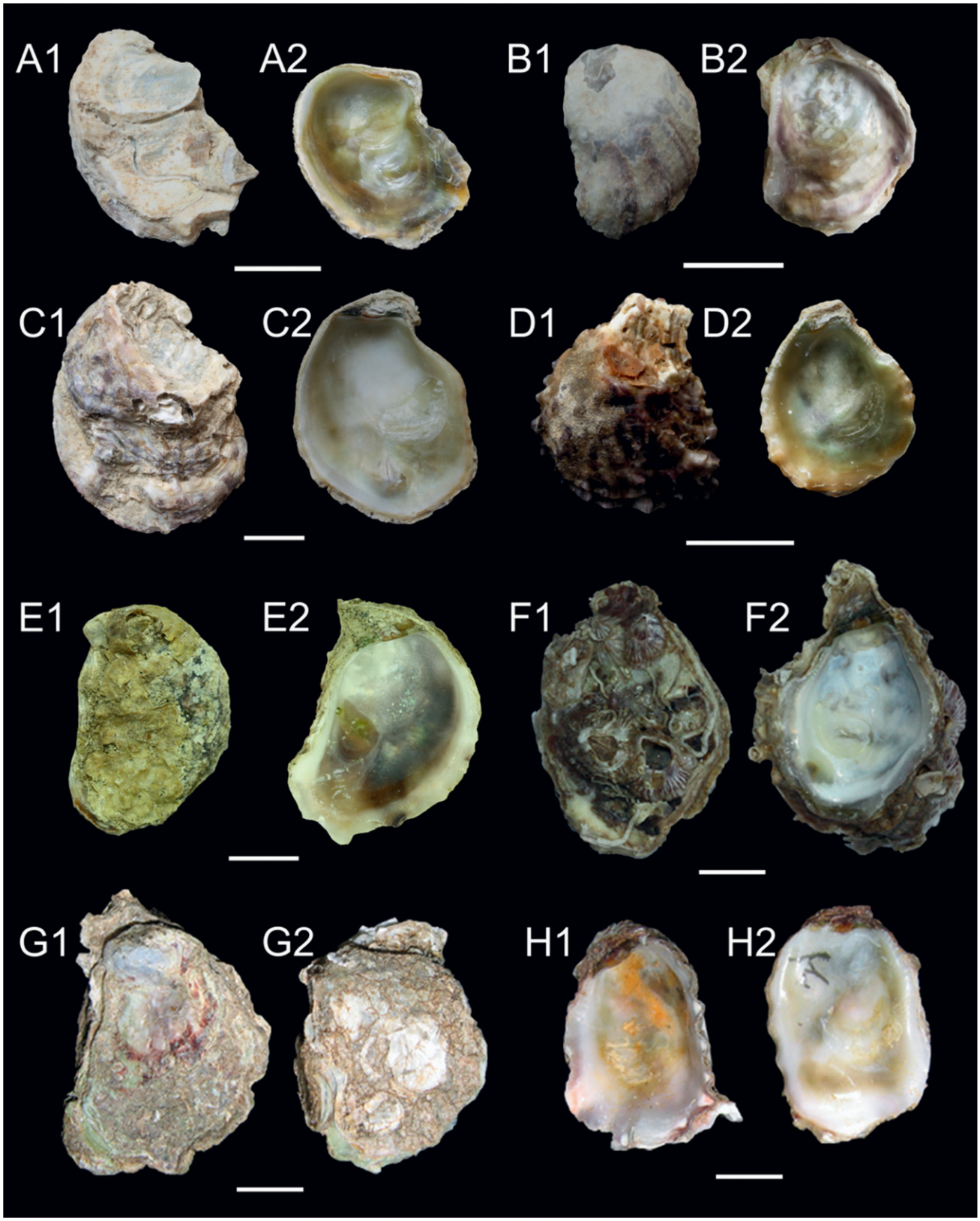

Figure 2. Shells of *Ostrea stentina* complex species collected in this study. A1–A2, *Ostrea neostentina*: Hong Kong, left external (A1) and right internal (A2) view. B–H, *Ostrea equestris*: Hong Kong, right external (B1) and left internal (B2); Daya Bay, left external (C1) and right internal (C2); Bohe, left external (D1) and right internal (D2); Fengjiacun, right external (E1) and left internal (E2); Full Moon Island, right external (F1) and left internal (F2); North Carolina, right external (G1, G2) and internal (H1, H2). Scar = 10 mm.

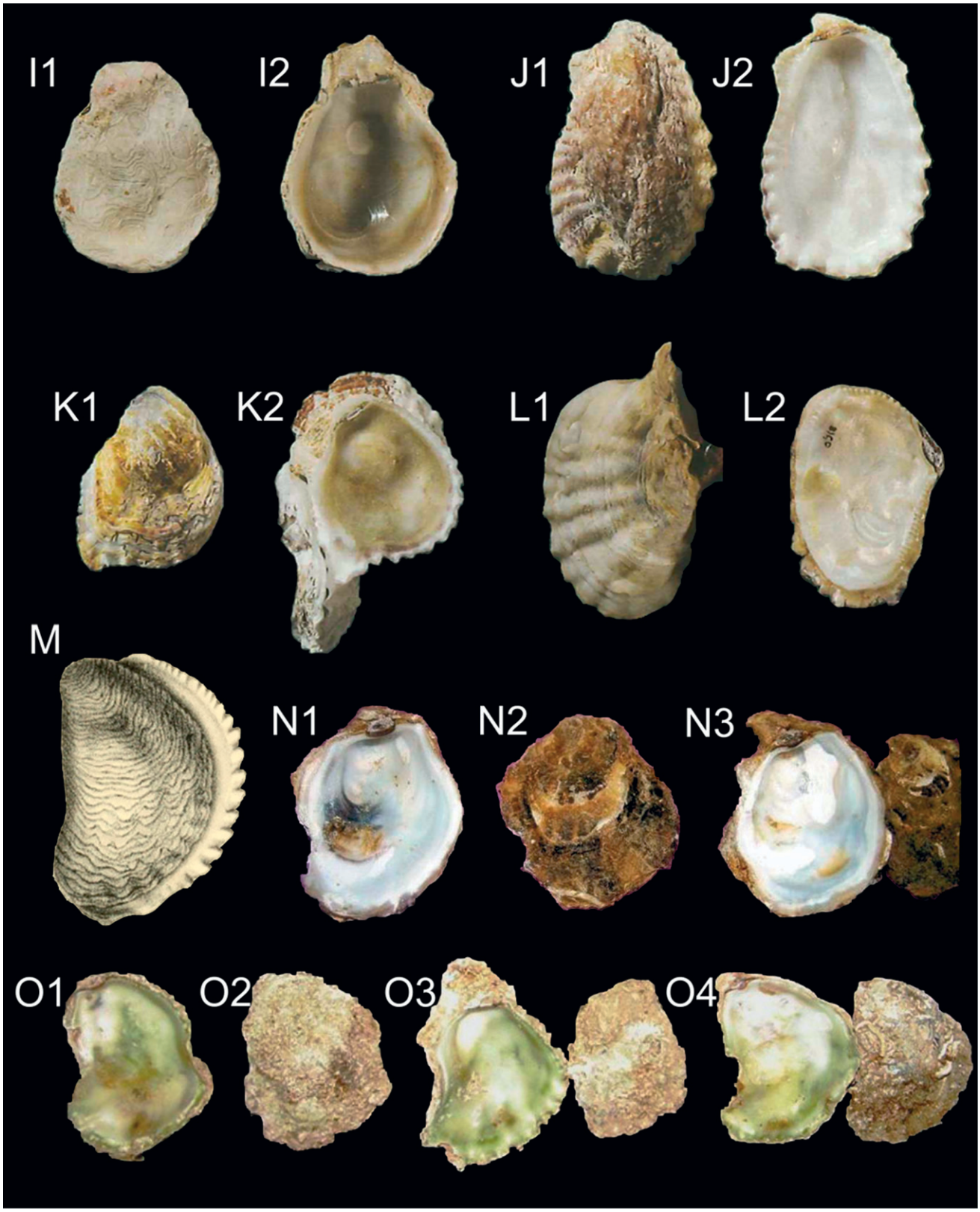

Figure 3. Shells of *Ostrea stentina* complex species from previous reports (Dridi et al. 2008, Huber 2010). I1 and I2: *Ostrea equestris* (name used by Huber: *Ostrea virescens*), Australia–NZ; J1 and J2: *O. equestris* (*O. equestris*), the United States–Martinique; K1 and K2: *O. equestris* (*Ostrea spreta*), Brazil–Argentina; L1 and L2: *O. stentina* (*Ostrea capsa*), Israel–Angola; M: *O. stentina* holotype, Mediterranean Corsica (Payraudeau 1826); N1–N3: *O. stentina*, Bizerte Lagoon; O1–O4: *Ostrea neostentina*, Gulf of Hammamet.

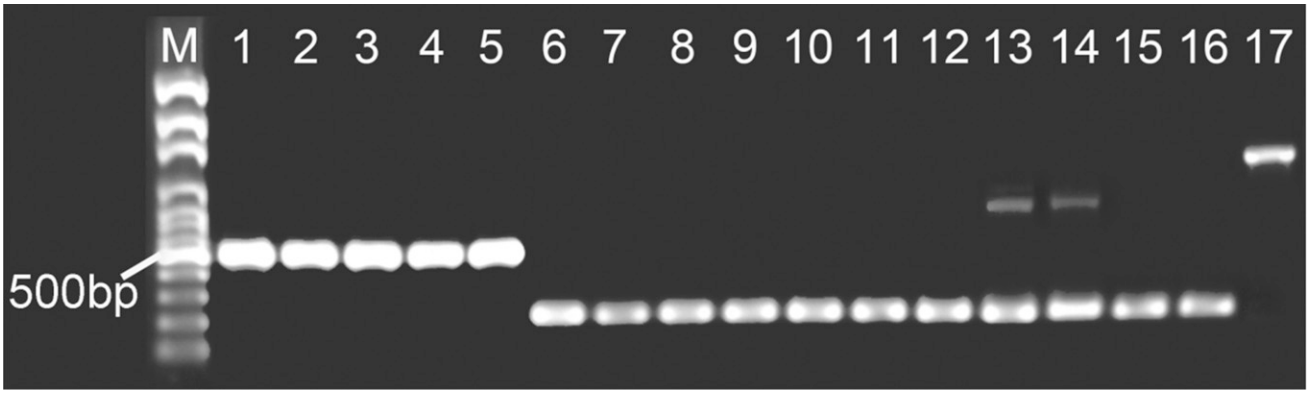

Figure 4. Multiplex genus-specific PCR with 7 primers from the 28S rRNA gene. Samples are labeled as follows: M, 100 bp DNA marker; 1: *Crassostrea gigas gigas*; 2: *C. gigas angulata*; 3: *Crassostrea sikamea*; 4: *Crassostrea iredalei*; 5: *Crassostrea nippona*; 6–9: *Ostrea stentina* complex; 10: *Ostrea angasi*; 11: *Ostrea edulis*; 12: *Ostrea denselamellosa*; 13: *Saccostrea echinata*; 14: *Saccostrea cucullata*; 15: *Saccostrea mordax*; 16: *Dendostrea crenulifera*; and 17: *Hyotissa hyotis*.

that among *Ostrea lurida*, *Ostreola conchaphila*, and *Ostrea angelica* (0.5%–1.2%); between *Ostrea permollis* and *Ostrea puelchana* (0.2%); and between *Ostrea edulis* and *Ostrea angasi* (0.5%) (Table 5).

Cytochrome oxidase subunit I sequences are more variable and provided higher resolution than 16S sequences. The K2P genetic distances between the COI haplotypes collected in this study (except Hap 2) and *Ostrea aupouria*/*Ostrea equestris* were 0%–1.2%, smaller than that between the haplotypes and *Ostrea stentina* (3.3%–4.3%) (Table 6). The largest distance within the *O. stentina* complex (4.3%) was larger than that between two subspecies *Crassostrea gigas gigas* and *Crassostrea gigas angulata* (2.22%–3.37%) (Wang et al. 2010), as well as that between *Ostrea permollis* and *Ostrea puelchana* (2.5%), and between *Ostrea edulis* and *Ostrea angasi* (1.9%) (Table 7).

Net average genetic distances (Tamura & Nei 1993) among the four groups are presented in Table 7. Giving the substitution rate of 2% (Marko et al. 2010) to 2.4% (Lazoski et al. 2011) for COI, the genetic distances correspond to divergences at about 0.25–0.31 million years ago (Mya) for groups 1 and 2, 0.72–1.04 Mya for groups 1 + 2 and groups 3 + 4, and 0.86–1.03 Mya between groups 3 and 4.

TABLE 3.

Distribution of COI haplotypes among sampling sites.

	Sampling sites						
Haplotype	**FM**	**ST**	**DY**	**XG**	**BH**	**FJ**	**NC**
1	1	0	7	5	1	0	0
2	0	0	0	1	0	0	0
3	0	0	0	1	0	0	0
4	0	0	3	1	0	0	0
5	0	0	0	1	0	0	0
6	0	0	0	1	0	0	0
7	0	0	0	1	0	0	0
8	0	0	4	1	1	0	0
9	0	0	2	0	0	0	0
10	0	0	1	0	0	0	0
11	0	0	2	0	0	0	0
12	0	0	1	0	0	0	0
13	0	0	1	0	0	0	0
14	0	0	1	0	0	0	0
15	0	0	2	0	0	1	0
16	0	0	1	0	0	0	0
17	0	1	0	0	0	0	0
18	0	0	0	0	0	1	0
19	0	0	1	0	0	0	0
20	0	0	0	0	0	0	1
21	0	0	0	0	0	0	1
22	0	0	0	0	0	0	2
23	0	0	0	0	0	0	1
24	0	0	0	0	0	0	1
25	0	0	0	0	0	0	1

DISCUSSION

Taxonomic Status

Phylogenetic analysis in this study shows that all sequences obtained in this study are closely related and belong to the *Ostrea stentina* species complex that includes *O. stentina*, *Ostrea equestris*, and *Ostrea aupouria*. The taxonomic status of flat oysters *O. stentina*, *O. equestris*, and *O. aupouria* has been

TABLE 4.

Distribution of 16S haplotypes among sampling sites.

	Sampling sites						
Hap	**FM**	**ST**	**DY**	**XG**	**BH**	**FJ**	**NC**
1	0	0	0	0	0	0	5
2	0	0	0	0	0	0	1
3	0	0	0	0	0	0	1
4	1	1	6	4	1	0	0
5	0	0	0	0	1	0	0
6	0	0	2	1	1	0	0
7	0	0	2	1	0	1	0
8	1	0	12	5	0	2	0
9	0	0	2	2	0	0	0
10	0	0	1	0	0	0	0
11	0	0	0	1	0	0	0
12	0	0	0	1	0	0	0
13	0	0	0	1	0	0	0

Figure 5. Neighbor-joining tree based on partial COI sequence. Nodes with bootstrap values greater than equal to 75 from both NJ and BI analyses are marked with solid circles. Groups 1 and 2, *Ostrea equestris*; group 3, *Ostrea neostentina*; and group 4, *Ostrea stentina*.

controversial. Kirkendale et al. (2004) examined the phylogenetic relationships between American *O. equestris* and New Zealand *O. aupouria* and showed they are synonymous species. Lapègue et al. (2006) recognized the close relationship among *O. stentina*, *O. aupouria*, and *O. equestris* and pointed out that their phylogenetic status deserved attention because of their geographic disjunction in New Zealand, Mexico, Gulf/Atlantic, and Mediterranean/African Atlantic. Shilts et al. (2007) and

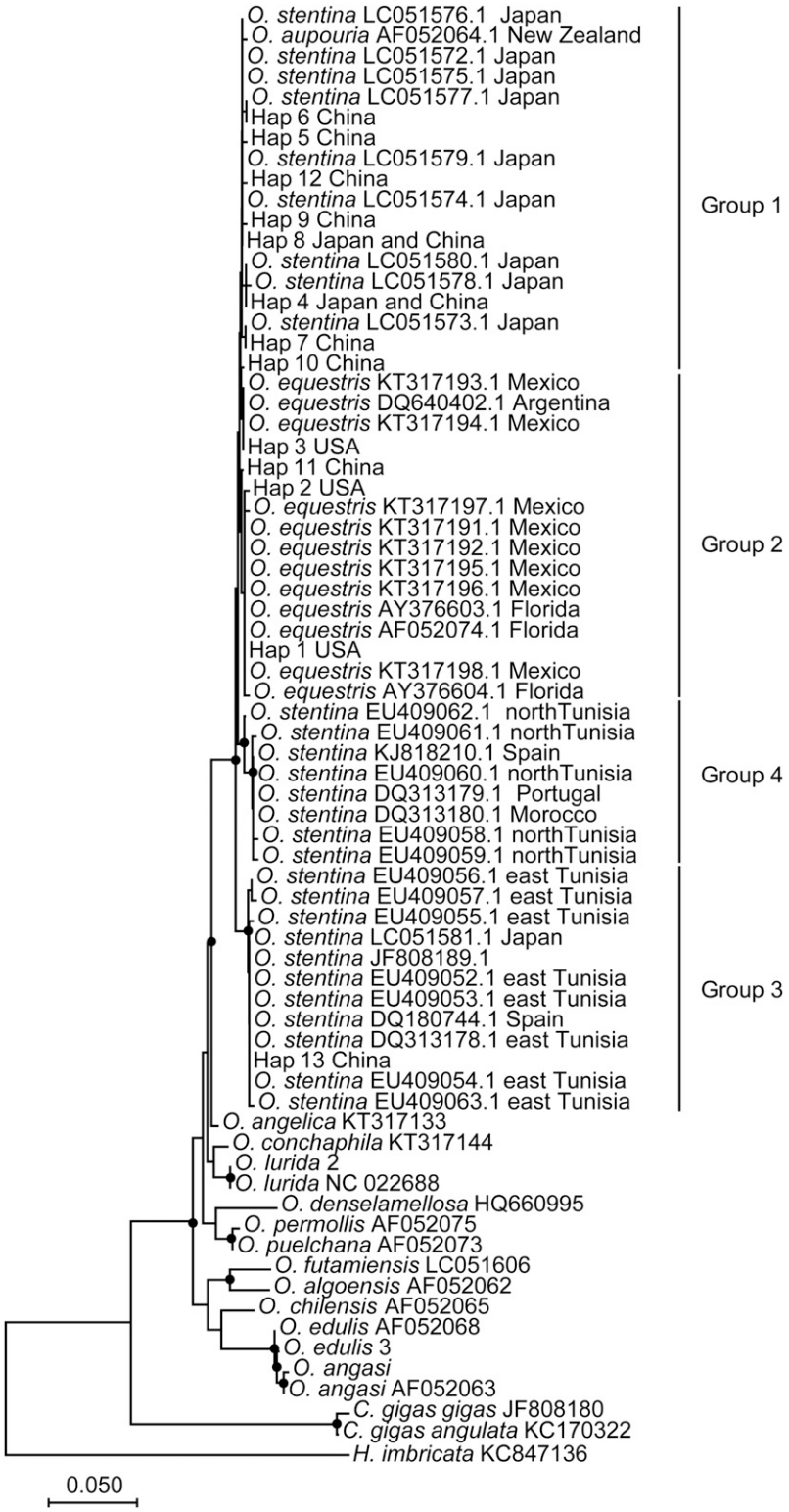

Figure 6. Neighbor-joining tree based on partial 16S sequence. Nodes with bootstrap values greater than equal to 75 from both NJ and BI analyses are marked with solid circles. Groups 1 and 2, *Ostrea equestris*; group 3, *Ostrea neostentina*; and group 4, *Ostrea stentina*.

Salvi et al. (2014) suggested that *O. stentina*/*O. equestris*/*O. aupouria* may belong to a single taxon. Hamaguchi et al. (2017) identified *O. stentina* in Japan and recognized significant divergence within the *O. stentina* complex. Guo et al. (2018) suggested that the *O. stentina* species complex may consist of three species: *O. equestris*, *O. stentina* A, and *O. stentina* B.

Phylogenetic analysis of all available sequences of the *Ostrea stentina* complex revealed four closely related groups, which

TABLE 5.

K2P genetic distances in partial 16S rDNA sequence among oyster species studied.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Hap 2*																		
2. Hap 4*	0.010																	
3. Hap 8*	0.007	0.002																
4. Hap 12*	0.010	0.005	0.002															
5. Hap 13*	**0.012**	**0.010**	**0.012**	**0.015**														
6. *Ostrea aupouria AF052064*	0.010	0.005	0.002	0.005	**0.015**													
7. *Ostrea equestris AY376603*	0.002	0.007	0.005	0.007	**0.012**	0.007												
8. *Ostrea stentina EU409056*	**0.012**	**0.012**	**0.015**	**0.017**	0.002	**0.017**	**0.012**											
9. *O. stentina KJ818210*	**0.014**	**0.012**	**0.010**	**0.012**	**0.017**	**0.012**	**0.015**	**0.019**										
10. *Ostrea lurida* 2*	0.035	0.035	0.032	0.035	0.042	0.035	0.032	0.042	0.040									
11. *Ostrea conchaphila KT317144*	0.032	0.029	0.032	0.034	0.034	0.034	0.034	0.034	0.039	**0.012**								
12. *Ostrea angelica KT317133*	0.022	0.024	0.022	0.024	0.027	0.024	0.022	0.027	0.027	**0.005**	**0.010**							
13. *Ostrea permollis AF052075*	0.045	0.047	0.045	0.047	0.050	0.047	0.045	0.050	0.042	0.035	0.037	0.029						
14. *Ostrea puelchana AF052073*	0.040	0.042	0.040	0.042	0.045	0.042	0.040	0.045	0.037	0.030	0.032	0.024	**0.002**					
15. *Ostrea edulis* 3*	0.080	0.080	0.080	0.077	0.083	0.083	0.080	0.083	0.083	0.069	0.069	0.061	0.069	0.064				
16. *Ostrea angasi**	0.086	0.086	0.086	0.083	0.088	0.089	0.086	0.088	0.088	0.075	0.074	0.067	0.074	0.069	**0.005**			
17. *Crassostrea gigas gigas JF808180*	0.192	0.192	0.192	0.189	0.189	0.195	0.189	0.189	0.205	0.186	0.189	0.189	0.202	0.200	0.186	0.183		
18. *Crassostrea gigas angulata KC170322*	0.185	0.185	0.185	0.182	0.179	0.189	0.182	0.179	0.199	0.179	0.182	0.183	0.195	0.193	0.186	0.183	**0.005**	
19. *Hyotissa imbricata KC847136*	0.340	0.339	0.343	0.347	0.335	0.347	0.343	0.335	0.350	0.331	0.314	0.331	0.352	0.353	0.341	0.350	0.403	0.399

Bold numbers refer interspecific distances among *Ostrea stentina* complex species.

* Sequences obtained in this study. Thirteen haplotypes were identified in this study, and five are presented here. Hap 13 is *Ostrea neostentina* and Hap 2/4/8/12 are *Ostrea equestris*.

TABLE 6.

K2P genetic distances in partial COI sequence among oyster species studied.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Hap 1*																		
2. Hap 2*	**0.036**																	
3. Hap 3*	0.008	**0.038**																
4. Hap 4*	0.003	**0.040**	0.012															
5. Hap 5*	0.002	**0.038**	0.010	0.005														
6. *Ostrea* sp. *JQ027291*	0.000	**0.036**	0.008	0.003	0.002													
7. *Ostrea aupouria DQ640080*	0.008	**0.034**	0.010	0.012	0.010	0.008												
8. *Ostrea stentina DQ226517*	**0.034**	0.002	**0.036**	**0.038**	**0.036**	**0.034**	**0.033**											
9. *O. stentina KJ818237*	**0.038**	**0.040**	**0.043**	**0.041**	**0.040**	**0.038**	**0.040**	**0.042**										
10. *Ostrea lurida KT317504*	0.135	0.133	0.137	0.135	0.137	0.135	0.133	0.131	0.133									
11. *Ostrea conchaphila KT317494*	0.135	0.133	0.137	0.135	0.137	0.135	0.129	0.131	0.133	**0.002**								
12. *Ostrea angelica KT317449*	0.135	0.146	0.137	0.130	0.137	0.135	0.133	0.144	0.124	0.098	0.098							
13. *Ostrea permollis DQ226526*	0.116	0.121	0.114	0.112	0.118	0.116	0.114	0.119	0.121	0.136	0.140	0.139						
14. *Ostrea puelchana DQ226521*	0.120	0.125	0.118	0.116	0.122	0.120	0.118	0.123	0.124	0.124	0.129	0.145	**0.025**					
15. *Ostrea edulis KJ818235*	0.212	0.223	0.209	0.209	0.214	0.212	0.215	0.220	0.210	0.192	0.197	0.190	0.212	0.194				
16. *Ostrea angasi AF540598*	0.222	0.233	0.214	0.220	0.225	0.222	0.220	0.230	0.225	0.192	0.197	0.197	0.204	0.187	**0.019**			
17. *Crassostrea gigas gigas JF700177*	0.282	0.282	0.282	0.282	0.279	0.282	0.277	0.279	0.279	0.287	0.284	0.274	0.298	0.289	0.308	0.294		
18. *Crassostrea gigas angulata KC170323*	0.285	0.280	0.285	0.285	0.282	0.285	0.279	0.277	0.271	0.288	0.285	0.274	0.287	0.287	0.314	0.306	**0.026**	
19. *Hyotissa imbricata AB076917*	0.532	0.509	0.538	0.532	0.538	0.532	0.521	0.504	0.532	0.515	0.520	0.542	0.519	0.492	0.542	0.542	0.578	0.573

Bold numbers refer interspecific distances among *Ostrea stentina* complex species.

* Sequences obtained in this study. Twenty-five haplotypes were identified in this study, and five are presented here. Hap 2 is *Ostrea neostentina* and Hap 1/3/4/5 are *Ostrea equestris*.

TABLE 7.

Net average K2P genetic distances in COI (below diagonal) and 16S (above diagonal) among *Ostrea stentina* complex species groups: 1 & 2, *Ostrea equestris*; 3, *Ostrea neostentina*; and 4, *Ostrea stentina*.

Groups	1	2	3	4
1	–	0.007	0.015	0.012
2	0.012	–	0.013	0.015
3	0.038	0.035	–	0.019
4	0.041	0.042	0.041	–

supports recent and ongoing speciation of *Ostrea* oysters (Guo et al. 2018). The genetic distances between groups 1 and 2 are 1.2% in COI and 0.7% in 16S, well below the level expected for interspecific divergence, considering the distances between subspecies *Crassostrea gigas gigas* and *Crassostrea gigas angulata* are 2.22%–3.37% in COI and 1.05%–1.32% in 16S (Wang et al. 2010). It has been suggested that oysters with a divergence in COI below 2% should be generally considered as the same species (Guo et al. 2018). Thus, oysters in groups 1 and 2 are accepted as one species. As the name *Ostrea equestris* (Say, 1834) predated *Ostrea aupouria* (Dinamani & Beu 1981), oysters of groups 1 and 2 should take the name of *O. equestris*. All *Ostrea* oysters collected from China belong to group 1—*O. equestris*, except for one sequence belonging to group 3, both are the first records for China. A sequence from a specimen from Taiwan submitted to GenBank under the name *Ostrea* sp. is also *O. equestris*. Results of this study indicate that *O. equestris* is widely distributed along the southern coast of China (Fig. 1).

These results greatly expand the known geographic distribution of *Ostrea equestris*, now including Asian specimens from China, Japan, and New Zealand, as well as American specimens from Argentina, Columbia, Florida, North Carolina, and Gulf of California on the Pacific side. There are clear and significant divergences between the Asian Pacific and American populations. All specimens from the Americas belonged to group 2, and all Asian Pacific specimens belonged to group 1, except for one specimen (16S Hap 11) from Hong Kong, which is clustered with the American samples. Hong Kong is an international hub of ship traffic, and whether the lone specimen represents a foreign introduction requires further studies. The wide distribution of *O. equestris* in both Atlantic and Pacific oceans, from Argentina, Columbia, the United States, Gulf of California, Japan, China, to New Zealand, is unusual and highlights wide connectivity of *Ostrea* species across oceans. The species of group 3 also had a wide distribution, ranging from the Mediterranean Sea to Hong Kong and Japan. Such a wide geographic distribution of a single species is rare for *Crassostrea* species (Guo et al. 2018). The unique biology and life history of these *Ostrea* species, such as hermaphroditism and small size, may increase the odds of colonization in nonnative environments during range expansion. The equatorial currents and Pacific Islands may be important in the range expansion of *O. equestris*. The circumglobal distribution of *Neopycnodonte cochlear* may be related to its deep-sea environment (Harry 1985, Guo et al. 2018). Understanding the wide geographic distribution of oyster species is an important part of marine phylogeography.

Genetic distances in COI among groups 1 + 2, group 3, and group 4 ranged from 3.5% to 4.2%, well above the 2% level commonly accepted for interspecific divergence (Guo et al. 2018) and the divergence between subspecies *Crassostrea gigas gigas* and *Crassostrea gigas angulata* (2.22%–3.37%) (Wang et al. 2010) and between *Ostrea permollis* and *Ostrea puelchana* (2.5%). Based on their significant divergence and their mostly distinctive distributions, groups 1 + 2, 3, and 4 should be accepted as separate species. As groups 1 and 2 are accepted as *Ostrea equestris*, groups 3 and 4 are considered as two species independent of *O. equestris*. This finding is consistent with the previous suggestion of three species in the *Ostrea stentina* species complex: *O. equestris*, *O. stentina* A, and *O. stentina* B (Guo et al. 2018).

The original record of *Ostrea stentina* was from Mediterranean Corsica described by Payraudeau (1826). It is characterized by strongly crenellated valves with the teeth fitting into each other. Shells of *O. stentina* holotype is shown in Figure 3M, and shells reported by Huber (2010), L1 and L2 in Figure 3, somewhat resemble the original description. In both reports, shells of *O. stentina* are characterized by regularly waved edges. Shells of group 3 oysters collected from the Gulf of Hammamet (O1–O4 in Figure 3; Dridi et al. 2008) and China (Fig. 2A1, A2) are clearly different from that of *O. stentina* holotype and Huber's specimen and should be accepted as a new species. Thus, group 3 is accepted as a new species and named *Ostrea neostentina* sp. nov. for its close relationship with *O. stentina*.

Systematics and Description of the New Species Ostrea neostentina *sp. nov.*

Order: Ostreida (Férussac, 1822).

Family: Ostreidae (Rafinesque, 1815).

Subfamily: Ostreinae (Rafinesque, 1815).

Genus: *Ostrea* (Linnaeus, 1758).

Ostrea neostentina sp. nov. new species (Fig. 2A1, A2 and 3O1–O4).

Type Locality

Hong Kong, China.

Material Examined

Holotype, 1 shell (SL 16 × SW 7 × SH 27 mm) (deposited in the Marine Biological Museum of the Chinese Academy of Sciences, accession number: MBM286577).

Description

Shells are elongate-ovate and small, with height greater than length. Exterior color is mainly pewter or close to the color of stone. The left valves adhere to the substrate over a large area. The interior of the right valve is green, and in the left valve, it is white with green. There are a series of fine crenellates on both sides of the ligament along the edges of the right valves. The adductor muscle scar is rounder and larger than that in other species. The umbo is closer to posterior.

Etymology

The species is closely related to *Ostrea stentina*, but a new species.

Habitat

The holotype specimen was collected by trawling from shallow water within 20 m depth.

Distribution

It is found in Hong Kong of China, Kagoshima and Ibusuki in Japan, and Gulf of Hammamet in Tunisia.

It should be noted that shells of group 4 (Fig. 3N1–N3, reference), which is widely accepted as *Ostrea stentina*, are quite different from the shells of the holotype (Fig. 3M). Therefore, the currently recognized *O. stentina*, as represented by multiple sequences in GenBank, may not be the original *O. stentina* of Payraudeau (1826), although they are from the same area. It is possible that there is another small flat oyster that matches the *O. stentina* holotype in shell morphology. If so, the currently accepted name *O. stentina* should be reconsidered.

Evolutionary History

According to the Paleobiology Database at Fossil Works (http://fossilworks.org/), *Ostrea stentina* was found in Algeria and Morocco during Miocene and in Angola, Italy, and Namibia during Quaternary. In western Atlantic, *Ostrea equestris* was in Panama and Venezuela during Miocene and in Brazil, Jamaica, and the United States (Florida) during Quaternary. The divergence time among the groups estimated based on COI sequence dated back to Pleistocene: 0.25–0.31 Mya between groups 1 and 2, 0.72–1.04 Mya between groups 1/2 and 3/4, and 0.86–1.03 Mya between groups 3 and 4. It is consistent with the suggestion of recent and ongoing speciation of living oysters (Guo et al. 2018). The eastern Atlantic and west Mediterranean region where groups 3 and 4 diverged first might be the ancestral home of the *O. stentina* complex. Over time, they expanded to east Mediterranean, western Atlantic, and Pacific via ocean currents, and subsequent isolation led to the speciation of *Ostrea neostentina* sp. nov. and *O. equestris*. Dridi et al. (2008) held that the Siculo-Tunisian Strait between eastern and northern Tunisia might act as a barrier effecting the differentiation of *O. stentina* and the speciation of *O. neostentina* sp. nov. *Ostrea neostentina* sp. nov. occurs in both eastern and western Mediterranean and also in Hong Kong and Japan. Its occurrence in Asia may be due to its range expansion or human activities as its haplotype diversity in Hong Kong and Japan is low. It is likely that other populations of *O. neostentina* exist along the coast of the Indian Ocean.

In conclusion, this study identified small *Ostrea* species from China, Japan, and the United States and clarified the taxonomy of the *Ostrea stentina* species complex. Phylogenetic analysis confirms that the *O. stentina* species complex consisted of three species: *Ostrea equestris* from the Americas and Asia, *O. stentina* from eastern Atlantic, and a new species *Ostrea neostentina* sp. nov. from the Mediterranean Sea and Asia. This study adds to the understanding of the diversity, global distribution, and evolution of *Ostrea* species, providing support for recent and ongoing speciation of oysters (Guo et al. 2018). It also highlights the need for global surveys to fully understand the diversity and evolution of living oysters.

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