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DNA barcoding of flat oyster species reveals the presence of *Ostrea stentina* Payraudeau, 1826 (Bivalvia: Ostreidae) in Japan

Masami Hamaguchi[1*], Miyuki Manabe[2], Naoto Kajihara[1], Hiromori Shimabukuro[1], Yuji Yamada[3] and Eijiro Nishi[4]

Abstract

Background: DNA barcoding is an effective method of accurately identifying morphologically similar oyster species. However, for some of Japan's *Ostrea* species there are no molecular data in the international DNA databases.

Methods: We sequenced the mitochondrial large subunit ribosomal DNA (LSrDNA) and cytochrome c oxidase subunit I (COI) gene of five known and two unidentified *Ostrea* species. Phylogenetic comparison with known *Ostrea* species permitted accurate species identification by DNA barcoding.

Results: The molecular data, which were deposited in an international DNA database, allowed for a clear distinction among native *Ostrea* species in Japan. Moreover, the nucleotide sequence data confirmed that *O. stentina* (Atsuhime-gaki) inhabits Kemi and Ibusuki, Japan.

Conclusions: This is the first record of *O. stentina* in Japan. These results provided for accurate species identification by DNA barcoding of the taxonomically problematic species *O. futamiensis*, *O. fluctigera*, *O. setoensis* and *O. stentina* in Japan.

Keywords: *Ostrea stentina*, *O. futamiensis*, *O. fluctigera*, *O. setoensis*, DNA barcoding

Background

Over the last half century, Japan's coastal ecosystems have been severely damaged by human activity. The Seto Inland Sea, which is surrounded by the Japanese main islands of Honshu, Shikoku and Kyushu, is located in the western part of Japan and is an area of human-induced ecological deterioration. The coastal areas were reclaimed for urban and industrial use during a period of rapid economic growth in the 1970s, leading to the loss of 63.7% of the natural coast (tidal flats, seagrass beds and estuary systems). Habitat loss, pollution, overfishing, invasive species and now global climate change are rapidly damaging the Seto Inland Sea. These factors have gradually decreased the biodiversity of the area, and many marine organisms have become endangered. Although many native and relict species of the last glacial epoch from the ancient East China Sea are found in the Seto Inland Sea (Botton et al. 1996; Futahashi 2011; Hamaguchi et al. 2013), other invasive alien and indigenous species have been discovered where human activity has led to the development of industrial areas along the coast (Iwasaki et al. 2004). Therefore, since 2008 we have been conducting a long-term study to monitor benthic species diversity at various tidal flats to promote the conservation of native marine fauna in the Seto Inland Sea and its adjacent marine areas supported by the Ministry of the Environment Monitoring Sites 1000 Project and the Japan Long Term Ecological Research Network.

We observed two morphologically different putative *Ostrea* species (*Ostrea* sp. A and *Ostrea* sp. B) during the field surveys. The external features of *Ostrea* sp. A were very similar to those of *O. futamiensis* Seki 1929

* Correspondence: masami@fra.affrc.go.jp
[1]National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan
Full list of author information is available at the end of the article

while some morphological features were not identical. We considered that *Ostrea* sp. A might be a juvenile form of another *Ostrea* species present in Japan. The external features of Ostrea sp. B were very similar to those of *Crassostrea gigas* Thunberg, 1793 and we misidentified the oyster as *C. gigas* at first. Some morphological features of *Ostrea* sp. B were similar to those of *O. stentina* Payraudeau, 1826 but this species has not previously been reported from Japan. Therefore, we performed accurate species identification of the oyster specimens.

In general, Ostreidae species are economically important marine organisms, but their morphological plasticity can cause taxonomic confusion. For example, shell morphology has been used as a primary feature to distinguish different species of oyster; however, the shell is affected by habitat and environment (Tack et al. 1992; Yamaguchi 1994; Lam and Morton 2004, 2006; Liu et al. 2011). In recent years, molecular analyses have been used to accurately identify Ostreidae species. Methods such as DNA barcoding (Hebert et al. 2003; Schindel and Miller 2005) have been used to detect hidden and cryptic species, determine their distributions, monitor the biodiversity of marine fauna and reconstruct the phylogeny of taxonomically confusing *Ostrea* species (Jozefowicz and O'Foighil 1998; Hurwood et al. 2005; Lapègue et al. 2006; Polson et al. 2009; Salvi et al. 2014). Moreover, DNA barcoding can be applied to all life stages of the oyster, e.g. planktonic larvae, spat and juvenile forms.

Five flat oyster species have been reported from Japan. *O. deselamellosa* Lischke, 1869 and *O. circumpicta* Pilsbry, 1904 are fishery and aquaculture species utilized in Japan. Molecular data of these two species have been deposited in the international DNA databases (DDBJ; DNA database of Japan/EMBL; European Molecular Biology Laboratory/GenBank DNA database).

The other three species recorded are small flat oysters, viz. *O. futamiensis*, *O. fluctigera* Jousseume in Lamy, 1925 and *O. setoensis* Habe 1957 about which there is little taxonomical or ecological information. The molecular data of these three species have not as yet been deposited in the international DNA databases.

Ostrea futamiensis Seki 1929 was first discovered in Futamigaura, Hyogo Prefecture, in the eastern part of the Seto Inland Sea (Seki 1929). The oyster has a small (20–35 mm in length), moderately thick and irregularly circular- or oval-shaped shell. The World Register of Marine Species (WoRMS; http://www.marinespecies.org/) lists *O. futamiensis* as a valid species. However, this species is not commercially important in Japan, and thus ecological and chorological research on this oyster is incomplete (Okutani 2000; Iijima 2007). Wada et al. (1996) recommended that *O. futamiensis* be designated a near-threatened species. However, Henmi et al. (2014) summarized claims made by other marine benthic researchers who maintain that *O. futamiensis* should not be designated as near-threatened because *O. futamiensis* is possibly a junior synonym of *O. denselamellosa*.

Ostrea fluctigera Jousseume in Lamy, 1925 is a hard-to-find and taxonomically problematic species. The species is small and settles on hermit crab shells. Inaba and Torigoe (2004) re-classified the species and concluded that *O. deformis* Lamarck, 1819 and *Nanostrea exigua* Harry 1985 were the synonyms of *O. fluctigera*. There has only been one paper (Kuramochi 2007) published on this topic since the reclassification by Inaba and Torigoe (2004).

Ostrea setoensis Habe 1957 is a small oyster and is also a hard-to-find species in Japan. Habe (1957) described the oyster as *O. sedea setoensis*, which is a subspecies of *O. sedea* Iredale, 1939 from Australia. However, he later transferred the oyster to the genus *Neopycnodonte* (Habe 1977). Torigoe (1983) claimed that it was an *Ostrea* species based on its anatomy and shell morphology and considered it *O. setoensis*.

As described above, species identification of *O. futamiensis*, *O. fluctigera*, *O. setoensis, Ostrea* sp. A and *Ostrea* sp. B by DNA barcoding has not been possible until now because no nucleotide sequence data from these oyster species has been deposited in international DNA databases.

In this study, we collected *O. futamiensis*, *O. fluctigera* and *Ostrea* sp. A from the Seto Inland Sea, and other *Ostrea* oysters including *Ostrea* sp. B and *O. setoensis* from Japanese waters elesewhere. We analyzed the nucleotide sequences of the mitochondrial large subunit ribosomal RNA (LSrRNA) and the cytochrome *c* oxidase subunit I (COI) gene to facilitate DNA barcoding of the members of the genus *Ostrea*.

Methods

Sample collection and morphological identification of Ostrea species in Japan

Ostrea sp. A and *O. fluctigera* specimens were sampled from the Kemi tidal flat in the Wakayama Prefecture. *Ostrea* sp. B were collected from Ibusuki in Kagoshima Bay. *Ostrea* sp. B was settled onto polyvinyl chloride plates used to culture *Crassostrea nippona* Seki, 1934 oysters at the Kagoshima Prefectural Fisheries Technology and Development Center. *O. futamiensis* specimens were sampled from five tidal flats (Nakatsu, Oiso, Hishiwo, Hinase and Kemi) in the Seto Inland Sea. *O. setoensis* specimens were sampled from the Tamanoura tidal flat in the Wakayama Prefecture. *O. circumpicta* and *O. denselamellosa* were collected from the Yamagata and Kumamoto Prefectures, Japan, respectively. All the oyster collection sites are shown in Fig. 1 and Table 1. *O. lurida* Carpenter, 1864 was collected from Willapa Bay, Washington State,

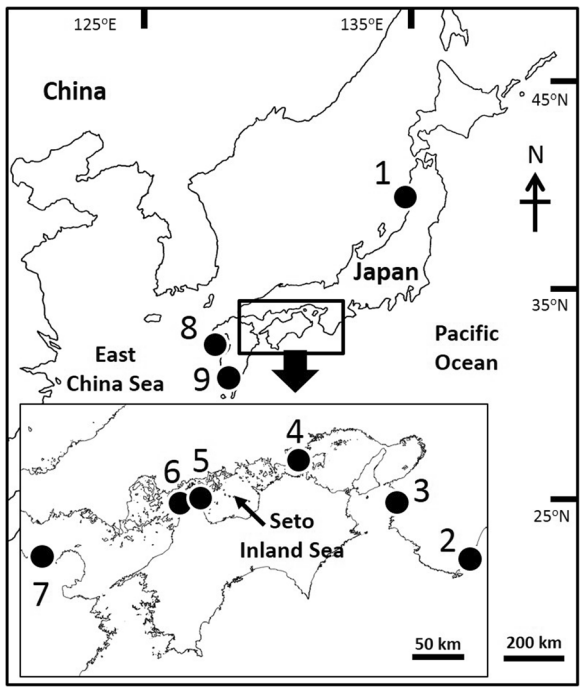

Fig. 1 Sampling sites of the *Ostrea* specimens used in this study

USA by Dr. Hori and Prof. Ruesink and compared with Japanese *Ostrea* species. We observed shell characteristics (i.e. shell shape and external features, growth lines, lamellae and ribs, umbo position and shape) and inner surface features (pallial sinus, adductor muscle scar shape and position and chomata), shell colour and hinge type. The oyster specimens were identified using these morphological features according to Seki (1929, 1930), Torigoe (1981), Inaba and Torigoe (2004) and Harry (1985). The specimens examined in this study were deposited in the Osaka Museum of Natural History (OMNH).

DNA preparation

All *O. futamiensis, O. fluctigera* and *O. setoensis* specimens were transported live to our laboratory in Hiroshima Prefecture, Japan. The adductor muscle of each individual organism was excised and preserved in 80% ethanol. The adductor muscle samples from *O. circumpicta*, *O. denselamellosa*, *Ostrea sp.* A, *Ostrea sp.* B and *O. lurida* obtained from each sampling site were preserved in 80% ethanol until DNA extraction. The total genomic DNA was extracted from all specimens using a DNeasy Blood & Tissue Kit (Qiagen, CA, USA) according to the manufacturer's instructions.

DNA barcoding on the basis of mitochondrial LSrRNA and COI

The mitochondrial LSrRNA and COI genes were subjected to polymerase chain reaction (PCR) amplification using our original primers (16SUF 5′-GAACTCGG CAAAATTAAACCTCGCCT-3′, 16SUR 5′-ARRGKWT TAARGGTCGAACAGA-3′) and universal primers (LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198 5′-TAAACTTCAGGGTGACCAAAA AATCA-3′) as reported by Hamaguchi et al. (2014) and Folmer et al. (1994), respectively. A MyCycler™ Thermal Cycler (Bio-Rad, CA, USA) was used to amplify PCR products in a total volume of 15 μL containing 5 U of Hot Taq™ (5 U/μL; Takara, Otsu, Japan), 10× Hot Taq™ buffer, 2.5 mM of each dNTP, 0.5–1.0 μM of each primer and 0.5 μL of template DNA. The PCR amplification cycles included denaturation at 94 °C for 1 min; 35 cycles of denaturation at 94 °C for 30 s followed by annealing at either 55 °C (LSrRNA) or 40 °C (COI) for 30 s and an extension at 72 °C for 45 s; and a final extension for 5 min at 72 °C. The PCR amplicons were checked by loading 3 μL of each sample with 3 μL of loading dye on a 2% agarose gel (Agarose S; Nippon Gene, Tokyo, Japan) containing

Table 1 Sampling sites in this study

Species	Year	Sampling site		Prefecture or State	Latitude	Longitude	N
Ostrea sp. A	2015	Kemi	Fig. 1-3	Wakayama, Japan	34.159493	135.183504	3
Ostrea sp. B	2015	Ibusuki	Fig. 1-9	Kagoshima, Japan	31.294740	130.604903	7
Ostrea futamiensis	2013	Nakatsu	Fig. 1-7	Oita, Japan	33.604920	131.237633	8
Ostrea futamiensis	2014	Hishiwo	Fig. 1-6	Hiroshima, Japan	34.380379	133.219520	12
Ostrea futamiensis	2014	Ooiso	Fig. 1-5	Hiroshima, Japan	34.398751	133.239540	12
Ostrea futamiensis	2014	Hinase	Fig. 1-4	Okayama, Japan	34.731732	134.276166	7
Ostrea futamiensis	2015	Kemi	Fig. 1-3	Wakayama, Japan	34.159493	135.183504	6
Ostrea fluctigera	2015	Kemi	Fig. 1-3	Wakayama, Japan	34.159493	135.183504	4
Ostrea setoensis	2015	Tamanoura	Fig. 1-2	Wakayama, Japan	33.568484	135.918252	3
Ostrea circumpicta	1999	Yura	Fig. 1-1	Yamagata, Japan	38.720467	139.675662	8
Ostrea denselamellosa	2008	Midori-River	Fig. 1-8	Kumamoto, Japan	32.720389	130.593348	16
Ostrea lurida	2013	Willapa Bay		Washington State, USA			12

0.5 μg/mL ethidium bromide. The remaining 12 μL of PCR product was subsequently purified using a QIAquick PCR Purification Kit (Qiagen, CA, USA).

The purified PCR amplicons were sequenced using the LSrRNA or COI primers as described above and the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) in a Genetic Analyzer 3130 *xl* automated DNA Sequencer (Applied Biosystems, CA, USA). The final LSrRNA and COI sequences were obtained from both strands for verification, and all newly obtained sequences were deposited in the DDBJ/EMBL/GenBank databases. The accession numbers were as follows: *Ostrea* sp. A, LC051572–LC051574; *Ostrea* sp. B, LC051575–LC051581; *O. futamiensis*, AB898267–AB898274, LC051592-051609; *O. fluctigera*, LC149503–LC149510; *O. setoensis*, LC149511–LC149516; *O. denselamellosa*, AB898275–AB898279; *O. circumpicta*, AB898279–AB898282; and *O. lurida*, AB898263–AB898266.

Comparison of the molecular data of native Japanese Ostrea species with those of known Ostrea species reported worldwide

The LSrRNA sequences of our samples were compared with those of the other known *Ostrea* species using the BLAST search in GenBank. The taxonomic separation among native and other *Ostrea* species was analysed by constructing a maximum parsimony tree for the LSrRNA sequences (424 bp). The 19 nominal *Ostrea* species of which the LSrRNA sequences were compared were (accession numbers in brackets) *Ostrea* sp. A (LC051572), *Ostrea* sp. B (LC051575), *O. futamiensis* (AB898267), *O. fluctigera* (LC149507), *O. setoensis* (LC149514), *O. denselamellosa* (AB898275), *O. circumpicta* (AB898279) and *O. lurida* (AB898263), as well as the LSrRNA sequences available in the international DNA databases for *O. angasi* Sowerby, 1871 (AF052063), *O. algoensis* Sowerby II, 1871 (AF052062), *O. aupouria* Dinamani, 1981 (AF052064), *O. chilensis* Philippi in Küster, 1844 (JF808186), *O. conchaphila* Carpenter, 1857 (FJ768527), *O. edulis* Linnaeus, 1758 (DQ093488), *O. equestris* Say, 1834 (AY376603), *O. puelchana* d'Orbigny, 1842 (AF052073), *O. stentina* (JF808189 and DQ180744), *O. spreta* d'Orbigny, 1846 (DQ640402) and *Ostrea* sp. JL-2011 (HQ661001). The LSrRNA sequence for *Saccostrea glomerata* Gould, 1850 (AF353101) was used as an outgroup. The sequences obtained for each region were aligned using ClustalW (Thompson et al. 1994; gap opening penalty, 15; gap extension penalty, 6.6; transition weight, 0.5), and the MP tree based on the Tamura 3-parameter model (Tamura 1992) was reconstructed in MEGA version 6 (Tamura et al. 2013).

Estimates of evolutionary divergence between sequences within the COI of each *Ostrea* species were calculated using the Kimura 2-parameter model (K2P; Kimura 1980) in MEGA version 6. The 19 nominal *Ostrea* species of which the COI sequences were compared were *Ostrea* sp. A (LC051584), *Ostrea* sp. B (LC051590), *O. futamiensis* (AB898290), *O. circumpicta* (AB898294), *O. fluctigera* (LC149507) and *O. setoensis* (LC149514), as well as *O. angasi* (AF540598), *O. aupouria* (AF112288), *O. chilensis* (AF112286), *O. edulis* (AF120651), *O. equestris* (AY376607), *O. conchaphila* (DQ464125), *O. denselamellosa* (NC015231), *O. lurida* (NC022688), *O. puelchana* (DQ226518), *O. stentina* (DQ226522), *Ostrea* sp. MS-2011 (JF915514) and *Ostrea* sp. STH-2012 (JQ027292) whose COI sequences were available from the international DNA databases (DDBJ/EMBL/GenBank). The COI sequence for *Saccostrea glomerata* (EU007483) was used as an outgroup.

Results

Morphological features of unknown Ostrea species in Japan

We compared *Ostrea* sp. A with native *Ostrea* species and their juvenile forms. Most of the important external features of *Ostrea* sp. A were very similar to those of *O. futamiensis*; for example, the samples OMNH-Mo38148 (*Ostrea* sp. A; Fig. 2-1) and OMNH-Mo38141 (*O. futamiensis*; Fig. 2-2) both had partially embedded stones on a sandy tidal flat attached to their undersides (Kemi tidal flat; Fig. 1-3). The right valves of *Ostrea* sp. A (OMNH-Mo38148; Fig. 3-1) and those of coexisting *O. futamiensis* (OMNH-Mo38141; Fig. 3-2) were also similar and are shown in Fig. 3. Shell shapes of *Ostrea* sp. A were elliptical and flat. The left valves were very thin, and shell height and length were less than 15 and 10 mm, respectively. Chomata, of which there were approximately 15–30, were inconspicuous and restricted to both ligament sides. The umbonal cavities were shallow. The adductor muscle scars were reniform, and the dorso-anterior borders were concave. External color of

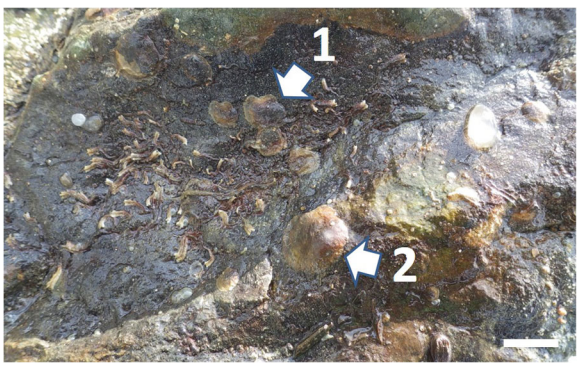

Fig. 2 *Ostrea* sp. A (1: OMNH-Mo38148) and *Ostrea futamiensis* (2: OMNH-Mo38141) in the Kemi tidal flat (Fig. 1. 3). Scale bar: 10 mm

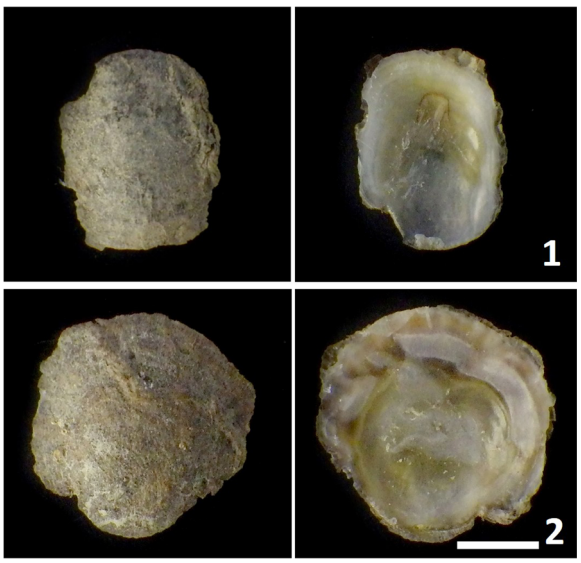

Fig. 3 The right valve of *Ostrea* sp. A (1: OMNH-Mo38148) and *Ostrea futamiensis* (2: OMNH-Mo38141) collected from the Kemi tidal flat. Scale bar: 10 mm

the right valves was opaque white to light brown with many dark brown streaks radiating from the ambo (Fig. 4). The interior shells were composed of olive to yellowish green conchiolin and a white calcareous layer, which was sometimes narrow (Fig. 4).

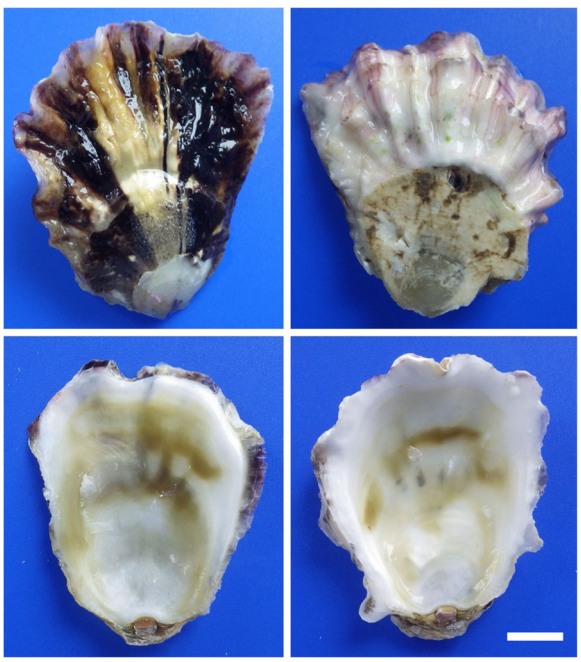

Fig. 4 Morphological features of *Ostrea* sp. B (OMNH-Mo38134) collected from the Kagoshima Prefectural Fisheries Technology and Development Center in Ibusuki. Scale bar: 10 mm

However, there were also several differences between *Ostrea* sp. A and *O. futamiensis* features. The shell shape of *Ostrea* sp. A was elliptical, and that of *O. futamiensis* was circular. Shape of the adductor muscle scar was very similar, but that of *Ostrea* sp. A was narrow compared with that of *O. futamiensis*. Position of the adductor muscle scar was below the center of the interior shell for *Ostrea* sp. A, whereas that of *O. futamiensis* was in the center of the interior shell. However, almost all morphological features of *Ostrea* species have been recorded from adult specimens. Therefore, we could not confirm by morphological features alone if *Ostrea* sp. A was a juvenile form of another *Ostrea* species.

The shell of *Ostrea* sp. B (OMNH-Mo38134) was orbicular and spatulate with many wrinkles, and with a height and length of less than 50 and 40 mm, respectively (Fig. 4). The external colour of the right valve was yellowish white to light brown with dark brown or black streaks radiating from the umbo. Initially, we misidentified the oyster as *C. gigas,* because the external colour and shape was very similar to that of *C. gigas.* However, the adductor muscle scar was colourless and reniform, and the dorso-anterior border was concave. The adductor muscle scar of *C. gigas* is light-coloured, purple, or brown. The chomata of the oysters were inconspicuous and restricted to each ligament side. These chomata features showed that the oyster belonged to genus *Ostrea*; *C. gigas* have no chomata. The colour of the interior shell was partly olive to yellowish green conchiolin in a white calcareous layer, whereas the interior shell of *C. gigas* is white. These morphological features clearly differed between *C. gigas* and *Ostrea* sp. B. Moreover, the external shell features of *Ostrea* sp. B were different from those of other known Japanese *Ostrea* oysters, but external and internal shell features, chomata, adductor muscle scar were similar to those of *O. stentina*.

Both *Ostrea* sp. A and *Ostrea* sp. B had inconspicuous chomata restricted to each ligament side, but other morphological features were different (Figs. 3 and 4). Although external shell features of *Ostrea* sp. B were similar to those of *O. stentina*, *O. stentina* has not been recorded from Japan. Therefore, we considered *Ostrea* sp. A and *Ostrea* sp. B to be different putative species based on morphological features, but molecular analysis by DNA barcoding is needed for accurate identification of these oysters.

DNA barcoding

The phylogenetic analysis of the LSrRNAs is shown in Fig. 5. The nucleotide sequences of both *Ostrea* sp. A and B clustered together in the *O. stentina* complex, which consisted of *O. stentina*, *O. aupouria*, *O. equestris* and *O. spreta. Ostrea* sp. A and B were clearly distinct

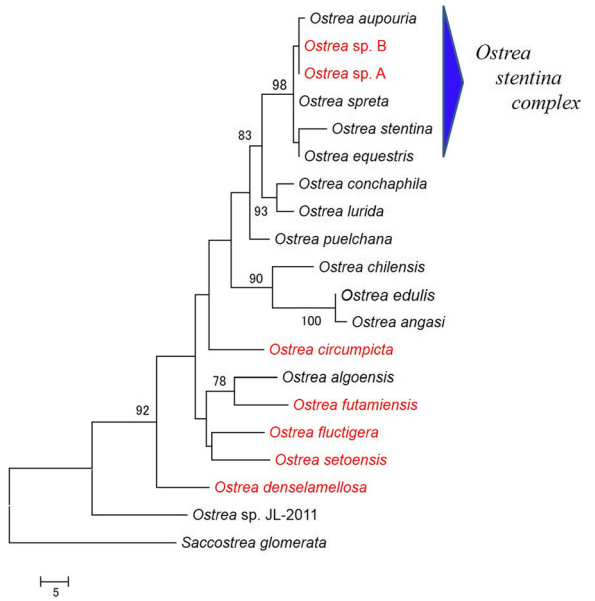

Fig. 5 Maximum parsimony tree of LSrRNA for *Ostrea* sp. rooted with *Saccostrea glomerata*. Bootstrap values above 70% are shown. *Ostrea* species native to Japan are indicated in red. Scale bar indicates five substitutions per 100 site

from the native *Ostrea* species cluster (*O. denselamellosa*, *O. circumpicta, O. futamiensis, O. fluctigera and O. setoensis). Ostrea* sp. A, *Ostrea* sp. B, *O. stentina*, *O. aupouria*, *O. equestris* and *O. spreta* were closely related. In contrast, the LSrRNA nucleotide sequences of *O. futamiensis*, *O. fluctigera* and *O. setoensis* revealed that these species were clearly distinct from all of the other *Ostrea* species.

Estimates of evolutionary divergence between the COI sequences obtained from the *Ostrea* species and *Saccostrea glomerata* as outgroup are shown in Table 2. The overall average evolutionary divergence was 0.205. The evolutionary divergences among *O. futamiensis*, *O. fluctigera, O. setoensis* and all of the other *Ostrea* species in the COI sequences ranged from 0.176 to 0.263, with clear differences between native Japanese species and known *Ostrea* species from elsewhere in the world. The *Ostrea* sp. A COI sequence was identical to that of *Ostrea* sp. B. The evolutionary divergences among *Ostrea sp.* A, *Ostrea sp.* B, *Ostrea* sp. STH-2012 (accession number JQ027292) and *O. aupouria* in the COI sequences ranged from 0 to 0.004. This indicated that these are the most closely related of the known *Ostrea* species. The WoRMS database gives *O. aupouria* Dinamani, 1981 as a synonym of *O. stentina* Payraudeau, 1826 at present. Consequently, we conclude that *Ostrea* sp. A and B are *O. stentina* Payraudeau, 1826. This is the first record of *O. stentina* from Japanese waters. Although *Ostrea* sp. A and B differed morphologically, the molecular data clearly showed that these are the same species, viz. *O. stentina*, according to both the LSrRNA and COI genes. We concluded that *Ostrea* sp. A was a juvenile form of *Ostrea stentina*.

The genetic analysis of the native Japanese *Ostrea* species *O. denselamellosa, O. circumpicta, O. futamiensis*, *O. fluctigera* and *O. setoensis* clearly distinguished them from known *Ostrea* species from around the world. Furthermore, molecular data indicated that *O. futamiensis* is distributed throughout the Seto Inland Sea.

Discussion

We identified two putative unidentified *Ostrea* species, *Ostrea* sp. A and B, during our long-term study of benthic species diversity in the Seto Inland Sea and its adjacent marine areas. Although *Ostrea* sp. A and B differed morphologically, the molecular data identified them as *Ostrea stentina*. This confirmed the difficulty in identifying *Ostrea* species by morphological features alone. The Olympia oyster, *O. lurida*, is a commercially important species on the Northwest Pacific Coast of the United States of America and Canada (Bulseco 2009) and is morphologically very similar to *O. conchaphila.* Harry (1985) proposed that these two species were synonymous because of common species-specific morphological features caused by high phenotypic plasticity. Polson et al. (2009) compared the species using molecular markers and post-hoc morphological characteristics and concluded that *O. lurida* and *O. conchaphila* were separate species. In this manner, DNA markers and molecular biological methods have been used to resolve taxonomic problems caused by species identification of flat oysters based on morphological features alone (O'Foighil et al. 1999; Jozefowicz and O'Foighil 1998; Hurwood et al. 2005; Lapègue et al. 2006; Lazoski et al. 2011; Pejovic et al. 2016).

In recent years, DNA barcoding, a term coined by Hebert et al. (2003), has been used effectively to identify many animal and plant species. Furthermore, this method allows accurate species identification of morphologically similar species. DNA barcoding has previously been used to identify a various oyster species as well as newly invasive alien and cryptic species (Banks and Hedgecock 1993; O'Foighil et al. 1998; Hedgecock et al. 1999; Boundry et al. 2003; Lam and Morton 2003; Lapègue et al. 2004; Chen et al. 2011; Liu et al. 2011; Melo et al. 2010; Hong et al. 2012; Crocetta et al. 2013a,b; Gal-Vao et al. 2013; Hamaguchi et al. 2013; Sekino and Yamashita 2013; Wu et al. 2013; Hamaguchi et al. 2014; Sekino et al. 2014; Xia et al. 2014).

We identified *O. stentina* in Japanese waters via DNA barcoding and propose "Atsuhime-gaki" as its Japanese name. While this is an important discovery, the question of whether or not *O. stentina* is a native or an invasive

Table 2 Estimates of evolutionary divergence between the COI Sequences obtained from *Ostrea* species and *Saccostrea glomerata*

Species	Accession No.	Abbriviation	Abbriviation																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Ostrea sp. A	LC051584	1																		
Ostrea sp. B	LC051590	2	0.002																	
Ostrea futamiensis	AB898290	3	0.249	0.246																
Ostrea fluctigera	LC149507	4	0.237	0.240	0.218															
Ostrea setoensis	LC149514	5	0.219	0.222	0.213	0.204														
Ostrea circumpicta	AB898294	6	0.252	0.256	0.237	0.262	0.231													
Ostrea denselamellosa	NC015231	7	0.227	0.224	0.212	0.240	0.250	0.246												
Ostrea conchaphila	DQ464125	8	0.162	0.164	0.222	0.219	0.176	0.234	0.222											
Ostrea stentina	DQ226522	9	0.009	0.011	0.234	0.234	0.216	0.237	0.221	0.159										
Ostrea aupouria	AF112288	10	0.004	0.005	0.249	0.234	0.216	0.249	0.227	0.164	0.013									
Ostreola equestris	AY376607	11	0.009	0.011	0.234	0.234	0.216	0.237	0.221	0.159	0.000	0.013								
Ostrea sp. STH-2012	JQ027292	12	0.000	0.002	0.249	0.237	0.219	0.252	0.227	0.162	0.009	0.004	0.009							
Ostrea puelchana	DQ226518	13	0.126	0.128	0.263	0.247	0.228	0.224	0.221	0.143	0.118	0.131	0.118	0.126						
Ostrea lurida	NC022688	14	0.146	0.149	0.228	0.210	0.187	0.234	0.195	0.034	0.143	0.149	0.143	0.146	0.136					
Ostrea edulis	AF120651	15	0.237	0.240	0.219	0.213	0.213	0.218	0.210	0.222	0.234	0.234	0.234	0.237	0.216	0.213				
Ostrea chilensis	AF112286	16	0.240	0.237	0.237	0.256	0.247	0.240	0.200	0.260	0.240	0.237	0.240	0.240	0.231	0.231	0.154			
Ostrea sp. MS-2011	JF915514	17	0.246	0.249	0.240	0.246	0.213	0.227	0.195	0.237	0.234	0.250	0.234	0.246	0.198	0.244	0.204	0.259		
Ostrea angasi	AF540598	18	0.250	0.253	0.219	0.225	0.225	0.218	0.207	0.222	0.240	0.247	0.240	0.250	0.207	0.213	0.020	0.159	0.207	
Saccostrea glomerata	EU007483	19	0.296	0.300	0.279	0.315	0.323	0.337	0.304	0.280	0.286	0.304	0.286	0.296	0.283	0.256	0.327	0.341	0.290	0.330

Red character show the native Ostrea oyster species in Japan

alien species remains unanswered. This species is widely distributed along Atlantic, Mediterranean, North African, New Zealand and South American coasts (Lapègue et al. 2006; Gofas et al. 2011; Crocetta et al. 2013a, b; Pejovic et al. 2016). In several cases, supposedly distinct *Ostrea* species in separate geographical areas have been revised to a single species by molecular analysis. Kenchington et al. (2002) reported that the European flat oyster *O. edulis* and *O. angasi* are conspecific based on their molecular analysis. Using mitochondrial COI sequences, O'Foighil et al. (1999) proved that *O. chilensis* is widely distributed from New Zealand to Chile, and they discussed genetic exchanges within transoceanic ranges that occur as a result of rafting. In DNA databases, the COI nucleotide sequences of an oyster collected from Taiwan (*Ostrea* sp. STH-2012, accession number JQ027292) were identical to those of *O. stentina* in Japan. The Kuroshio Current flows past Taiwan Island to the southern part of Japan. In recent years, as a result of global warming, a northward shift in the distribution patterns of tropical marine benthic species has been observed in Japan. Ibusuki in the Kagoshima Prefecture and Kemi in the Wakayama Prefecture, where the *O. stentina* were collected for this study, are located in the southern part of Japan, where subtropical and tropical oyster species have been observed (Hamaguchi et al. 2014). Thus, *O. stentina* from Taiwan could ride the warm Kuroshio Current to Ibusuki and Kemi either by dispersion of planktonic larvae or rafting (O'Foighil et al. 1999). If this is the case, it is likely that *O. stentina* is a native oyster in Japan. Our preliminary survey, in which *O. stentina* was identified along coasts exposed to the Kuroshio Current, supports this hypothesis.

However, many invasive alien species of marine organism have been introduced in Japan by various human activities (Iwasaki et al. 2004); many of these have been introduced by ballast water, hull fouling and sea chests via shipping (Otani 2004). The *O. stentina* used in this study, for example, were collected from the Kemi tidal flat and Ibusuki. An oil storage facility and a private steel plant are located near these sites, and either oil tankers or iron ore ships may have introduced *O. stentina* to the area from the Arabian Sea or from countries bordering the Indo-Pacific Ocean such as Asia, South America and Oceania. In the near future, we will survey the distribution of *O. stentina* in Japan to determine if the oyster is a native or an invasive alien species. If *O. stentina* is a newly invasive alien species, it will undoubtedly impact Japan's native ecosystems (Ruesink et al. 2005).

Another aim of this study was to develop DNA barcoding for the taxonomically confusing species *O. futamiensis, O. fluctigera* and *O. setoensis*. Habe and Itoh (1965), Habe and Kosuge (1967) claimed, based on morphological similarities, that *O. futamiensis* was an ecological variant of the sympatric *O. denselamellosa.* Torigoe and Inaba (1975) compared the electrophoretic patterns of muscle proteins and some morphological features of larvae and of adult shells of three native *Ostrea* species (*O. denselamellosa*, *O. circumpicta* and *O. futamiensis*) and concluded that these were separate species. *O. fluctigera* and *O. setoensis* are small oysters and were re-classified by Torigoe (1983), Inaba (1995) and Inaba and Torigoe (2004). The taxonomic status of both these oyster species is currently unknown.

In this study, we determined the nucleotide sequences of *Ostrea* LSrRNA and COI regions, which are widely used for DNA barcoding. The data confirmed that *O. futamiensis*, *O. fluctigera*, *O. setoensis*, other native *Ostrea* species and the newly found *O. stentina* were distinct from each other. These results strongly support the findings of Torigoe and Inaba (1975) and Inaba and Torigoe (2004). Moreover, the LSrRNA and COI nucleotide sequences both proved that *O. futamiensis,*

O. fluctigera and *O. setoensis* were distinct from the known foreign *Ostrea* species deposited in the DNA database. Habe (1957) reported that *O. setoensis* was a subspecies of *O. sedea*. Iredale, 1939, for which sequence information was not available to us. The molecular data in this study indicated that *O. setoensis* was a separate species. If molecular data for *O. sedea* become available, the taxonomic status of Japan's *O. setoensis* can be confirmed.

We suggest that for the accurate identification of *Ostrea* species with high phenotypic plasticity, both traditional morphological methods and current molecular methods should be used. At present, information on the distribution patterns and ecology of four oysters, *O. stentina, O. futamiensis*, *O. fluctigera* and *O. setoensis*, is incomplete. Additionally, planktonic *O. futamiensis* larvae have distinctive morphological features and coloration and are easy to distinguish from other *Ostrea* species (Torigoe and Inaba 1975). We found *O. futamiensis*-like larvae in planktonic samples collected from Matsushima Bay, in the northern part of Japan. Bussarawit and Cedhagen (2010, 2012) reported that they detected *O. futamiensis*-like larvae in samples collected from Phuket, Thailand; however, surprisingly, the adult species could not be found in any of the samples. In fact, these oysters may be widely distributed from Japan to Southeast Asia. Therefore, DNA barcoding by using our new molecular data of small *Ostrea* oyster species could be useful in surveys of these *Ostrea* species inhabiting Korea, China and other Asian countries.

In the near future, we intend to revise the taxonomic status of the Japanese *Ostrea* species using more molecular data than was included in this study, e.g. the nucleotide sequences of complete mitochondrial DNA, multilocus analysis of mitochondrial DNA and nuclear DNA, and rRNA sequence-structure models (Milbury and Gaffney 2003; Wu et al. 2010; Ren et al. 2009, 2010; Danic-Tchaleu et al. 2011; Wu et al. 2012; Salvi et al. 2014).

Conclusions

In addition to clearly establishing that *O. futamiensis*, *O. fluctigera, O. setoensis* and *O. stentina* are species distinct from the other native oyster species, we also reported the occurrence of *O. stentina*, a new oyster species to Japanese waters. Furthermore, the nucleotide sequence data obtained in this study, which provides significant information on *O. stentina*, *O. futamiensis, O. fluctigera and O. setoensis,* may prove useful for monitoring species diversity in marine fauna. Finally, we offer our results as proof of the need to more fully incorporate the use of DNA barcoding in field studies and monitoring efforts conducted on oyster species.

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Availability of data and materials
Our molecular data are available in international DNA databases (DDBJ/EMBL/GenBank) under the accession numbers given in the text. The specimens examined in this study are available in the Osaka Museum of Natural History (OMNH).

Authors' contributions
MH carried out the molecular analysis on all of the specimens and drafted the manuscript. MM discovered and collected the *Ostrea* sp. B specimens in Kagoshima Prefecture. NK, HS and EN carried out the morphological identification of specimens. All authors collected specimens at various collection sites in Japan. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Availability of supporting data
The dataset supporting the conclusions of this article are included in the text of the article and the molecular data was deposited in the DDBJ/EMBL/GenBank DNA databases.

Author details
[1]National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan. [2]Kagoshima Prefectural Fisheries Technology and Development Center, 160-10 Takada-ue, Ibusuki, Kagoshima 891-0315, Japan. [3]Kurashikiminami High School, 330 Yoshioka, Kurashiki, Okayama 710-0842, Japan. [4]Yokohama National University, 79-1 Tokiwadai, Yokohama, Kanagawa 240-8501, Japan.

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