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

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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
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’Foghlil 1998; Hurwood et al. 2005; Lapégue 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. circumpecta 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 Joussemé 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, Henni 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. deselamellosa.

Ostrea fluctigera Joussemé 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 elsewhere. 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. circumpecta 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,
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).

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            | 34.159493 | 135.183504 | 3  |
| Ostrea sp. B        | 2015 | Ibusuki       | Fig. 1-9            | 31.294740 | 130.604903 | 7  |
| Ostrea futamiensis  | 2013 | Nakatsu       | Fig. 1-7            | 33.604920 | 131.237633 | 8  |
| Ostrea futamiensis  | 2014 | Hishiw0       | Fig. 1-6            | 34.380379 | 133.219520 | 12 |
| Ostrea futamiensis  | 2014 | Osaka         | Fig. 1-5            | 34.398751 | 133.239540 | 12 |
| Ostrea futamiensis  | 2014 | Hine          | Fig. 1-4            | 34.731732 | 134.276166 | 7  |
| Ostrea fluctigera   | 2015 | Kemi          | Fig. 1-3            | 34.159493 | 135.183504 | 6  |
| Ostrea setoensis    | 2015 | Tamanoura     | Fig. 1-2            | 34.356848 | 135.918252 | 3  |
| Ostrea circumpicta  | 1999 | Kemi          | Fig. 1-1            | 38.720467 | 139.675662 | 8  |
| Ostrea denselamellosa| 2008 | Midori-River  | Fig. 1-8            | 32.720389 | 130.593348 | 16 |
| Ostrea lurida       | 2013 | Willapa Bay   |                     | 32.720389 | 130.593348 | 12 |

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 DNAse Blood & Tissue Kit (Qiagen, CA, USA) according to the manufacturer’s instructions.

DNA barcoding on the basis of mitochondrial LSU rRNA and COI

The mitochondrial LSU rRNA and COI genes were subjected to polymerase chain reaction (PCR) amplification using our original primers (16SUF 5′-GAACTCGG CAAAATTAACCTCGCC-3′, 16SUR 5′-ARRGKWT TAARGTGAACAGA-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), 10x 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 (LSU rRNA) 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 Fig. 1 Sampling sites of the Ostrea specimens used in this study.
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. algensis Sowerby II, 1871 (AF052064), O. aequostriata Gould, 1850 (AF052073), O. stentina (JF808189 and DQ180744), O. spreta d’Orbigny, 1846 (DQ464125), O. denselamellosa (NC015231), O. lurida (NC022688), O. puelchana (DQ226518), O. testacea (DQ226522), Ostrea sp. MS-2011 (JF915154) 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
the right valves was opaque white to light brown with many dark brown streaks radiating from the umbo (Fig. 4). The interior shells were composed of olive to yellowish green conchiolin and a white calcareous layer, which was sometimes narrow (Fig. 4).

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.
Ostrea sp. A and B differed morphologically, the sequences obtained from the Ostrea of the other revealed that these species were clearly distinct from all. Consequently, we conclude that Ostrea Payraudeau, 1826 at present. Ostrea Dinamani, 1981 as a synonym of species. The WoRMS database gives that these are the most closely related of the known COI sequences ranged from 0 to 0.004. This indicated (accession number JQ027292) and Ostrea aupouria A, Ostrea sp. Ostrea sp. B. The evolutionary divergences among sp. A COI sequence was identical to that of Ostrea sp. from elsewhere in the world. 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
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 *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 dispersal 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). The taxonomic status of both these oyster species is currently unknown.

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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.

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