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Mitochondrial and nuclear DNA analyses of *Saccostrea* oysters in Japan highlight the confused taxonomy of the genus

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ABSTRACT

The systematics of oysters in the genus *Saccostrea* remains unclear, mainly because their taxonomy has been based on their highly variable shell forms. To obtain insights into the taxonomy of this genus, we evaluated the phylogenetic diversity of Japanese *Saccostrea* oysters on the basis of the nucleotide sequences of four mitochondrial genes and the ribosomal first internal transcribed spacer in the nuclear genome. Our specimens ($N = 226$) were divided into seven mitochondrial lineages. However, the nuclear DNA data provided strong evidence of reproductive independence for only three. For most of the lineages, it was difficult to identify a lineage-specific shell feature, thereby preventing us from establishing a decisive link between the observed lineages and morphology-based species names. Given the extreme difficulty in determining the species of *Saccostrea* oysters on the basis of shell morphology, we suggest that the systematics of this genus should be re-established by laying more emphasis on DNA evidence.

INTRODUCTION

In true oyster species (superfamily Ostreoidea), the taxonomic framework has been established mainly on the basis of their shell morphology and anatomical features (e.g. Stenzel, 1971; Harry, 1985). However, shell form is known to be highly variable depending on life stages as well as habitat characteristics, and is therefore a poor basis for species-level taxonomy (Lam & Morton, 2004, 2006; Reece *et al.*, 2008; Liu *et al.*, 2011; Salvi *et al.*, 2014). This problem is acute in the genus *Saccostrea* Dollfus & Dautzenberg, 1920, in which the shell is highly plastic (Lam & Morton, 2006) and taxonomy very confused.

The supraspecific classification of the true oysters is also controversial. Members of the Ostreoidea have been variously grouped into the two families Gryphaeidae and Ostreidae (e.g. Stenzel, 1971; Harry, 1985; Salvi *et al.*, 2014), into the three Gryphaeidae, Ostreidae and Pycnodonteidae (Inaba & Torigoe, 2004) and into the four Arctostreidae, Flemingostreidae, Gryphaeidae and Ostreidae (Carter *et al.*, 2011). Within Ostreidae, Inaba & Torigoe (2004) defined 11 extant genera, whereas Huber (2010) recognized 18 genera. Of these genera, the major ones are *Crassostrea*, *Dendrostrea*, *Lopha*, *Ostrea*, *Saccostrea* and *Striostrea*, although Inaba & Torigoe (2004) considered *Striostrea* as a synonym of *Saccostrea* (Table 1). The genus *Saccostrea* has been placed in either the subfamily Ostreinae or Crassostreinae, but Salvi *et al.* (2014) erected a new subfamily (Saccostreinae) for *Saccostrea* (Table 1). Raith *et al.* (2015) have recently defined another subfamily (Striostreinae). The tribe Striostreini was proposed for *Saccostrea*, *Striostrea* and *Striostrea* (*Parastriostrea*) by Harry (1985). In contrast, Carter *et al.* (2011) recognized four tribes including Ostreini, Lophini, Crassostreini and Striostreini (without specifying generic composition of each), of which the last two composed the family Flemingostreidae. However, this classification is not supported by more recent phylogenetic studies in Ostreidae (Salvi *et al.*, 2014; Raith *et al.*, 2015). There is clearly a need for further research into the taxonomic status of the genus *Saccostrea*.

The taxonomy within the genus *Saccostrea* is even more complicated (see Table 2 for list of nominal species with authorities). Harry (1985) claimed that the Indo-West Pacific (IWP) *Saccostrea* oysters belong to a single species, *S. cucullata*. Other authors recognize many nominal *Saccostrea* species in this region, but the identities and synonymies of these species remain unclear (Table 2). The morphology-based diagnoses of *Saccostrea* species have focused on external features such as shell size, pattern of radial ribs and grooves, plication of the valve margin, external lamellae (or squamae), internal chomata (marginal wrinkles), hyote (tubular) spines, adductor muscle scar and area of valve attachment (e.g. Inaba & Torigoe, 2004; Lam & Morton, 2006; Huber, 2010). These shell features are variable and do not necessarily serve for the accurate diagnosis of species. For example, Lam & Morton (2006) showed that shell morphology was capable only of separating *S. mordax* from other IWP *Saccostrea* species (see below). Moreover, even with the recent accumulation of mitochondrial DNA (mtDNA) data (Lam & Morton, 2006; Liu *et al.*, 2011; Sekino & Yamashita, 2013; Hamaguchi *et al.*, 2014), which can be used for DNA barcoding (Hebert *et al.*, 2003), their ambiguous shell morphology has

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Table 1. Alternative classifications of six major genera in the family Ostreidae.

References	Family	Subfamily	Tribe	Genus
Stenzel (1971)	Ostreidae	Ostreinae	–	*Ostrea*
				Crassostrea
				Saccostrea
				Striostrea
		Lophinae	–	*Lopha*[*]
Harry (1985)	Ostreidae	Ostreinae	Ostreini	*Ostrea*
		Crassostreinae	Crassostreini	*Crassostrea*
			Striostreini	*Saccostrea*
				Striostrea
		Lophinae	Lophini	*Lopha*
				Dendrostrea
Inaba & Torigoe (2004)	Ostreidae	Ostreinae	–	*Ostrea*
				Crassostrea
				Saccostrea[†]
		Lophinae	–	*Lopha*
				Dendrostrea
Carter *et al.* (2011)	Ostreidae	Ostreinae	Ostreini	–
		Lophinae	Lophini	–
	Flemingostreidae	Crassosatreinae	Crassostreini	–
			Striostreini	–
Salvi *et al.* (2014)	Ostreidae	Ostreinae/ Lophinae[‡]	–	*Ostrea*
				Lopha
				Dendrostrea
		Crassostreinae	–	*Crassostrea*
		Saccostreinae	–	*Saccostrea*
Raith *et al.* (2015)	Ostreidae	Ostreinae/ Lophinae[‡]	–	*Ostrea*
				Lopha
				Dendrostrea
		Crassostreinae	–	*Crassostrea*
		Saccostreinae	–	*Saccostrea*
		Striostreinae	–	*Striostrea*

–, not specified.

[*]Including *Dendrostrea* as a synonym of *Lopha*.

[†]Including *Striostrea* as a synonym of *Saccostrea*.

[‡]Salvi *et al.* (2014) and Raith *et al.* (2015) noted that the relationship between Ostreinae and Lophinae needed further study.

hindered the establishment of links between original morphology-based species names and DNA-based monophyletic groups of individuals.

Lam & Morton (2006) classified the IWP *Saccostrea* species using a combination of shell morphology and sequences of the mitochondrial 16 S rRNA gene (*16 S*). They proposed a 'superspecies' of *S. cucullata*, under which nine mitochondrial lineages (not 'species') were referred to as follows: lineages A–G of *S. cucullata*, *S. kegaki* and *S. glomerata*. Their data also allowed the unambiguous distinction of the *S. cucullata* superspecies from the *S. mordax* superspecies, which comprises mitochondrial lineages A, B and C (lineage C was reported in subsequent studies; Sekino & Yamashita, 2013). Another *16S*-based study focused on Japanese *Saccostrea* species (Hamaguchi *et al.*, 2014) and revised the lineage definitions, also recognizing *S. mytiloides* and two lineages of *S. malabonensis* (although the authorities of these species names were not specified by Hamaguchi *et al.*, 2014; see Discussion).

During our field surveys to determine the distribution of *Saccostrea* species along the coasts of the southern subtropical islands in Japan (Amami and Ryukyu Islands; Fig. 1), we found individuals that did not conform to the shell forms of the proposed nominal species or those given in previous DNA studies (Lam & Morton, 2006; Sekino & Yamashita, 2013; Hamaguchi *et al.*, 2014). This finding motivated us to re-evaluate the phylogenetic diversity of the IWP *Saccostrea* species.

In the present study, we investigated the phylogenetic diversity of the so-called *S. cucullata* superspecies (see below for a discussion of the validity of this species name), because the taxonomy is more confused than that of the *S. mordax* superspecies (Lam & Morton, 2006). To that end, we analysed the nucleotide sequences of four mitochondrial genes. Assessing the presence of reproductive isolation between *S. cucullata* lineages was not possible in previous DNA studies (Lam & Morton, 2006; Sekino & Yamashita, 2013; Hamaguchi *et al.*, 2014), because they exclusively examined uniparentally inherited mtDNA. Thus, we also screened the ribosomal first internal transcribed spacer (*ITS1*) in the nuclear genome, which has been used to substantiate the synonymy of two nominal Australian *Saccostrea* species, *S. glomerata* and *S. commercialis* (Anderson & Adlard, 1994).

In the following sections, we avoid using the species name of *S. cucullata*. Many authors have used *S. cucullata* to denote the IWP *Saccostrea* oysters. However, the type locality of *Ostrea cucullata* is the West Indies and Ascension Island in the Atlantic (Born, 1780; Iredale, 1924; Iredale & Roughley, 1933). Consistent with this fact and given the characteristics of the shell of *O. cucullata*, Inaba & Torigoe (2004) and Huber (2010) defined the geographical distribution range of *S. cucullata s.s.* from the West Indies to West Africa and from West Africa to the Arabian Peninsula, respectively. *Saccostrea cucullata* is a large oyster, with a shell height (SH, the maximum shell dimension) ranging from 80 to 140 mm (Arkihpkin *et al.*, 2014). The type specimen has a SH of 127 mm (Inaba & Torigoe, 2004: pl. 6, fig. 4), with acutely angled and raised radial ribs on the left valve, and an internally thickened edge in the left valve. In the Pacific, we have found no *Saccostrea* oyster with these features. Moreover, *S. cucullata* inhabits exposed rocky shores on Ascension Island, its type locality (Arkihpkin *et al.*, 2014), whereas members of the '*S. cucullata* superspecies' (*sensu* Lam & Morton, 2006) favour estuaries and sheltered bays, except for *S. kegaki* (see Results).

Use of *S. cucullata* for *Saccostrea* oysters in the IWP should therefore be discontinued. Instead, we refer to Lam & Morton's (2006) '*S. cucullata*' superspecies as 'non-*mordax*' oysters, because they can be separated from *S. mordax* on the basis of both their mtDNA sequences and their shell morphology (Lam & Morton, 2006).

MATERIAL AND METHODS

Oyster specimens

We collected *Saccostrea* oysters from four subtropical islands in Japan: Amami-Oshima, Kakeroma, Okinawa and Iriomote Islands (Fig. 1 and Table 3). We also analysed specimens from two temperate areas of Japan including Ehime Prefecture (Shikoku Island) and Wakayama Prefecture (mainland Honshu). For each specimen, we preserved a piece of mantle or adductor muscle tissue in >99% ethanol at below –20 °C for subsequent DNA extraction (Quick Gene-810, Kurabo). We deposited shells of 64 specimens in the Osaka Museum of Natural History (OMNH; Supplementary Material S1A).

In the field, we excluded *S. mordax*-like oysters on the basis of our empirical observations of the shell shapes of *Saccostrea* oysters. In subsequent statistical analyses, we set aside the few individuals of *S. mordax* that contaminated the studied specimens, which were easily identified by *16S*-based DNA barcoding (see below). We also included results from a previous study (Sekino & Yamashita, 2013), in which we determined the sequences of *16 S* and the mitochondrial cytochrome *c* oxidase subunit I (*coxI*) genes for specimens from Okinawa Island (sites A-Sa, G-Sa and K-Sa; Fig. 1 and Table 3). These Okinawa specimens were grouped into two of Lam & Morton's (2006) non-*mordax* lineages: all of the A-Sa

Table 2. Summary of nominal *Saccostrea* species.

	Nominal species name	Type locality	Accepted in literature	
			Huber (2010)	Inaba & Torigoe (2004)
1	*S. circumsuta* (Gould, 1850)	Fiji and Samoa Islands	Yes	Yes
2	*S. commercialis* (Iredale & Roughley, 1933)	New South Wales	Syn. of 6	Yes
3	*S. cucullata* (Born, 1778)	West Indies; Ascension Island	Yes	Yes
4	*S. echinata* (Quoy & Gaimard, 1835)	Amboina (Indonesia)	Yes	Yes
5	*S. forskhalii* (Gmelin, 1791)	Red Sea	Syn. of 3	Yes
6	*S. glomerata* (Gould, 1850)	New Zealand	Yes	Syn. of 1
7	*S. kegaki* (Torigoe & Inaba, 1981)	Mukaishima (Hiroshima, Setouchi Inland Sea)	Yes	Yes
8	*S. malabonensis* (Faustino, 1932)	Malabon, Rizal, Philippines	Yes	Yes
9	*S. margaritacea* (Lamarck, 1819)	American seas?*	?†	Yes
10	*S. mytiloides* (Lamarck, 1819)	Southern ocean of East Indies	Syn. of 4	Syn. of 4
11	*S. mordax* (Gould, 1850)	Fiji Islands	Syn. of 15	Yes
12‡	*S. palmula* (Carpenter, 1857)	Mazatlan, Mexico	Yes	Yes
13‡	*S. prismatica* (Gray, 1825)	Guacomayo, Central America	?†	Yes
14	*S. pseudangulata* (Lamy, 1930)	Manila (Philippines)	?†	Yes
15	*S. scyphophilla* (Peron & Lesueur, 1807)	Australia	Yes	–
16	*S. spathulata* (Lamarck, 1819)	Not given	Yes	–
17‡	*S. stellata* (Gmelin, 1791)	Guinea	Syn. of 3	Yes
18	*S. subtrigona* (G. B. Sowerby II, 1871)	Australia	Yes	Syn. of 5
19‡	*S. tubulifera* (Dall, 1914)	Gulf of California	Yes	Yes

*A question mark is added as it is in the original species description.

†Huber (2010) tentatively assigned *S. margaritacea* and *S. prismatica* to *Striostrea* and *S. pseudangulata* to *Pustulostrea*.

‡These are not considered as IWP *Saccostrea* species.

specimens into lineage C, and those from G-Sa and K-Sa into lineage F (Table 4).

DNA sequencing

To determine the mitochondrial lineages of our specimens in accordance with the lineage or species names used in previous *16S*-based studies (Lam & Morton, 2006; Hamaguchi *et al.*, 2014), we first screened the *16 S* gene for comparison with the *16 S* sequences available in the EMBL/DDBJ/GenBank database (ref-seq; for accession numbers, see Supplementary Material S2). Using Primer3 v. 0.4.0 (Untergrasser *et al.*, 2012), we designed a novel *16 S* primer set for PCR on the basis of the complete *S. mordax* mitogenome (acc. no. FJ841968). The primers (F: 5′-attaaagtatggcaaaaggaactcg-3′; R: 5′-aaacttcctctttcagcagtcct-3′) are located at the 3′-end region of split *16 S* (Milbury & Gaffney, 2005). The protocol for touchdown PCR (Don *et al.*, 1991) was followed, as described by Sekino *et al.* (2015). The basal annealing temperature for touchdown PCR (T_{base}) ranged from 52 to 58 °C depending on the specimens.

We also determined the sequences of three additional mitochondrial genes, *coxI*, cytochrome *c* oxidase subunit III (*coxIII*) and NADH dehydrogenase subunit 3 (*nad3*). For the touchdown PCR amplification of *coxI* and *nad3*, we used previously reported PCR primers (*coxI*: Folmer *et al.*, 1994; *nad3*: Sekino *et al.*, 2012), where T_{base} was 48 °C during thermal cycling. By referring to the *S. mordax* mitogenome, we developed a novel *coxIII* primer set, which flanks the whole *coxIII* gene (F: 5′-tgtcttccaaacaaaaggtatcc-3′ within the $tRNA^{Gly}$ gene; R: 5′-cataatcttcgacatcaacaaagc-3′ within the $tRNA^{Ile}$ gene; T_{base}, 52–55 °C).

We conducted enzymatic purification of the PCR products before DNA sequencing (Sekino & Yamashita, 2013). The sequences were determined by reading from both directions using 3730*xl* DNA Analyzer (Applied Biosystems by Thermo Fisher Scientific) in combination with DNASIS Pro v. 2.02 (Hitachi Software Engineering). All of the four primer sets allowed direct sequencing of the PCR products. For specimens with poor sequence quality, we re-purified the PCR products using the agarose-gel excision method (MagExtractor, Toyobo).

All of the mtDNA sequences were deposited in the GenBank database (Supplementary Material S1A).

Nuclear ribosomal ITS1

We followed the method described by Sekino *et al.* (2012) for *ITS1*-PCR amplification, TA cloning of the PCR amplicons and sequencing of the resulting clones. We obtained sequences of at least seven clones per specimen. All singleton clones within specimens were omitted, in order to reduce the risk of including clones with erroneous nucleotide bases. Thus, for each specimen, we defined an *ITS1* type as an identical sequence found in multiple clones (acc. nos in Supplementary Material S1B).

Multiple sequence alignment

We used MAFFT v. 7.222 (Katoh & Standley, 2013) with the E-INS-i algorithm to align the sequences. We removed ambiguously aligned segments in *16 S* and *ITS1* sequences (see below) using Gblocks v. 0.91b (Castresana, 2000), with default settings, except that a gap was defined as a position where ≥50% of sequences exhibited a gap (i.e. 'with half' option for allowed gap positions).

Classification of mitochondrial lineages

Using MEGA v. 6.06 (Tamura *et al.*, 2013), we summarized the results of *16S*-based DNA barcoding as a neighbour-joining (NJ) tree (Saitou & Nei, 1987) with Kimura's two-parameter (K2P) distance (Kimura, 1980). This approach was consistent with those adopted in previous DNA barcoding of *Saccostrea* oysters (Lam & Morton, 2006; Sekino & Yamashita, 2013; Hamaguchi *et al.*, 2014). Gaps or missing sites were omitted in calculating the genetic distances. We evaluated the confidence of the resulting tree with bootstrap resampling (BS; 1,000 replicates).

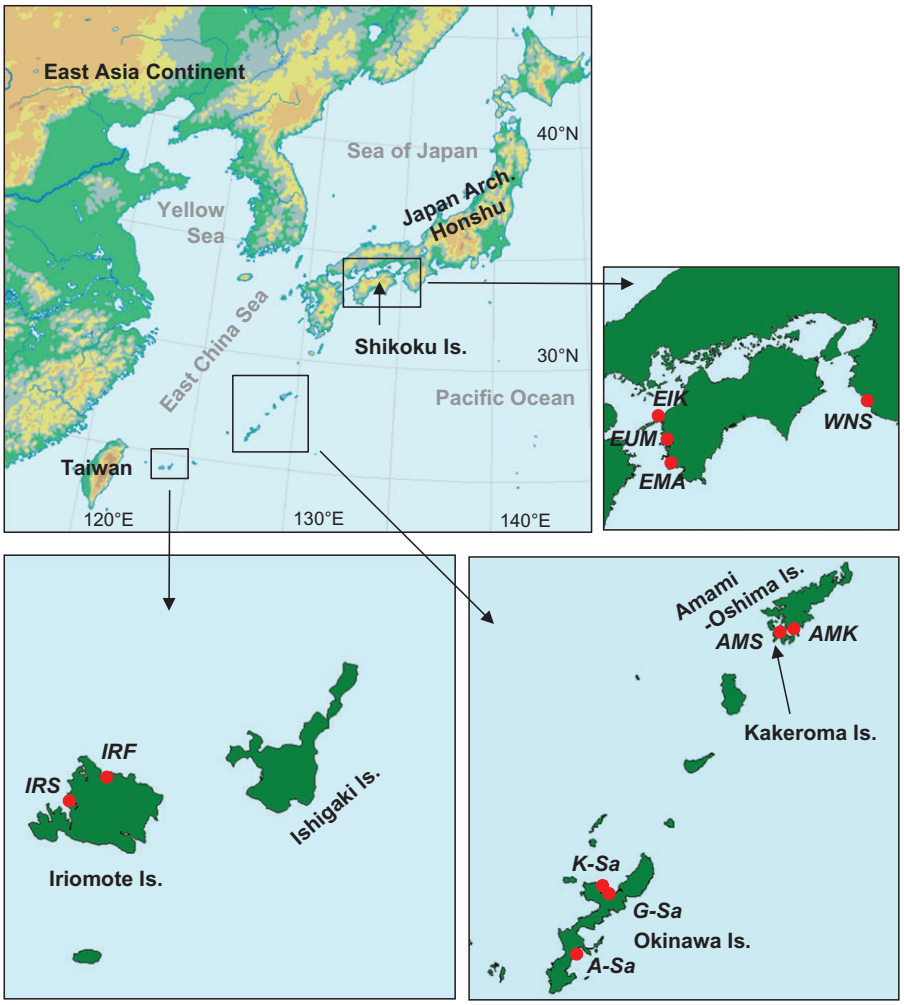

Figure 1. Sampling locations.

After the *16 S* analysis, we added sequences of *coxI*, *coxIII* and *nad3*, concatenating the four genes for each specimen. First, we evaluated divergence between lineages by estimating the net K2P distance (with MEGA) and the number of net substitutions per site (d_A; Nei, 1987) using DnaSP v. 5.10.01 (Librado & Rozas, 2009). Using DnaSP, we also obtained the estimates of nucleotide diversity within lineages (π; Nei, 1987).

For tree-building, three clustering methods were used, NJ, maximum parsimony (MP) and Bayesian inference (BI). For the MP analysis with MEGA, we selected the subtree pruning and regrafting algorithm with search level 3 (Nei & Kumar, 2000). Branch lengths were estimated on the basis of the average pathway method (Nei & Kumar, 2000). Nucleotide sites with less than 5% alignment gaps or missing sites were allowed for MP calculation. The resulting trees were evaluated with 1,000 BS replicates.

For the BI calculation, we used MrBayes v. 3.2 (Ronquist *et al.*, 2012). First, we searched for the best-fit substitution model for each gene using jModelTest v. 2.1.7 (Darriba *et al.*, 2012). We set the number of substitution schemes to three (24 models) and selected the most appropriate model according to the Bayesian information criterion (Schwarz, 1978). During BI analysis with the gene-specific models, the Markov chain Monte Carlo (MCMC) simulation was run for up to 10^7 generations. The resulting trees were saved every 500 generations with an initial burn-in of 5,000 trees (25% of saved trees). We evaluated the convergence of two parallel MCMC runs (four chains per run) on the basis of the standard deviation of split frequencies (below 0.01).

Comparison of nuclear ITS1 *among lineages*

We compared the sequences of *ITS1* by eye to search for lineage-specific structure. We constructed NJ, MP and BI trees as described above. In addition, we visualized the relationships among *ITS1* types in a median-joining network (Bandelt *et al.*, 1999), constructed using Network v. 5.0.0.0 (Fluxus-engineering; http://www.fluxus-engineering.com/).

RESULTS

DNA barcoding using 16 S

We retrieved 21 ref-seqs of *16 S* gene from GenBank (Supplementary Material S2), representing all of the non-*mordax* lineages or species proposed in previous studies (Lam & Morton, 2006; Hamaguchi *et al.*, 2014), an unspecified *Saccostrea* oyster, *S. mordax* and *Ostrea denselamellosa* Lischke, 1869. The length of the alignment of the ref-seqs and our 226 specimens was 533 bp, reduced to 470 bp after removal of ambiguous segments (Supplementary Material S3A). Three ref-seqs (Ref-ech2, EF122390; Ref-mal, EF122391; Ref-myt, EF122392) were missing the first 52 and last 121 bp. There were 130 variable sites, among which 55 were singletons.

In the NJ tree, our specimens fell into seven clades (Fig. 2, Table 4), four of which corresponded to non-*mordax* lineages C, F, G and *S. kegaki* (Lam & Morton, 2006). However, the support for relationships among lineages was low.

Table 3. Sampling locations of *Saccostrea* specimens.

Site abbrev.	*N*	Island name	Location	Lat./Long.	Sampling date
AMS	39	Kakeroma	Setouchi, Oshima, Kagoshima Pref.	28°08′35″N/129°14′53″E	9 July, 2012
AMK	83	Amami-Oshima	Setouchi, Oshima, Kagoshima Pref.	28°08′20″N/129°20′44″E	10 July, 2012
A-Sa	6	Okinawa*	Awase, Okinawa, Okinawa Pref.	26°18′44″N/127°49′44″E	26 October, 2011
G-Sa	5		Goga, Nago, Okinawa Pref.	26°38′07″N/128°00′11″E	28 October, 2011
K-Sa	2		Gabui, Kunigami, Okinawa Pref.	26°39′06″N/127°58′60″E	28 October, 2011
IRF	13	Iriomote	Taketomi, Yaeyama, Okinawa Pref.	24°23′46″N/123°49′16″E	6 March, 2012
IRS	53		Taketomi, Yaeyama, Okinawa Pref.	24°21′36″N/123°44′60″E	28 June, 2012; 28 July, 2015
EIK	4	Shikoku	Ikata, Nishi-Uwa, Ehime Pref.	33°29′35″N/132°18′60″E	21 May, 2012
EUM	5		Miura, Uwajima, Ehime Pref.	33°10′06″N/132°29′36″E	1 May, 2013
EMA	7		Ainan, Minami-Uwa, Ehime Pref.	32°57′48″N/132°33′05″E	29 April, 2013
WNS	9	Honshu	Shirahama, Nishi-Muro, Wakayama Pref.	33°41′41″N/135°20′11″E	16 October, 2012

*Mitochondrial sequences previously published by Sekino & Yamashita (2013; see text).

Table 4. Observed number of specimens from each non-*mordax* lineage at each location.

Site abbr.	Island name	Lineage C	*S. kegaki*	F	G	H	I	J	No. of lineages	Total
AMS	Kakeroma	3	32	4	–	–	–	–	3	39
AMK	Amami-Oshima	67	–	12	4	–	–	–	3	83
A-Sa	Okinawa	6	–	–	–	–	–	–	1	6
G-Sa		–	–	5	–	–	–	–	1	5
K-Sa		–	–	2	–	–	–	–	1	2
IRF	Iriomote	–	–	3	–	–	10	–	2	13
IRS		6	1	21	7	14	–	4	6	53
EIK	Shikoku	–	4	–	–	–	–	–	1	4
EUM		–	–	5	–	–	–	–	1	5
EMA		–	–	7	–	–	–	–	1	7
WNS	Honshu	–	8	1	–	–	–	–	2	9
	Total	82	45	60	11	14	10	4		226

The ref-seq of *S. malabonensis* (Ref-mal, EF122391) was nested in the lineage F clade, as reported by Hamaguchi *et al.* (2014). Four individuals from Iriomote Island (IRS) clustered with the ref-seqs of *Saccostrea* sp. (Ref-SacSP, JF915522), *S. echinata* (Ref-ech2, EF122390) and lineage 1 of putative *S. malabonensis* (Ref-IshSac11, AB898233); we refer to this clade as non-*mordax* lineage J. Another ref-seq of *S. echinata* (Ref-ech1, AF463493) had a close relationship with lineages A and B of Lam & Morton (2006). Hamaguchi *et al.* (2014) proposed two lineages of putative *S. malabonensis* (lineages 1 and 2), but we found no specimen that corresponded to lineage 2. The specimens from Iriomote Island included two additional lineages (lineages H and I). Lineage H did not belong to any of the lineages proposed by Lam & Morton (2006) or Hamaguchi *et al.* (2014), and its closest relationship was with *S. kegaki*. Lineage I was grouped with the ref-seqs of *S. mytiloides* (Ref-myt, EF122392; Ref-Iri12, AB898228).

Among the observed lineages, lineage F had the widest spatial distribution (9 of 11 sampling sites), followed by lineage C and *S. kegaki* (4 sites each) (Table 4). All seven lineages were observed at Iriomote Island (IRF and IRS).

Trees of four mitochondrial genes

The additional genes *coxI*, *coxIII* and *nad3* were not available for the non-*mordax* lineages represented in GenBank, so we used *S. mordax* and *O. denselamellosa* as ref-seqs, each of which has a completely sequenced mitogenome (Supplementary Material S2).

From the alignments, none of the three genes contained indel polymorphisms, giving constant lengths of 561 bp for partial *coxI*, 765 for partial *coxIII* and 354 for the whole *nad3*. For *16 S*, we repeated multiple alignment and trimming using the dataset with reduced number of species/lineages, which slightly increased the number of available positions to 477 bp (Supplementary Material S3B).

Before phylogenetic reconstruction using the concatenated sequences of the four genes, we examined if the topology of the *16S*-based NJ tree (Fig. 2) was supported by NJ analysis of each of the three protein-coding genes (Supplementary Material S4). The same lineages were retrieved in each case. However, there were several incongruences in the relationships among lineages. For example, lineage G was positioned apart from lineage C in the *16S*- and *nad3* trees, but these two lineages were connected first in the *coxI*- and *coxIII* trees. Another notable discrepancy was the clustering of the *S. mordax* ref-seq within the non-*mordax* clades in the *coxI* tree. As with the *16 S* NJ tree, most nodes between lineages were not statistically supported, most likely due to the limited phylogenetic signal from the short DNA segments.

For the BI analysis of the 228 concatenated sequences (2 ref-seqs and 226 specimens), the best-fit model was HKY (Hasegawa *et al.*, 1985) with invariable sites and rate variation among sites (Γ) for *coxI* and *coxIII*, and HKY with Γ for *16 S* and *nad3*.

Overall, the NJ, MP and BI methods gave a congruent tree topology (Fig. 3 shows BI tree; for NJ and MP trees see Supplementary Material S5), although there were many inconsistencies in the clustering patterns within major clades. Seven major clades were retrieved, as in all the single-gene trees. Most of the nodes between lineages were statistically supported by the different clustering methods, providing better resolution of relationships among lineages than the single-gene trees.

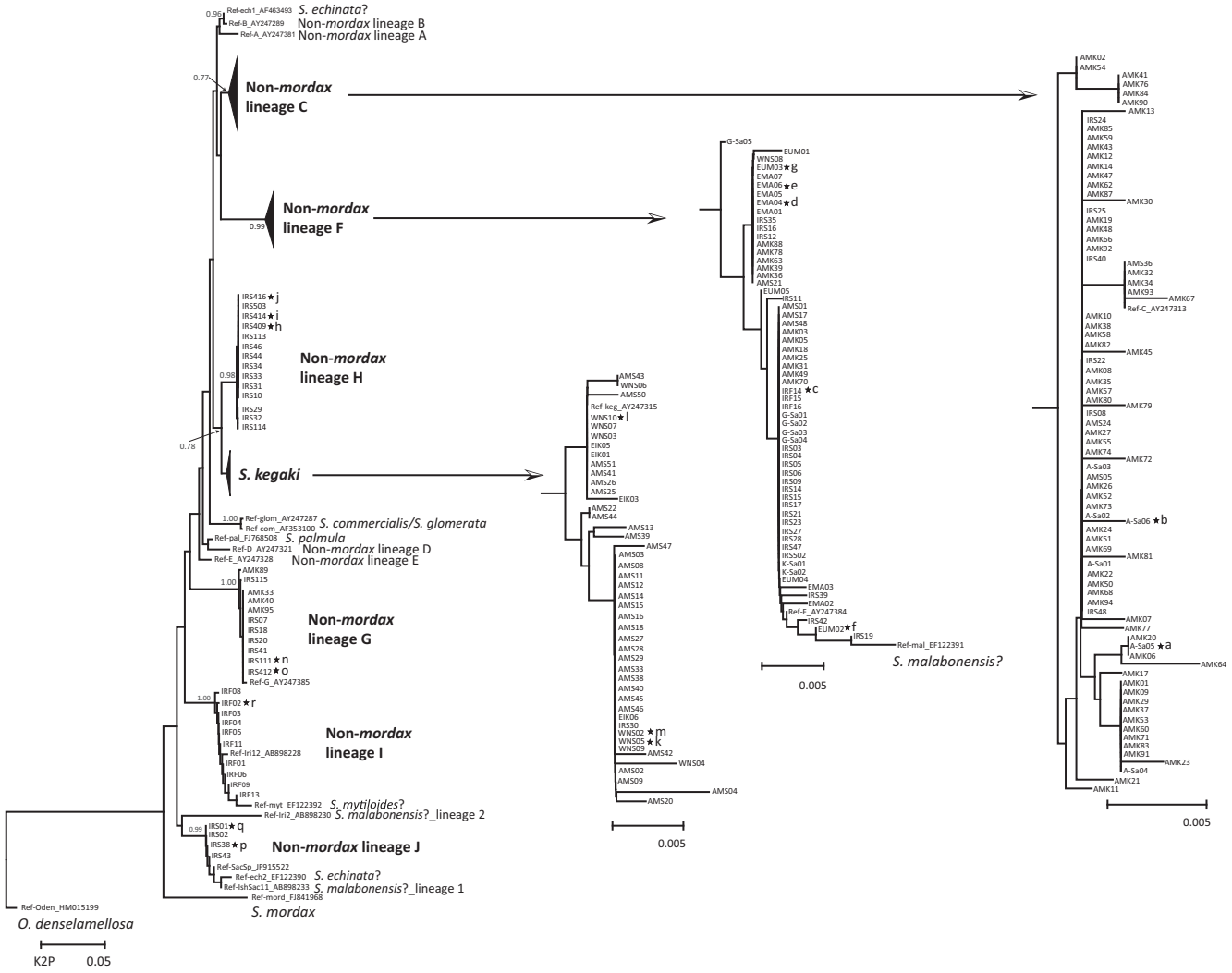

Figure 2. Neighbour-joining tree for *Saccostrea* oysters reconstructed using mitochondrial 16 S rRNA gene. Bootstrap values (≥0.70) are given on nodes. For details of the sources of the reference sequences (prefix 'Ref'), see Supplementary Material S2. A reference sequence from *Ostrea denselamellosa* was set as outgroup. Shells of 18 specimens are illustrated in Figure 7 (indicated by asterisk and alphabetical identification label as used in Fig. 7).

We estimated pairwise divergences between lineages (Table 5). A combination of lineage H and *S. kegaki* gave the smallest difference (K2P 4.4%; d_A 4.2%). The largest difference was observed between lineages G and J (K2P 19.3%; d_A 16.5%). All lineage combinations except the closest pair yielded a difference of more than 10%.

Comparison of ITS1 *sequences among lineages*

We sequenced 1,138 *ITS1* clones derived from 72 specimens, which covered all of the observed non-*mordax* lineages (lineage C, $N = 11$; F, 25; G and H each, 8; I, 10; J, 4; *S. kegaki*, 6). The number of sequenced clones per specimen ranged from seven to 27. After the removal of singletons and duplicated clones for each specimen, 136 *ITS1* sequences were retained. Before multiple alignments, the lengths of these sequences, including the last 11 bp of the 18 S rRNA gene (at the 5′-end) and the first 30 bp of the 5.8 S rRNA gene (3′-end), ranged from 457 to 583. Among the specimens, 21 exhibited no sequence variation, whereas, in the remaining 51, the number of different *ITS1* types per specimen varied between two and four. After the removal of ambiguously aligned segments, the sequence length was 397 including gaps (Fig. 4).

Lineage G was characterized by unique fixed deletions and a fixed substitution (Fig. 4). Lineages I and J both had many fixed substitutions and deletions. Among the remaining four lineages (C, F, H and *S. kegaki*), we failed to find a lineage-specific character.

In terms of the structure of *ITS1*, a long insertion (84 bp) was observed in one individual each from *S. kegaki* and lineage F (Fig. 5; this block was removed after trimming with Gblocks, so is not included in Fig. 4). The long insertion could be divided into two segments, each homologous to the adjacent upstream segment (Fig. 5: segment A) and downstream segment (segment B). However, the order of the A- and B-homologous segments was inverted in the insertion. According to BLAST search of the GenBank database, this idiosyncratic structure was not exclusive to our specimens; for example, a similar structure occurs in the East Pacific *S. palmula* (acc. no. KF370473).

For the *ITS1* trees (Fig. 6A for BI, Supplementary Material S6 for NJ and MP), the best-fit substitution model, inferred using all the *ITS1* types, was K80 (K2P) with Γ. Consistent with the lineage-specific features of lineages G, I and J, these formed separate clusters, although support for the lineage G clade was low in the NJ and MP trees.

The separation of lineages G, I and J from the other lineages was also shown in the *ITS1* network (Fig. 6B), despite many uncertainties in the topology. None of the *ITS1* types from lineages G, I and J was shared with the other lineages, from which they were

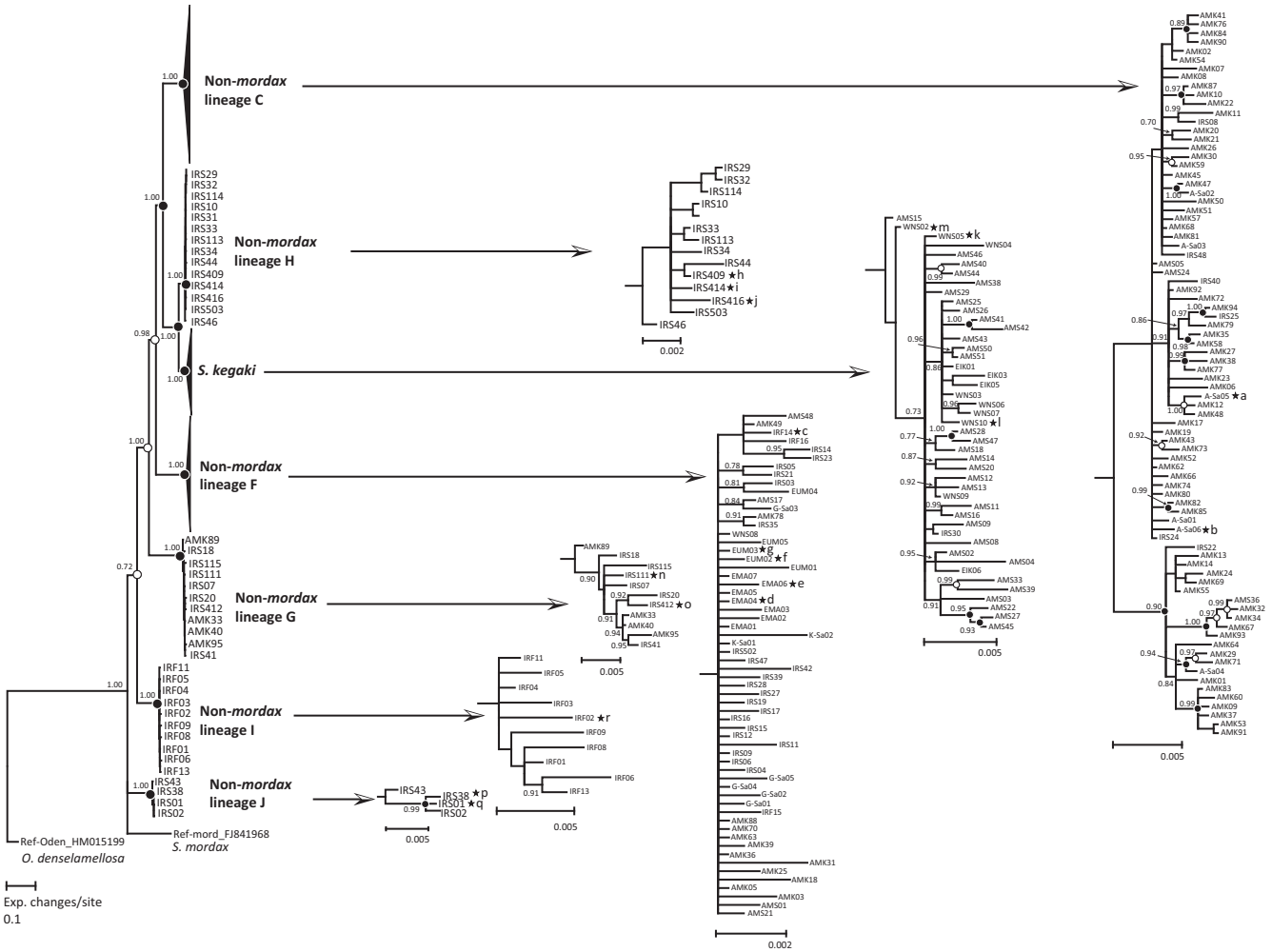

Figure 3. Bayesian phylogenetic tree for *Saccostrea* oysters reconstructed using concatenated sequences of mitochondrial *coxI*, *coxIII*, *nad3* and *16 S* genes. The lineages were defined according to results of *16S*-based DNA barcoding (Fig. 2). Numbers on nodes are posterior probability values. Solid spots are nodes that were consistently supported by BI, MP and NJ with confidence level ≥0.70 (see Supplementary Material S5 for NJ and MP trees). Open spots represent nodes that were supported by BI and either MP or NJ. For details of the sources of the reference sequences (prefix 'Ref'), see Supplementary Material S2. A reference sequence from *Ostrea denselamellosa* was used as outgroup. Shells of 18 specimens are illustrated in Figure 7 (indicated by asterisk and alphabetical identification label as used in Fig. 7).

Table 5. Nucleotide divergence between non-*mordax* lineages.

	Lineage C	*S. kegaki*	Lineage F	Lineage G	Lineage H	Lineage I	Lineage J
Lineage C	0.005	0.106	0.125	0.138	0.109	0.133	0.149
S. kegaki	0.119	0.004	0.125	0.144	0.042	0.135	0.145
Lineage F	0.142	0.142	0.002	0.146	0.125	0.138	0.154
Lineage G	0.158	0.166	0.168	0.006	0.143	0.141	0.165
Lineage H	0.123	0.044	0.142	0.165	0.002	0.134	0.148
Lineage I	0.151	0.154	0.158	0.162	0.152	0.006	0.138
Lineage J	0.172	0.166	0.177	0.193	0.171	0.157	0.005

Below diagonal: net K2P between lineages (Kimura, 1980); on diagonal: nucleotide diversity (π; Nei, 1987) within lineages; above diagonal: number of net nucleotide substitutions per site between lineages (d_A; Nei, 1987).

separated by more than 10 mutational steps. Obviously, the *ITS1* types from lineages C, F, H and *S. kegaki* were not randomly distributed among these lineages, but they were closely related, three of them even being shared by two or three lineages. Given these patterns, we consider that the number of shared *ITS1* types was underestimated owing to the small sample sizes.

Shell features

Shell features of the non-*mordax* lineages are summarized in Table 6 and representative shells are shown in Figure 7. The shell form of each lineage was more or less variable, preventing us from defining lineage-specific features in most cases.

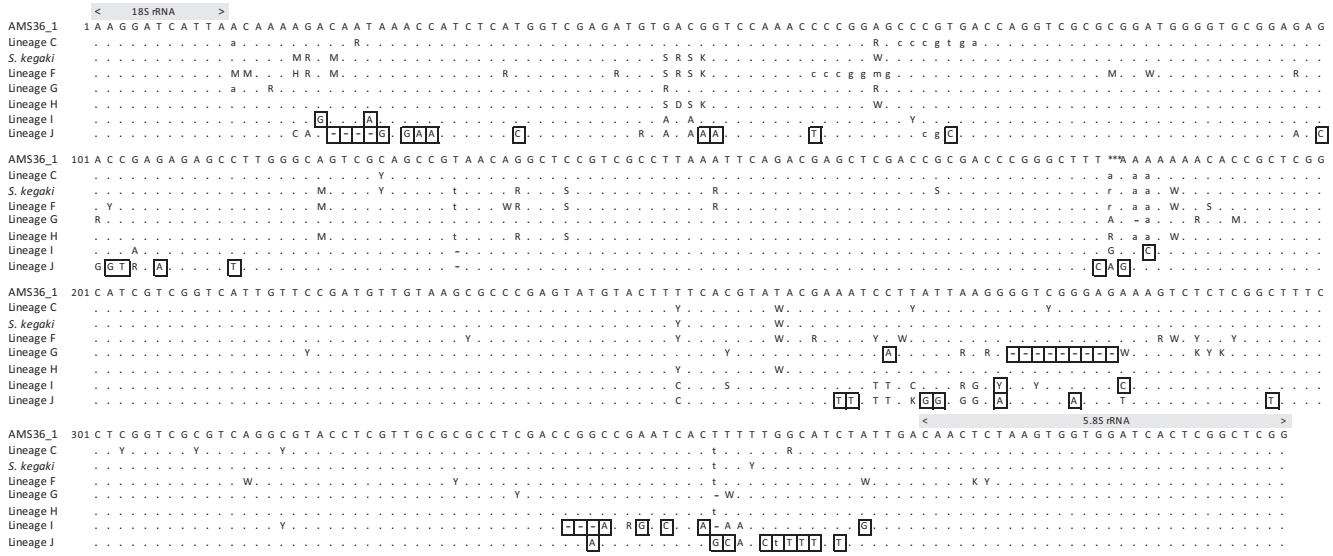

Figure 4. Aligned sequences for the nuclear first internal transcribed spacer (*ITS1*) region. Sequence of specimen AMS36, lineage C, is placed at top. Dots indicate nucleotides identical to those in top sequence. Hyphens represent gaps. Ambiguously aligned segments were removed (see text). Site numbers were added after removing ambiguously aligned segments. Substitution polymorphisms within lineages are represented by the IUPAC ambiguity codes (D = A↔G↔T; H = A↔C↔T; K = G↔T; M = A↔C; R = A↔G; S = C↔G; W = A↔T and Y = C↔T). Indel polymorphisms are in lowercase letters. For each of lineages G, I and J, the lineage-specific features are enclosed within solid lines.

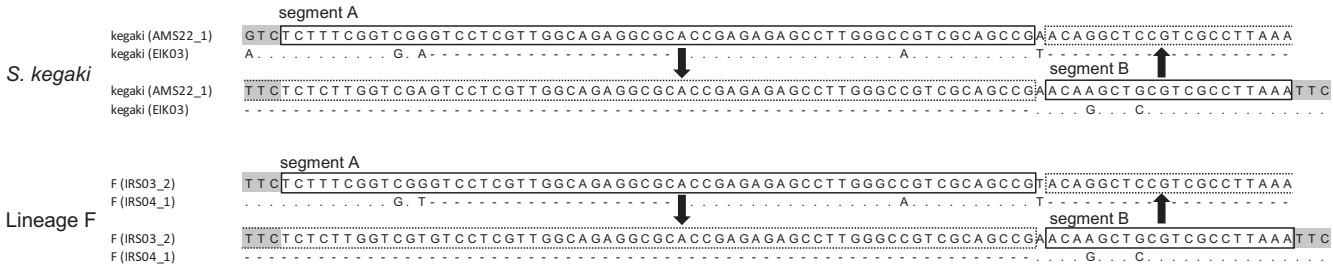

Figure 5. The sequence structure of the long insertion observed in *ITS1*. A long insertion (84 bp) was found commonly in *S. kegaki* (AMS22) and lineage F (IRS03). For each lineage, the sequence from another specimen was aligned (*S. kegaki*, EIK03; lineage F, IRS04). Dots indicate nucleotide identity. Hyphens represent gaps. The insertion is enclosed within dotted lines. This long insertion comprised two segments: one homologous to the adjacent upstream segment (segment A), the other homologous to the downstream segment (segment B) (enclosed within solid line), but in the inverse order in the insertion (indicated by bold arrows). Grey-shaded nucleotides in insertion (TTC) were homologous to the three nucleotides both before segment A (GTC or TTC) and after segment B (TTC).

Overall, the shell sizes were small to medium (SH < 80 mm; Table 6), except for lineage J (SH to 123 mm; Fig. 7: p1, p2). Lineages G, H and *S. kegaki* had a relatively narrow shell (width ≤ 18 mm), with a weakly upturned margin of the left valve (Fig. 7: k). In many lineages, the anterior part of the left valve was frequently raised; this pattern contrasts with that of *S. mordax*, where the raised left valve margin is more conspicuous posteriorly (Inaba & Torigoe, 2004). Without exception, hyote spines were observed in *S. kegaki* (Fig. 7: k, l1, l2, m; Table 6). A few specimens of lineage F, as well as small specimens of lineage H, also had hyote spines (Fig. 7: j1). Lineages C and F had particularly well-developed ribs on the left valve. The edge of the left valve margin was smooth, wavy or saw-like in the non-*mordax* lineages (Table 6). In contrast to *S. mordax*, radial ribs and grooves on the right valve were absent. The protrusion of the ligamental area was not extensive compared with *S. mordax* and *Crassostrea* species. All of the non-*mordax* lineages possessed chomata, the strength and continuity of which varied within lineages. Harry (1985) and Hamaguchi *et al.* (2014) reported a few *Saccostrea* specimens with no chomata, but this appears to be rare. The adductor muscle scar exhibited continuous variation among reniform, D-shaped and oval forms, and was more deeply coloured in the right valve. Some specimens exhibited a conspicuous quenstedt muscle insertion (Fig. 7: o2). The disjunct pallial lines were remarkable in lineages C (Fig. 7: a2) and G. Chalk deposits were found in all lineages, but the degree of development was not lineage-specific. Squamae or lamellae were observed on the outer surface of the right valve in most specimens, but these were often removed by erosion except at the shell margin, or remained only as commarginal threads (Table 6). Lineages F, I and J had well-developed squamae. Lineages H, J and *S. kegaki* formed calcareous lamellae (Fig. 7: p1). The inner shell colour was essentially white, but the interior of the right valve was occasionally silver or silver-grey (Fig. 7: o2). The internal margin of the left valve was often stained black or lilac (Fig. 7: n2, p2). Yellowish green, ochre or black spots or layers were sometimes present on the inner surfaces. The internal edges of the lamellae in the right valve were usually a mixture of ochre and black (Fig. 7: d2, h2, j2, q).

Habitat

According to the present and previous studies (Lam & Morton 2006; Hamaguchi *et al.*, 2014), the non-*mordax* lineages are

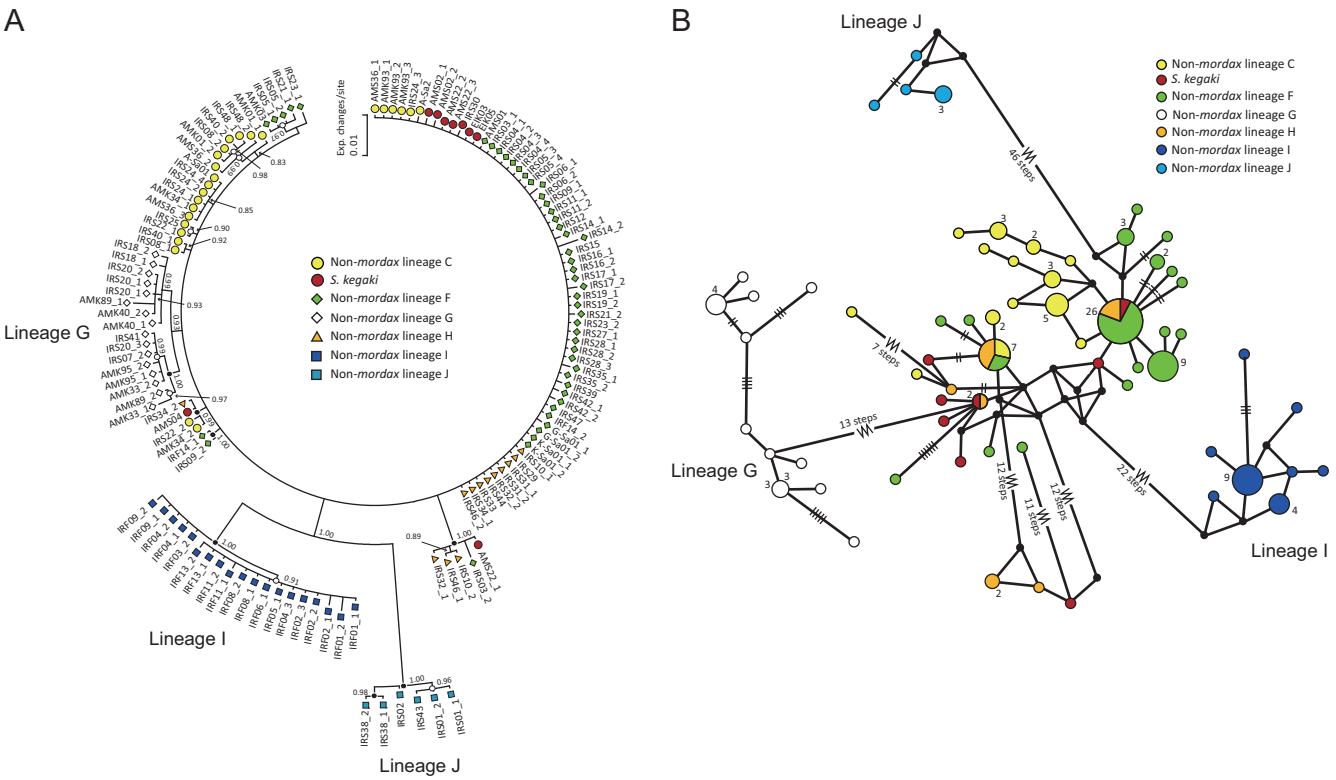

Figure 6. A. Bayesian phylogenetic tree reconstructed using *ITS1* sequences. The majority of specimens (51 of 72) had multiple *ITS1* types (indicated by underscore plus number after each specimen ID). Lineages defined according to results of *16S*-based DNA barcoding. Numbers on nodes are posterior probability values. Solid spots are nodes that were consistently supported by BI, MP and NJ with confidence levels ≥0.70. Open spots are nodes supported by BI and either NJ or MP. The NJ and MP trees are provided in Supplementary Material S6. **B.** Median-joining network among observed *ITS1* types. Circles represent *ITS1* types. Lineages are distinguished by different colours. The frequency of each type is shown numerically, except for singletons. The numbers of mutational steps between *ITS1* types are shown as bars or (if more than 6) as figures; branches with no bar represent a single mutational step. Black dots are median vectors.

Table 6. Shell characteristics of each non-*mordax* lineage.

				Left valve				Right valve	
	Size*	SH† (mm)	SW† (mm)	Attachment area‡	Ribs§	Rib form	Shell-margin form	Growth lamellae	Hyote spines§
Lineage C	M	60	28	>50%	±	Rounded or moderately sharp	Smooth or saw-like	Thread-like	–
S. kegaki	M	68	17	>70%	±	Rounded or sharp	Smooth, wavy or saw-like	Undeveloped	+
Lineage F	S, M	59	27	>10%	±	Rounded or sharp	Smooth, wavy or saw-like	Thread-like or small squamae	±¶
Lineage G	M	56	18	>70%	±	Rounded	Smooth, wavy or saw-like	Thread-like or small squamae	–
Lineage H	M	64	14	>70%	–	No ribs	Smooth, wavy or saw-like	Undeveloped or small squamae	±
Lineage I	M	77	26	>50%	±	Rounded	Smooth or wavy	Well developed	–
Lineage J	M, L	123	59	>10%	±	Rounded	Smooth or wavy	Well developed	–

*According to SH of adults: S, <30 mm; M, ≤80 mm; L, >80 mm.

†SH, maximum shell height; SW, maximum shell width.

‡Attachment area as proportion of whole outer surface of left valve.

§–, invariably absent; +, invariably present; ±, absent or present.

¶Only one specimen exhibited hyote spines.

distributed mainly in tropical and subtropical zones (Table 7). *Saccostrea kegaki* is the northernmost lineage and inhabits temperate coasts. As noted by Lam & Morton (2006), our specimens were found in estuarine or sheltered environments, with the exception of *S. kegaki*, which occurred on open and exposed shores. Most specimens were found attached to rocks at mid and low tide, but lineage I was found exclusively on the trunks or roots of mangrove trees in the mid-tidal zone (Table 7).

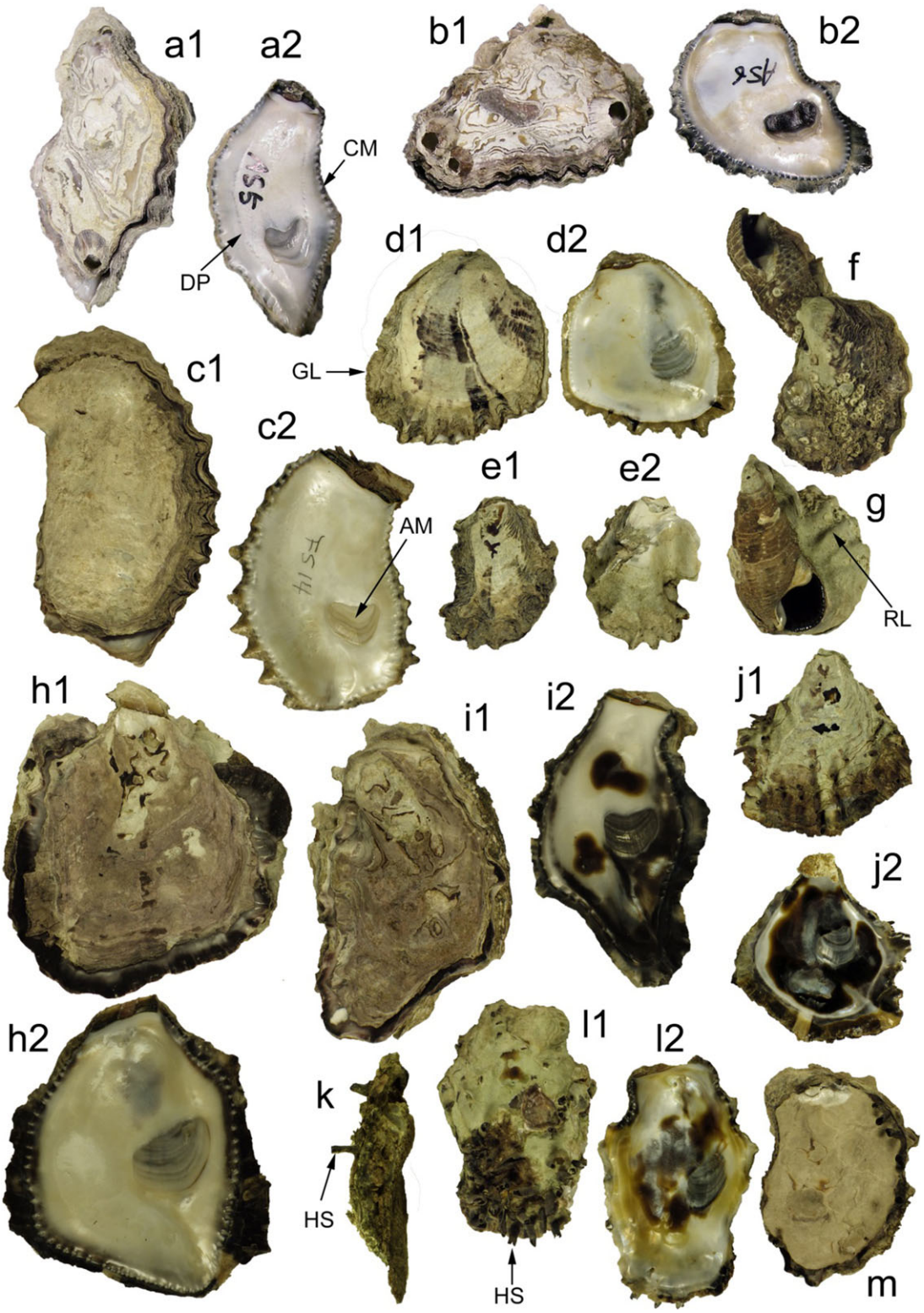

Figure 7. Shells of non-*mordax* lineages of *Saccostrea* oysters. Each specimen, often shown in two views, is indicated by a unique letter and Osaka Museum of Natural History (OMNH) registration number. Lineage C (a1–b2): a, OMNH-Mo 38402, A-Sa (a1, BV, SH 57 mm; a2, IRV); b, OMNH-Mo 38403, A-Sa (b1, BV, SH 45 mm; b2, IRV); lineage F (c1–g): c, OMNH-Mo 38431, IRF (c1, BV, SH 60 mm; c2, IRV); d, OMNH-Mo 38472, EMA (d1, BV, SH 41 mm; d2, IRV); e, OMNH-Mo 38474, EMA (e1, BV, SH 33 mm; e2, OLV); f (OMNH-Mo 38465, EUM, BV, SH 30 mm) and g (OMNH-Mo 38466, EUM, OLV, SH 27 mm), which were attached to living *Batillaria multiformis*; lineage H (h1–j): h, OMNH-Mo 38507, IRS (h1, BV, SH 61 mm; h2, IRV); i, OMNH-Mo 38512, IRS (i1, BV, SH 60 mm; i2, IRV); j, OMNH-Mo 38514, IRS (j1, ORV, SH 43 mm; j2, IRV); *S. kegaki* (k–m): k, OMNH-Mo 38458, WNS (anterior side of BV, SH 43 mm); l, OMNH-Mo 38463, WNS (l1, ORV, SH 52 mm; l2, IRV); m, OMNH-Mo 38455, WNS (BV, SH 38 mm); lineage G (n1–o2): n, OMNH-Mo 38481, IRS (n1, BV, SH 53 mm; n2, ILV); o, OMNH-Mo 38510, IRS (o1, BV, SH 56 mm; o2, IRV); lineage J (*S. spathulata*; p1–q): p, OMNH-Mo 38436, IRS (p1, ORV; p2, ILV, SH 123 mm); q, OMNH-Mo 38434, IRS (IRV, SH 107 mm); lineage I: r, OMNH-Mo 38419, IRF (BV, SH 50 mm). Abbreviations on figure: AM, adductor muscle scar; CM, chomata; DP, disjunct pallial line; GL, growth lamella; HS, hyote spine; LA, ligamental area; QM, quenstedt muscle insertion; RL, radial rib of the left valve; UC, umbonal cavity. Abbreviations in caption above: BV, both valves; ILV, interior left valve; IRV, interior right valve; OLV, outer left valve; ORV, outer right valve. Sampling sites abbreviated as in Table 3.

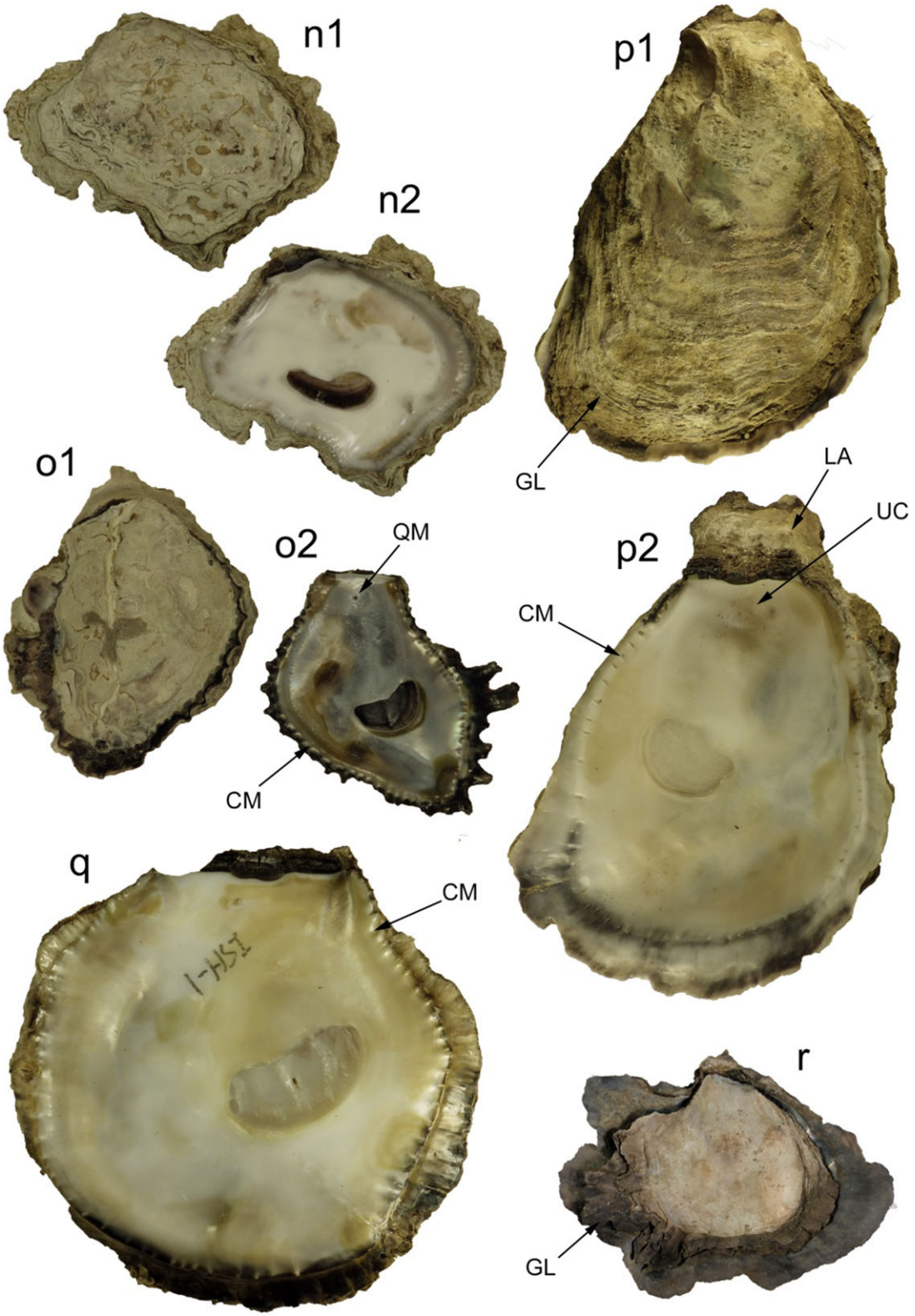

Figure 7. *Continued*

Table 7. Summary of geographical distribution and habitat of non-*mordax* lineages.

Lineage	Habitat*	Tidal zone	Substrate	Geographic distribution (references†)	Climatic zone
Lineage C	RM, SB, MS	Low to middle	Rock	Southern Honshu, Amami to Iriomote Islands (a–c)	Temperate to subtropical
S. kegaki	OB, OS	Low to middle	Rock	Northern Honshu to Amami, Iriomote Island, South Korea (a–e)	Temperate to subtropical
Lineage F	RM, SB, MS	Low to middle	Rock, shell, mangrove	Southern Honshu to Southeast Asia (a–c)	Temperate to tropical
Lineage G	RM, SB, MS	Low to middle	Rock	Ishigaki and Iriomote Islands, Taiwan (a–c)	Subtropical
Lineage H	RM, SB, MS	Low to middle	Rock	Iriomote Island (a)	Subtropical
Lineage I	MS	Middle	Mangrove	Ishigaki and Iriomote Islands (a,c)	Subtropical
Lineage J‡	RM, SB, MS	Low to middle	Rock	Ishigaki and Iriomote Islands, tropical IWP (a,c,f)	Subtropical to tropical

*RM, river mouth; SB, sheltered bay; MS, mangrove swamp; OS, oceanic shore; OB, open bay.

†a, this study; b, Lam & Morton (2006); c, Hamaguchi et al. (2014); d, Inaba & Torigoe (2004); e, Min (2004); f, Huber (2010).

‡Inferred assuming that this lineage corresponds to *S. spathulata* (see text).

DISCUSSION

Cryptic phylogenetic diversity

Our analysis of mitochondrial genes allowed a robust separation of the studied non-*mordax Saccostrea* specimens into seven lineages, irrespective of the method of tree reconstruction. Almost all the lineage pairs exhibited more than 10% nucleotide difference (Table 5). For comparison, we estimated the divergence among *Crassostrea* species (analysing data from Sekino *et al.*, 2015; four mitochondrial genes; K2P). The divergence among non-*mordax* lineages was comparable with the level of interspecific divergence in *Crassostrea* (e.g. 10% difference between *C. gigas* and *C. sikamea*, 15% between *C. gigas* and *C. hongkongensis*, 17% between *C. gigas* and *C. ariakensis* and 20% between *C. gigas* and *C. bilineata*). Even the minimum difference observed between non-*mordax* lineages (4% between H and *S. kegaki*) exceeded the 2% difference between *C. gigas* and *C. angulata*.

Among our seven non-*mordax* lineages, lineages H, I and J were not described by Lam & Morton (2006). Given the high site-specificity of molluscan fauna in the Japanese subtropical islands (Nawa, 2008), previous failures to find these lineages could be attributed simply to insufficient geographical coverage during sampling. However, our study areas were also limited, so the present study should be affected by the same problem. For example, unlike Lam & Morton (2006), we failed to identify lineage A at Okinawa Island, probably because we did not survey their sampling site (Itoman, southern Okinawa I.). Similarly, lineage 2 of *S. malabonensis* described by Hamaguchi *et al.* (2014) was not found in our specimens, whereas we found the new lineage H. Thus, the phylogenetic diversity of IWP *Saccostrea* oysters may still be underestimated and further study is required.

*Geographical distribution of non-*mordax *lineages*

Among the observed lineages, lineage F was found over the widest geographical range, followed by lineage C and *S. kegaki* (Table 4). The frequent occurrence of lineage C and *S. kegaki* was in agreement with Lam & Morton (2006) and Hamaguchi *et al.* (2014), who showed that these lineages were limited to subtropical and temperate zones of the northern hemisphere. On the other hand, *S. glomerata*, which was not identified in the present study, is mainly distributed in temperate southeastern Australia (Lam & Morton, 2006). These results imply that climatic adaptation and geographic separation have contributed to the phylogenetic diversity of non-*mordax* oysters.

Lam & Morton (2006) showed that, among their study sites, the highest phylogenetic diversity was observed in Taiwan (with at least five non-*mordax* lineages). This is geographically close to Iriomote Island (Fig. 1), where we found the highest phylogenetic diversity, with all seven lineages present (Table 4). This site is located at the lowest latitudinal position among the surveyed islands, agreeing with the suggestion that *Saccostrea* species are most diverse in low-latitudinal tropical zones (Harry, 1985). Furthermore, since Iriomote Island lies towards the northern limit of the tropical zone, the environment is still suitable for lineage C and *S. kegaki*, which apparently favour northern subtropical or temperate waters (Lam & Morton, 2006).

*Nuclear DNA differences among non-*mordax *lineages*

Overall, the separation among the mitochondrial lineages was not supported well by the nuclear *ITS1* data. However, lineages G, I and J were distinct in the *ITS1* data (Figs. 4, 6), even in Iriomote Island where all seven lineages co-occurred. Although the small sample sizes should be taken into consideration, our results suggest that these lineages are reproductively isolated, thereby meriting the status of biological species (Mayr, 1963). Thus, we argue against the lumping of these 'species' as a 'non-*mordax* superspecies' (or '*S. cucullata* superspecies' as by Lam & Morton, 2006).

By contrast, we failed to find consistent differences in *ITS1* among lineages C, F, H and *S. kegaki*. Neither fixed substitutions nor indels were observed among these lineages (Fig. 4), even in the removed segments (data not shown). The long insertion (Fig. 5) was common in two lineages (lineage F and *S. kegaki*). Our attempted phylogenetic tree reconstruction produced an unresolved pattern (Fig. 6A). The network analysis (Fig. 6B) revealed an uneven distribution of their *ITS1* types among these lineages, but some types were shared by different lineages. Moreover, it is likely that future surveys will find more shared *ITS1* types. If these lineages are reproductively isolated at present, then our results can be ascribed to incomplete lineage sorting after their reproductive isolation (e.g. Xiao *et al.*, 2010), as a consequence of the larger effective population size of the nuclear than mitochondrial genomes. Another likely scenario is the occurrence of ongoing hybridization and subsequent introgression among the lineages.

However, it is also possible that our data from the short *ITS1* region underestimate the underlying phylogenetic divergence among lineages. Further sequencing of the entire *ITS* region (*ITS1* and *ITS2*) may provide a finer resolution of lineages (Nolan & Cribb, 2005); the *ITS2* region has proved useful for phylogenetic separation among Ostreidae oysters (Salvi *et al.*, 2014). On the other hand, the presence of multiple ribosomal DNA arrays in the genome complicates *ITS*-based phylogenetic inference (reviewed by Álvarez & Wendel, 2003). Thus, multiple single-copy genes may be more suitable for future studies (e.g. Cronn *et al.*, 2002). The draft genome of *C. gigas* (Zhang *et al.*, 2012) offers the potential for discovery of such genes.

Correspondence between species names and mitochondrial lineages

The species assignment was unclear for some of the ref-seqs. For example, two ref-seqs of *S. echinata* belonged to different clades (lineage A/B or lineage J). The ref-seq of *S. malabonensis* was grouped with lineage F. However, Hamaguchi *et al.* (2014) concluded that this ref-seq was misidentified and instead they assigned the name *S. malabonensis* (based on their lineage 1 of *S. malabonensis*) to a clade corresponding to lineage J (Fig. 2). In addition, lineage I included *S. mytiloides* determined by Hamaguchi *et al.* (2014) (see below for the validity of this species name). However, we could not verify these species assignments, because photographs of their *S. mytiloides* and *S. malabonensis* specimens are not available. In any case, the highly variable shells even within lineages make it difficult to define lineage-specific morphological traits. Thus, it was not possible to definitely associate most of the non-*mordax* lineages with a nominal species (see Table 2 for nominal species and authorities). We present below our inferences of the species name for each lineage.

Saccostrea kegaki (Fig. 7: k–m): the well-developed hyote spines allowed relatively easy separation of this lineage from the others. With this conspicuous trait, *S. kegaki* should be assigned to this lineage, in agreement with Lam & Morton (2006). However, our nuclear data failed to support the genetic integrity of *S. kegaki*. In Japan, *S. kegaki* can be found in temperate and subtropical zones, from northern Honshu (Inaba & Torigoe, 2004) to Iriomote Island, but is scarcer at lower latitudes.

Non-*mordax* lineage F (Fig. 7: c–g): this is one of the most common lineages, with a wide geographical distribution from temperate southern Japan to tropical Southeast Asia (present study; Lam & Morton, 2006; Hamaguchi *et al.*, 2014). SH was variable but generally small (as little as 22–28 mm in Shikoku). The left valve was sometimes cupped with a narrow attachment area (Fig. 7: e2, g) and attachment was sometimes to shells of *Batillaria* (Fig. 7: f, g). According to the original species description of *S. malabonensis*, it is a small oyster (SH 25–45 mm) with cupped left valve and thick ribs and attaches to substrates, including other shells, with narrow attachment area (Faustino, 1932). We therefore consider that *S. malabonensis* may correspond to lineage F, although Hamaguchi *et al.* (2014) assigned *S. malabonensis* to lineage J (see below). The shell shapes of *S. malabonensis sensu* Torigoe (1981), Inaba & Torigoe (2004) and Huber (2010), i.e. with a wide attachment area and narrow, planar shells, do not conform to the original species description. Lineage F also resembled one of the syntypes of *S. circumsuta* (Inaba & Torigoe, 2004: pl. 4, fig.1); however, the type locality of that species is Fiji and Samoa, whereas lineage F has never reported from the South Pacific (Lam & Morton, 2006).

Non-*mordax* lineage H (Fig. 7: h–j): our smaller specimens of lineage H occasionally had hyote spines. Interestingly, this lineage and *S. kegaki*, which was characterized by distinctive hyote spines, were phylogenetically close (Figs. 2, 3). *Saccostrea ehinata* is also characterized by the occurrence of hyote spines (Quoy & Gaimard, 1835). Since hyote spines are present in some juveniles of non-*mordax* lineages A and B, Lam & Morton (2006) proposed that *S. echinata* was a juvenile form or ecotype of '*S. cucullata*' (i.e. non-*mordax* lineage A or B). On the other hand, *S. echinata* with hyote spines has been reported from Ishigaki Island, which neighbours Iriomote Island (Fig. 1) (Torigoe, 1981; Inaba & Torigoe, 2004). As non-*mordax* lineages A and B appear not to be major lineages in the subtropical islands of Japan (present study; Hamaguichi et al., 2014), we consider that *S. echinata* could also correspond to lineage H. It seems that the *S. echinata sensu* Torigoe (1981) and Inaba & Torigoe (2004) included lineages A, B or H (with hyote spines) and another large oyster, *S. spathulata* (lineage J; see below).

Non-*mordax* lineage J (Fig. 7: p–q): it is appropriate to assign *S. spathulata* to this lineage, because of its large size (SH 90–123 mm in our specimens), wide commissural shelf and the presence of violet squamae or lamellae on the outer right valve. This nominal species has long been neglected, but Huber (2010) regarded it as valid. Hamaguchi *et al.* (2014) applied *S. malabonensis* to lineage J, but the shell features of our specimens did not agree with its original description.

For each of the lineages C, G and I, our data were not sufficient to infer a likely species name. Lineage C (Fig. 7: a–b) resembled lineage F, but typically had conspicuous disjunct pallial lines. It is distributed mainly in the subtropical zone, although Hamaguchi *et al.* (2014) reported it from central Honshu. The specimens of lineage I (Fig. 7: r) were attached to mangrove trees and had distinctive shells with an extended margin of the left valve, as if mimicking tree bark. Our review of the original species descriptions of the nominal species (Table 2) failed to find a species with a similar shell. Hamaguchi *et al.* (2014) claimed that lineage I corresponds to *S. mytiloides*; however, if this '*S. mytiloides*' refers to *S. mytiloides* (Lamarck, 1819), then the name is invalid, because *Ostrea mytiloides* Lamarck, 1819 is a homonym of *O. mytiloides* Hermann, 1781 and *O. mytiloides* Gmelin, 1791. We also note that Inaba & Torigoe (2004) and Huber (2010) synonymized *O. mytiloides* Lamarck, 1819 with *S. echinata* (Table 2).

Concluding remarks

We reaffirm the extreme difficulty of species diagnosis in *Saccostrea* species of the non-*mordax* group. In our specimens, only *S. kegaki* and lineage J had diagnostic shell features, thereby allowing us to associate them with proposed species names. Further attempts at species assignment based on DNA evidence and shell morphology in non-*mordax* oysters, or *Saccostrea* species generally, will be error-prone in most cases, even with reference to the original species descriptions and type specimens.

Because shells are of limited use as a taxonomic guide, the genus *Saccostrea* should be resystemized by laying more weight on DNA evidence; the parallel analyses of mitochondrial and nuclear DNA are prerequisite for adequate definition of these species.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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