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Molecular phylogenetic affinities of *Scarus obishime* Randall & Earle, 1993, Endemic to the Pacific Ocean archipelago of Ogasawara (Japan)

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Abstract

Scarus obishime is a rare parrotfish endemic to the Ogasawara Islands, located in the Northwestern Pacific Ocean roughly 1,000 km south of the main Japanese Archipelago. It was described as a new species in 1993. Since then, it has been speculated to be closely related to *Scarus ovifrons* occurring on southern coasts of the main Japanese Archipelago, but this has never been formally tested. To identify the closest relative of this rare parrotfish, we determined one nuclear (S7 ribosomal protein gene intron 1 [S7I1], ca. 600 bp) and two mitochondrial (control region [CR], ca. 400 bp and 16SrRNA, ca. 600 bp) partial DNA sequences for two specimens of the species and conducted molecular phylogenetic analyses using the recently published sequences from 45 of the 52 described species of the genus and 16 of the 18 described species of *Chlorurus*. The nuclear and mitochondrial sequences were analyzed separately based on the results of Basic Local Alignment Search Tool (BLAST) searches using the newly obtained sequences of *S. obishime* as queries. The phylogenies resulting from two single-region analyses (S7I1 and 16SrRNA) and the concatenation of the two mitochondrial regions (CR + 16SrRNA) supported a close phylogenetic affinity between *S. obishime* and *S. ovifrons*. In addition, nuclear (S7I1) analyses demonstrated that the two "East Asian" species formed a robust monophyletic group with the Arabian species *Scarus arabicus*, whereas mitochondrial datasets significantly rejected the monophyly of the three species. This result seemed to indicate a partially shared evolutionary history between the "East Asian" and Arabian lineages.

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Introduction

The Ogasawara Islands are located in the Northwestern Pacific Ocean roughly 1,000 km south of the main Japanese Archipelago (Fig. 1). These subtropical islands harbor many endemic plants and animals due to their isolated location (UNESCO's World Heritage List: http://whc.unesco.org/en/list/1362). In total, 801 species of reef and shore fishes have been recorded in the ocean around the islands (Randall et al. 1997). However, only a few fish species are recognized as endemic, which is probably because reef fish eggs and/or larvae may disperse over long distances. *Scarus obishime* Randall & Earle, 1993 is one of the few examples of an endemic fish species (the others include *Genicanthus takeuchii* and *Ammodytoides kimurai*).

Scarus obishime (*obishime* in Japanese) was described as a new species by Randall and Earle in 1993. Adult males of this species reach >70 cm standard length (SL) and have characteristic coloration (terminal phase [TP] coloration): a bright yellow bar on a deep blue body (see Fig. 1a; in the name *obishime*, *obi* means colorful sash of traditional costume, and *shime* means "wearing"). It seems surprising that such a large species with distinctive coloration could remain undescribed until the end of the twentieth century. However, it is endemic around these isolated islands, and is rare, particularly the males, which usually inhabit water >25 m and are difficult to approach. That is why the species remained undescribed until relatively recent times (Randall and Earle 1993).

The closest relative of the rare parrotfish *S. obishime* remains unclear. The genus *Scarus* includes 52 described species (Parenti and Randall 2000, 2011), which are widely distributed from temperate to tropical areas in the Pacific and throughout the Indian and Atlantic oceans. Among the species, Randall and Earle (1993) mentioned *S. ovifrons* as possibly the closest relative to *S. obishime* based on some morphological characters (including six median dorsal scales, 15 pectoral rays, and a strong convex forehead in large males). However, no

phylogenetic analysis of *S. obishime* has been conducted at the morphological or molecular levels.

A recently published comprehensive molecular phylogenetic study of the parrotfish genera *Scarus* and *Chlorurus* (Choat et al. 2012) provides a good basis for inferring the closest relatives of *S. obishime*, but the published phylogenetic analysis does not include *S. obishime*. As a preliminary analysis, we determined the nucleotide sequences of one *S. obishime* specimen (NSMT-P 95345) for the same DNA regions as in that published study, and conducted Basic Local Alignment Search Tool (BLAST) searches using the obtained sequences as queries in the NCBI nucleotide database. As a result, the most similar species to *S. obishime* in sequence similarity differed among the DNA regions (Table 1).

Thus, we conducted molecular phylogenetic analyses based on the published DNA sequences used in Choat et al. (2012) and those of *S. obishime* newly determined here to identify the closest relative of *S. obishime* more precisely. In the published study, sequences from mitochondrial and nuclear DNA regions were concatenated for each species when analyzed. However, in the present study, we analyzed them separately, which resulted in significantly different phylogenies for the members of the close relatives of *S. obishime*. In addition to the molecular phylogenetic analyses, we searched for species that were morphologically similar to *S. obishime* with the methods and data used by Choat et al. (2012).

MATERIALS AND METHODS

Taxon sampling

Two specimens of *S. obishime* (NSMT-P 95302 and 95345: vouchers deposited at the National Museum of Nature and Science, Tokyo; for the latter specimens, see Fig. 1b) were sequenced.

Both were initial phase (IP) specimens of about 50 cm in SL, collected by pole spear at Ani-jima and Ototo-jima Islands in the Ogasawara Islands in August and September 2009.

Phylogenetic analyses incorporated 62 species, including one that was newly sequenced in the present study (*S. obishime*) and 61 species used in Choat et al. (2012). The 62 species included 46 of the 52 described species of *Scarus* and 16 of the 18 described species of *Chlorurus*, collectively covering 89% of the described *Scarus/Chlorurus* species (Parenti and Randall 2000, 2011). Each species was represented by a single specimen except for *S. rubroviolaceus* and *S. ghobban*. These two species were each represented by four specimens from four geographic locations because of their wide geographic ranges spanning the Pacific and Indian oceans.

Another species (*Hipposcarus harid*) was included as an outgroup. In Choat et al. (2012), four species, including the one that we used, were used as outgroups. However, we did not include the other three species (*Bolbometopon muricatum*, *Hipposcarus longiceps*, and *Cetoscarus bicolor*) because doing so greatly reduced the sequence regions that we could align unambiguously.

DNA extraction, polymerase chain reaction, and sequencing

Total genomic DNA of *S. obishime* was extracted from the two ethanol-preserved muscle tissue specimens using an AquaPure Genomic DNA Isolation kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. Three DNA markers, two mitochondrial (control region [CR], 16SrRNA) and one nuclear (S7 ribosomal protein gene intron1: S7I1) DNA region, were sequenced. These fragments were amplified using EmeraldAmp PCR Master Mix (TaKaRa, Ohtsu, Japan) following the manufacturer's protocol, with the same primer sets as used in Choat et al. (2012). The polymerase chain reaction (PCR) proceeded for 30 cycles for all three

fragments on a Model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA), with denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min; the final cycle was followed by an extension at 72°C for 2 min.

The PCR products were electrophoresed on a 1% agarose gel for band characterization under ultraviolet trans-illumination. Double-stranded short PCR products were then purified using the Exosap-IT enzyme reaction (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). They were subsequently used for direct cycle sequencing with dye-labeled terminators (BigDye terminator ver. 3.1, Applied Biosystems) using the same primers as those used in the PCR. All sequencing reactions were carried out according to the manufacturer's instructions. Labeled fragments were analyzed on an ABI PRISM 3130xl genetic analyzer (Applied Biosystems). All fragments were sequenced for both strands.

Because the chromatograms obtained for the nuclear intron (S7I1) were double-peaked after probable insertion/deletion sites, these fragments from the two *S. obishime* specimens were cloned before sequencing. A standard cloning procedure (pGEM-T Easy Vector Systems; Promega, Madison, WI, USA) was used in accordance with the manufacturer's instructions.

Sequence editing and alignment

Sequence editing was conducted with the computer program ATSQ ver. 5.1 (Genetyx Corp., Richmond, CA, USA). The newly determined sequences from *S. obishime* were deposited in DNA Data Bank of Japan (DDBJ) under the accession numbers AB811973–AB811976. The obtained nucleotide sequences for all three DNA regions were aligned using Proalign ver. 0.5 software (Löytynoja and Millinkovitch 2003) with default parameter settings. Regions with posterior probabilities ≤70% were excluded from the subsequent phylogenetic analyses.

Phylogenetic analyses

Phylogenetic analyses were conducted separately for the nuclear and mitochondrial DNA regions. Each of the two mitochondrial (CR, 16SrRNA) and one nuclear (S7I1) regions was examined for the best-fit model using KAKUSAN4 (Tanabe 2007) and the Akaike information criterion. Bayesian inference (BI) and maximum-likelihood (ML) analyses were performed first for each of the three regions to estimate phylogeny under the sequence evolution model (GTR + Gamma) determined by KAKUSAN4. Then, two mitochondrial regions (CR and 16SrRNA) were concatenated, and partitioned BI and ML analyses were carried out using the combined data.

BI analyses were conducted using MrBayes, version 3.1.2 (Huelsenbeck and Ronquist 2001), with the GTR + Gamma model for nucleotide substitution. The Markov chain Monte Carlo (MCMC) process was set so that four chains (three heated and one cold) ran simultaneously. We conducted two independent runs for each dataset and continued for 500,000 cycles, and 1 in every 100 trees was sampled. "Stationarity" (lack of improvement in the likelihood score) was checked graphically using TRACER, version 1.5 (http://tree.bio.ed.ac.uk/software/tracer/), and all trees and parameters before stationarity were discarded as "burn-in." After confirming agreement of the estimated parameters between the two independent runs using Tracer, we pooled all post-burn-in trees from the two runs. The posterior probabilities of phylogenies and their internal branches were estimated based on these pooled trees.

ML analyses were carried out using RAxML 7.2.6 (Stamatakis 2006), a program that implements a novel, rapid-hill-climbing algorithm. For each dataset, a rapid bootstrap analysis (–f a) and search for the best-scoring ML tree were conducted in one single program run using the GTR + Gamma model. The rapid bootstrap analyses were conducted with 1000 replications, with

four threads running in parallel. The program finally conducted ML optimization for every fifth bootstrapped tree to search for the best-scoring ML tree.

Testing alternative phylogenetic hypotheses

Alternative tree topologies were compared to the best-scoring ML tree obtained using the likelihood-based AU test (Shimodaira 2002). We first created constraint topologies considering the monophyly of alternative hypotheses using MacClade (Maddison and Maddison 2000) and conducted ML analyses using RAxML with those constraints. The resulting constrained ML trees were used to compute the per-site log likelihoods using RAxML (–f g option), and outputs were subjected to AU tests using CONSEL (Shimodaira and Hasegawa 2001). A $p < 0.05$ was considered significantly different.

Molecular phylogenetic analyses based on the sequence alignment in Choat et al. (2012)

To determine the phylogenetic position of *S. obishime* in the previously published tree, BI analyses were conducted as in Choat et al. (2012), using their sequence alignment. Their aligned sequences spanned 1727 bp, with two mitochondrial and one nuclear sequences being concatenated for each species. The new sequences for *S. obishime* were added and aligned to the original data matrix using the online version of MAFFT (http://mafft.cbrc.jp/alignment/server/) with the option "mafft –add", which produced a matrix of 1728 bp in which the original alignment was preserved. BI analysis was conducted using MrBayes as described above. The software KAKUSAN4 selected a separate model with the nucleotide substitution model for each of the three partitions as follows: 16SrRNA, SYM + Gamma; CR, HKY + Gamma; and S7I1, GTR + Gamma. The MCMC process was continued for 5,000,000 cycles, and 1 in every 100

trees was sampled. Two species, *C. bicolor* and *B. muricatum*, were used to root the resulting tree.

Morphological analysis based on the data matrix in Choat et al. (2012)

To identify the species that was most morphologically similar to *S. obishime*, we conducted a hierarchical cluster analysis using five morphometric (i-v) and three meristic (vi-viii) variables as in Choat et al. (2012). The five morphometric variables were i) fork length, ii) degree of exposure of dental plates (angle), iii) total length / body depth, iv) head length / cheek depth, and v) snout profile (profile length from start of spinous dorsal to upper margin of dental plate / shortest distance between the two points). Measurements for these variables (except for fork length) were obtained from digital images of specimens using ImageJ (http://imagej.nih.gov/ij/), while fork length was measured by examining the original specimens. Three meristic variables, which were of major importance in separating scarine species, were the numbers of vi) median pre-dorsal scales, vii) cheek scale rows, and viii) pectoral rays. They were counted on the original specimens. Four specimens of *S. obishime* (NSMT-P35477, 35478, 95302, and 95345) were measured and counted to estimate average values for each variable in this species. The average values for *S. obishime* were added to the original data matrix of mean values used by Choat et al. (2012), and the values were standardized across species. The resulting data matrix was analyzed using hierarchical cluster analysis of Euclidian distances between the species with the group average method in the statistical software R (http://www.r-project.org/).

RESULTS

Sequences from S. obishime

Identical mitochondrial DNA sequences were obtained from the two specimens of *S. obishime* for the partial 16SrRNA gene region (AB811976, 594 bp) and CR (AB811975, 403 bp). The sequences obtained differed slightly between the two specimens and among cloned fragments for the nuclear intron region (S7I1). Because the observed sequence variations (including insertions/deletions) within individuals were minor, each of the two specimens was represented by the most frequently observed fragment within each individual: AB811973 (616 bp) for NSMT-P 95302; AB811974 (608 bp) for NSMT-P 95345. An 8-bp insertion/deletion site was observed between the two sequences.

Alignments and datasets

Sequence alignments among 69 (or 68) sequences from 62 *Scarus*/*Chlorurus* species (61 for S7I1, because the *C. gibbus* sequence was absent from the database) and one outgroup species of *Hipposcarus* were difficult for the mitochondrial control and nuclear S7I1 regions. In other words, about half of these regions were filtered out because of alignment ambiguities. The lengths of the aligned regions (after filtration) were 192 bp for mitochondrial CR and 330 bp for the nuclear S7I1 region, whereas that for the mitochondrial 16S rRNA gene region was 570 bp (only *ca*. 20 bp were filtered out by alignment ambiguity). In the sequence regions used in later phylogenetic analyses, 105, 78, and 65 bp were parsimoniously informative for the S7I1, CR, and 16S rRNA regions, respectively.

Nuclear DNA phylogenies

BI analysis for the nuclear S7I1 region (330 bp) resulted in the tree in Figure 2A; statistical support (posterior probabilities [PPs] from the BI analysis and bootstrap probabilities [BPs] from the ML analysis) is indicated on/near each internal branch. Although many of the phylogenetic relationships remained unresolved, which was the case for the ML tree (not shown), both BI and ML analyses reproduced a relatively highly supported clade including *S. obishime*. In both analyses, two S7I1 sequences from the two specimens of *S. obishime* formed a monophyletic group with those from *S. ovifrons* (endemic to southern Japan) and *S. arabicus* (endemic to the southern coast of the Arabian Peninsula), and their monophyly was supported by 0.99 PP and 90% BP.

Mitochondrial DNA phylogenies

BI and ML analyses of the 16S rRNA gene region (570 bp) yielded only partly resolved phylogenies (see Fig. 2B for the BI tree; ML tree not shown). In these trees, the sequences from *S. obishime* and *S. ovifrons* were monophyletic with support values of 0.54 PP and 37 BP. In contrast, *S. arabicus*, which formed a robust clade with the two species in the nuclear S7I1 analysis, was not located near the two species but was included in a clade almost identical to the SC10 clade shown in the S7I1 tree (lacking *S. zufar* in the 16S rRNA tree). In other words, *S. arabicus* constituted "SC10 minus the *S. zufar*" clade (0.83/72), together with the two Arabian species, *S. collana* and *S. fuscopurpureus*. The monophyly of *S. obishime*, *S. ovifrons*, and *S. arabicus*, which was robustly supported in the nuclear S7I1 trees, was confidently rejected by the AU test ($P = 0.016$) in the 16S rRNA dataset.

Analyses of CR (192 bp) also yielded only partly resolved phylogenies (not shown). *S. obishime*, *S. ovifrons*, and *S. arabicus* were scattered in both the BI and ML trees. However,

monophyly of the former two species, and that of the three species, was not rejected by AU tests (P = 0.307 and 0.121, respectively). The SC10 clade included *S. arabicus*.

The partitioned BI analysis based on the concatenated data (16S rRNA + CR; 762 bp) resulted in the tree shown in Figure 2C, with a BP value from the ML analysis, as well as a PP value from the BI analysis indicated on/near each internal branch. Although *S. obishime* and *S. ovifrons* were paraphyletic in both the BI and ML trees, their monophyly was not rejected by AU tests (P = 0.166). However, *S. arabicus* was not located near the two species but was included in the "SC10 minus *S. zufar*" clade with relatively high support values (1.00/76). The monophyly of *S. obishime*, *S. ovifrons*, and *S. arabicus* was confidently rejected by the AU test (P = 0.003).

Molecular phylogeny using the sequence alignment in Choat et al. (2012)

The partitioned BI analysis based on concatenated data (16S rRNA + CR + S7I1; 1728 bp) produced the tree shown in Figure 3A (only one clade that included all of the *Chlorurus* and *Scarus* clades is shown). All of the 5 clades of *Chlorurus* (CH1-5) and 7 of the 10 clades of *Scarus* (SC2-7 and 9) [named in Choat et al. (2012)] were recovered. Two of the ten *Scarus* clades, SC1 and SC8, were respectively divided into three and two clades (SC1-a, b and c; SC8-a and b). The clade SC8-a (*S. arabicus*) was nested within SC10 (located next to *S. collana*), and the remaining clade SC8-b (*S. ovifrons*) formed a monophyletic group with the newly added sequences of *S. obishime*.

Cladogram based on morphological data

The obtained dendrogram is shown in Figure 3B. The topology was almost identical to the tree produced by Choat et al. (2012). The newly added *S. obishime* was located next to *S. ovifrons*. The mean values of the five morphometric and three meristic variables, based on the four

specimens of *S. obishime*, were as follows: i) 583 mm, ii) 90°, iii) 3.06, iv) 2.25, v) 1.12, vi) 6.3, vii) 3.0, and viii) 15.0.

DISCUSSION

Relationship between S. obishime and S. ovifrons

Both the nuclear and mitochondrial datasets indicated a close relationship between *S. obishime* and *S. ovifrons*. In the preliminary analysis by BLAST search (Table 1), the nuclear S7I1 sequence from one specimen of *S. obishime* (AB811974) was almost identical to that of *S. ovifrons* (JX026639.1) (>99% identity [606/608 bp]). In accordance with the BLAST result, the present BI and ML analyses demonstrated that the nuclear S7I1 sequences of *S. obishime* (representing two specimens) formed a robust monophyletic group with that of *S. ovifrons* (Fig. 2A). In contrast, the BLAST search using the mitochondrial 16S rRNA (AB811976) and CR (AB811975) sequences of *S. obishime* as queries did not identify *S. ovifrons* as the most similar species (Table 1). However, phylogenetic analyses of 16S rRNA sequences reproduced the monophyly of the two species (Fig. 2B; 0.54/37). While the two species were dispersed in the mitochondrial CR trees (not shown), their monophyly was not rejected by the AU test (P = 0.307). The two species were paraphyletic in the trees of the concatenated dataset (16S rRNA + CR) (Fig. 2C). However, monophyly was not rejected by AU tests (P = 0.166). Taken together, these results seem to reveal a close phylogenetic affinity between *S. obishime* and *S. ovifrons*. The close relationship between the two species was recovered in the analysis that used the aligned data of Choat et al. (2012) (Fig. 3A).

Although possibility remains that any of the six described species not included in the present analysis is the true closest relative to *S. obishime*, the probable close relationship between *S. obishime* and *S. ovifrons* appears to be reasonable considering the following three

observations. First, they are morphologically similar in the characters that are useful for separating species of *Scarus*, as is shown in Figure 3B. Second, they share unique TP coloration: body deep blue with a pale blue-green or green-blue spot on the cheek (see Fig. 1a and c). Third, their geographic ranges are both restricted to the sea along and/or off coasts of East Asia (Fig. 1). More precisely, they are almost allopatric within the area (sympatric only at Hachijyo-jima island), with *S. obishime* occupying peripheral oceanic islands (Shimada 2013). Such geographic relationships may indicate that *S. obishime*, which inhabits the waters of oceanic islands, diverged from *S. ovifrons*, which inhabits waters around the mainland, by peripatric speciation, a kind of allopatric speciation (for details, see Coyne and Orr 2004).

In the present study, the following six described species of the genus *Scarus* were not included: *S. caudofasciatus* (Red Sea and Western Indian Ocean), *S. chinensis* (China), *S. fuscocaudalis* (East Indies, Palau, Guam, Philippines, and Ryukyu Islands), *S. gracilis* (China), *S. hypselopterus* (Ryukyu Islands to Indonesia and Palau), and *S. maculipinna* (East Indies) (Parenti and Randall 2000, 2011). Among them, two Chinese species, *S. chinensis* (Steindachner, 1867) and *S. gracilis* Steindachner, 1869, are known only from type material (Parenti and Randall 2000). None of the remaining four species is similar to *S. obishime* or *S. ovifrons* in TP coloration.

Relationship between the S. obishime + S. ovifrons *clade and* S. arabicus

The phylogenetic relationships between *S. obishime*, *S. ovifrons*, and *S. arabicus* were not straightforward, as nuclear and mitochondrial analyses produced different phylogenies. As shown in Table 1, the nuclear S7I1 sequence from one specimen of *S. obishime* (AB811974) was almost identical to that of *S. arabicus* (JX026612.1) (>99% identity [605/608 bp]), as well as that of *S. ovifrons*. In accordance with the BLAST result, the present BI and ML analyses of the S7I1

region recovered the monophyly of these three species (Fig. 2A; except for *S. obishime*, this clade corresponded to SC8 in Choat et al., 2012). However, the BLAST search using the mitochondrial 16S rRNA or CR sequences of *S. obishime* as queries did not identify *S. arabicus* as a similar species (Table 1). Furthermore, *S. arabicus* was located far from the *S. obishime* + *S. ovifrons* clade in the mitochondrial 16S rRNA and 16S rRNA + CR trees (Fig. 2B and C, respectively), and the monophyly of these three species was confidently rejected by AU tests (P = 0.016 and 0.003, respectively for 16S rRNA and 16S rRNA + CR datasets). Hence, *S. arabicus* was closely related to *S. obishime* and *S. ovifrons* based on the nuclear S7I1 dataset, but distantly related to the latter two based on the mitochondrial datasets. The analysis using Choat et al.'s (2012) original dataset with the *S. obishime* sequences added supported the latter (mitochondrial) relationship (Fig. 3A). We supposed this was because the phylogenetic signals from the mitochondrial sequences surpassed those from the nuclear sequences in the analysis.

The phylogenetic positions of *S. arabicus* in the mitochondrial analyses appeared to be more reasonable than those in the nuclear S7I1 analyses. Although *S. arabicus* was closely related to the two East Asian species in the nuclear analyses, the geographic distribution of *S. arabicus* is almost restricted to the southern coasts of the Arabian Peninsula, far from East Asia (see Fig. 1). Considering the geographic ranges, it seems reasonable that *S. arabicus* was included in the "SC10 minus *S. zufar*" clade with phylogenetic affinity to the Red Sea species *S. collana* (see Fig. 1 for range), as was demonstrated in the mitochondrial analyses (see Fig. 2B and C) and the analysis using the dataset of Choat et al. (2012) and the new *S. obishime* sequences (Fig. 3A).

However, the phylogenetic affinities between the one Arabian and the two East Asian species indicated by the nuclear S7I1 analyses (Fig. 2A) are not so enigmatic because there are some examples of close relationships between Arabian and East Asian reef fish species. A labrid

species *Thalassoma cupido* endemic to East Asian waters is said to be closely related to a similar species *Thalassoma loxum* endemic to Arabian waters (Randall 1995). Their close relationship was supported by a molecular analysis conducted by Bernaldi et al. (2004). An apogonid species *Ostorhincus hortulanus*, which is widely distributed in the Western Indian Ocean including the Arabian Sea, is thought to be closely related to a similar species occurring in East Asian waters (Yoshida et al. 2010; Liu and Dai 2012) (taxonomic problems yet to be resolved for the scientific name of the latter species).

The cause of the mitochondrial-nuclear discordance for the phylogenetic positions of *S. arabicus* remains unclear. Discordance between mitochondrial and nuclear trees can frequently occur by introgression of heterospecific genes via interspecific hybridization or by stochastic lineage sorting of ancestral polymorphisms (e.g., Avise 2004). To discriminate the former from the latter, it will be indispensable to reconstruct robust phylogenies with reliable branch lengths both for mitochondrial and multiple nuclear genes (Holder et al. 2001).

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Science, Sports, and Culture, Japan (22770089), and CREST “Development of marine ecosystem evaluation methods in the high throughput sequencing era” funded by Japan Science and Technology Agency.

TABLE 1

Results of BLAST Searches Using Sequences from *Scarus obishime* (NSMT-P 95345) as Queries in the NCBI Nucleotide Database

Rank	Species	Accession Nos.	Identities
Query: nuclear S7I1 region sequence (AB811974, 608 bp)			
1	*Scarus ovifrons*	JX026639.1	606/608 (99%)
2	*Scarus arabicus*	JX026612.1	605/608 (99%)
3	*Scarus xanthopleura*	JX026659.1	583/616 (95%)
Query: mitochondrial 16S rRNA gene region sequence (AB811976, 594 bp)			
1	*Scarus globiceps*	JX026492.1	588/594 (99%)
2	*Scarus ovifrons*	JX026499.1	588/594 (99%)
3	*Scarus* aff. *rubroviolaceus* OM	JX026508.1	587/594 (99%)
Query: mitochondrial control region sequence (AB811975, 403 bp)			
1	*Scarus zufar*	JX026591.1	368/410 (90%)
2	*Scarus globiceps*	JX026561.1	370/412 (90%)
3	*Scarus perrico*	JX026570.1	363/408 (89%)

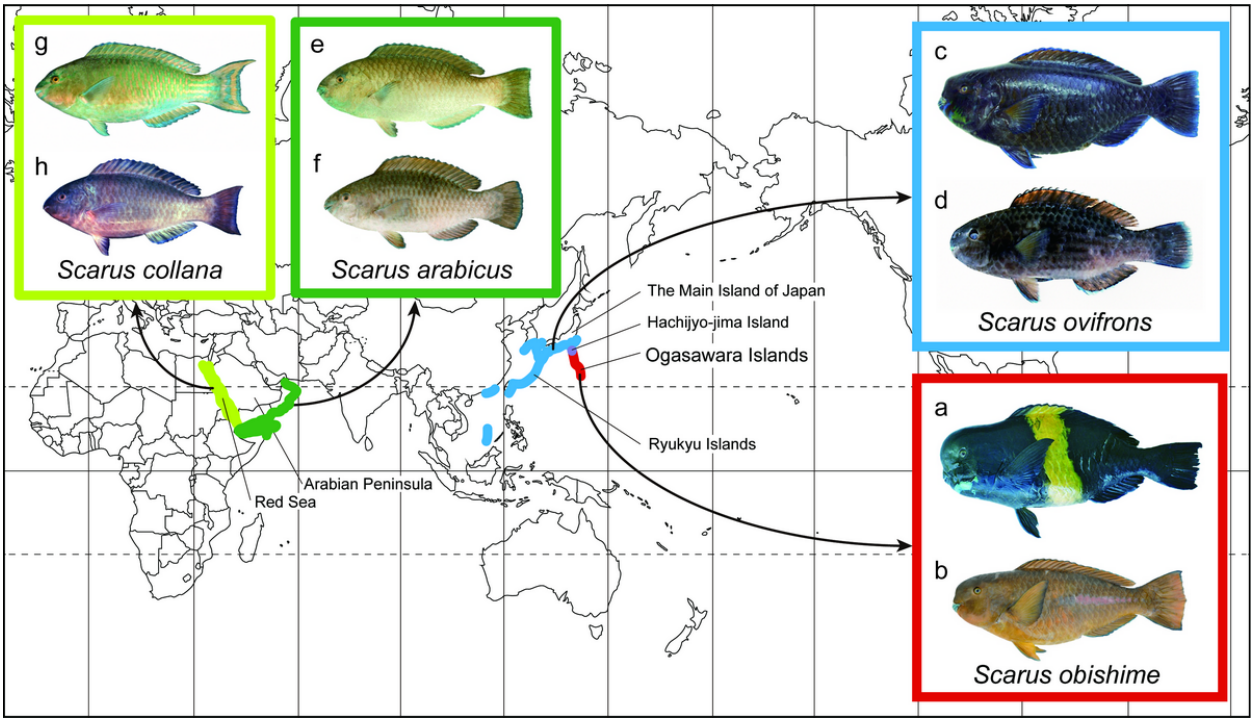

Figure 1. Distributions and body coloration of *Scarus obishime*, *S. ovifrons*, *S. arabicus*, and *S. collana*. *a*, terminal phase (TP) of *S. obishime*, holotype, Chichi-jima Island, Ogasawara Islands, Japan; *b*, initial phase (IP) of *S. obishime*, NSMT-P 95345, Ototo-jima Island, Ogasawara Islands, Japan; *c*, TP of *S. ovifrons*, Seto Inland Sea, Ehime Prefecture, Japan; *d*, IP of *S. ovifrons*, Uchiura Bay, Izu Peninsula, Japan; *e*, TP of *S. arabicus*, Mutrah, Oman; *f*, probable IP of *S. arabicus*; *g*, TP of *S. collana*, Suakin, Sudan; *h*, TP? of *S. collana*, Suakin, Sudan. Images *a*, *e*, *f*, *g*, and *h* are from Randall (1997): *a* by K. Kato; *e*, *f*, *g*, and *h* by J. E. Randall. Images *c* and *d* from FishPix (http://fishpix.kahaku.go.jp/fishimage-e/index.html): *c* by Takaaki Shimizu, KPM-NR0059239; *d* by Hiroshi Senou, KPM-NR0052104. Image *b* by Kaoru Kuriiwa. Geographic distributions of *S. obishime* and *S. ovifrons* are based on Shimada (2013), and those of *S. arabicus* and *S. collana* are based on Choat et al. (2012).

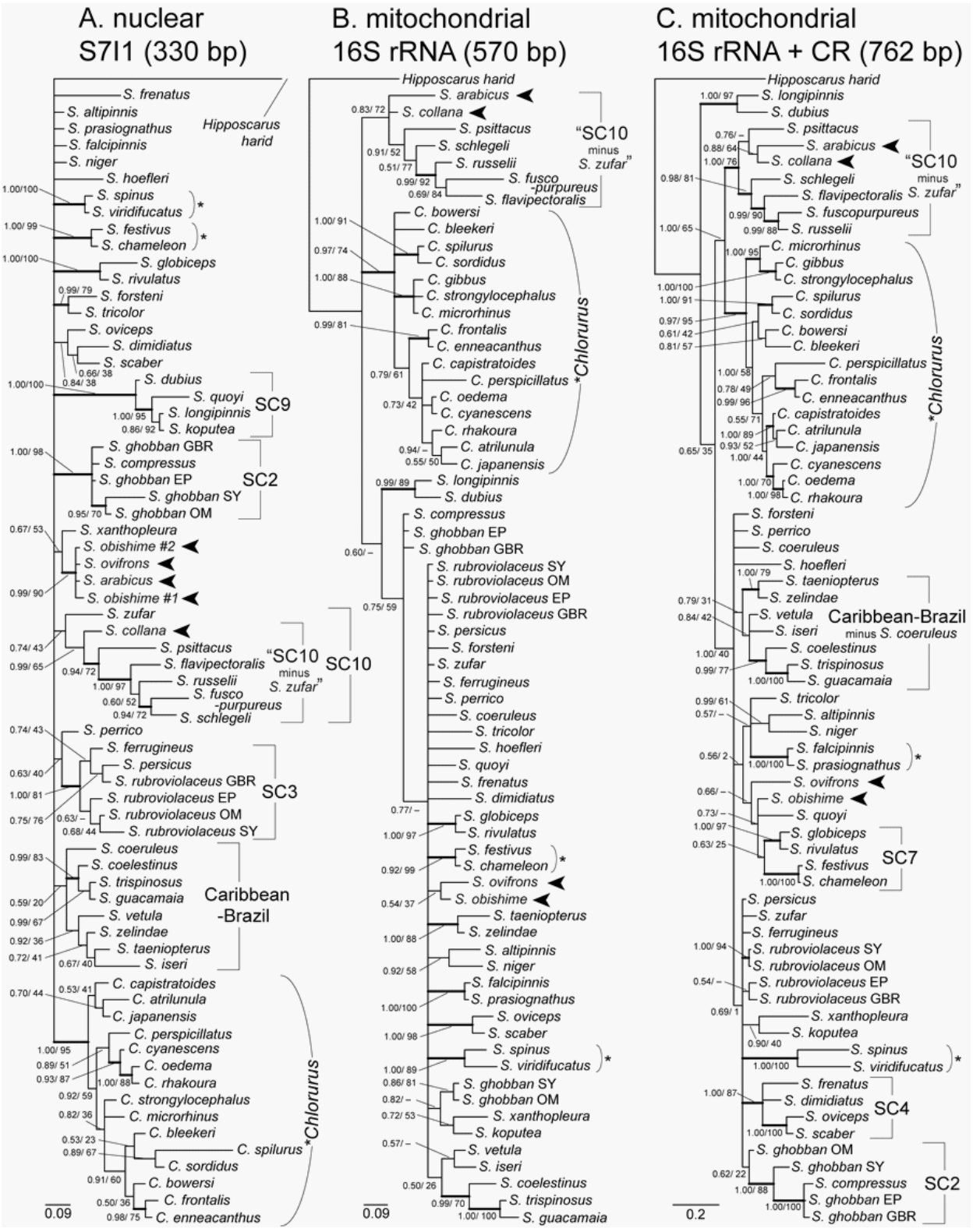

Figure 2. Bayesian trees. *A*, nuclear S7I1; *B*, mitochondrial 16S rRNA; *C*, concatenated mitochondrial (16S rRNA + control region [CR]) dataset. Support values on/near internal branches are Bayesian posterior probabilities (PP, shown as rates)/maximum likelihood (ML) bootstraps (BP, shown as percentages). ‘‘–’’ indicates node not shown in the ML trees. Thick

[Fig 2 caption, cont'd]
branches supported by 0.9 PPs and 70% BPs. Scale bar indicates the number of substitutions per site. Species shown in Fig. 1 are indicated by arrowheads. Species pair reproduced throughout all the four datasets (S7I1, 16S rRNA, CR, and 16S rRNA + CR) are denoted by asterisks.

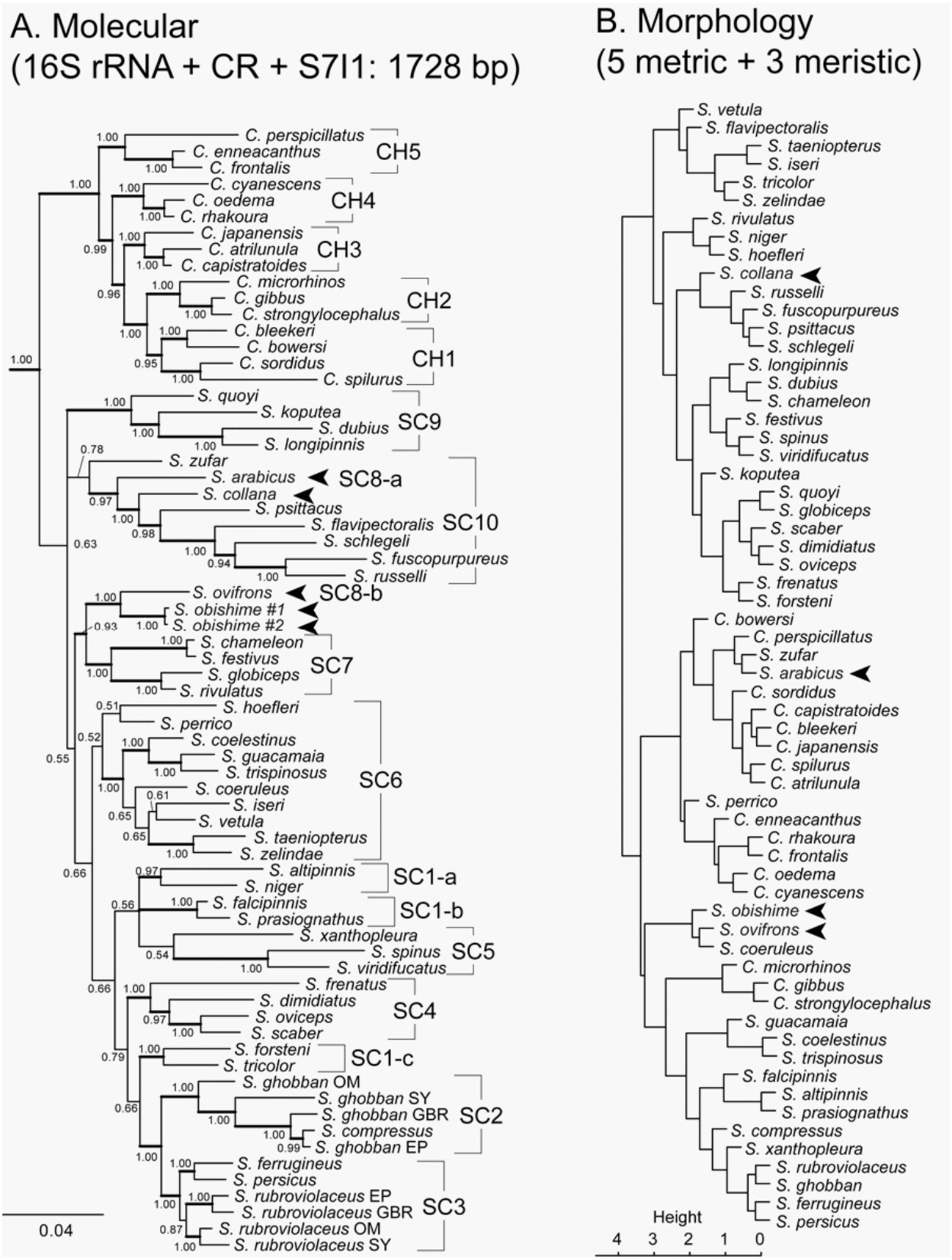

Figure 3. *A*, Bayesian tree based on the original sequence alignment in Choat et al. (2012) combined with *S. obishime* sequences. Support values on/near internal branches are

[Fig 3 caption, cont'd]

Bayesian posterior probabilities (PP, shown as rates). Thick branches are supported by 0.9 PPs. Scale bar indicates the number of substitutions per site. *B*, Cladogram constructed using a hierarchical cluster method based on five morphometric and three meristic variables as in Choat et al. (2012). Mean values for each of the eight variables for *S. obishime* were calculated based on four specimens (NSMT-P35477, 35478, 95302, and 95345), and the values were added to the original data matrix of Choat et al. (2012). After standardizing mean values across species, the resulting data matrix was analyzed using hierarchical cluster analysis of Euclidian distances among the species using the group average method. Species shown in Fig. 1 are indicated by arrowheads.

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