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Assessing the effectiveness of DNA barcoding for exploring hidden genetic diversity in deep-sea fishes

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ABSTRACT: The numbers of deep-sea fish species and their genetic diversities are poorly understood because of taxonomic confusion and the lack of robust diagnostic features. However, DNA barcoding using mitochondrial DNA sequences may offer an effective approach to identifying cryptic species and characterizing their genetic diversities. To validate the genetic differentiation identified by DNA mitochondrial barcoding, it is necessary to show that these reflect variations present in nuclear genomic markers. Here, we performed DNA barcoding using cytochrome *c* oxidase subunit I (COI) sequences and also carried out multiplexed intersimple sequence repeat genotyping by sequencing (MIG-seq) for mesopelagic and demersal fish species from the continental shelf and upper slope of the northwestern Pacific Ocean. We obtained the COI sequences of 115 species from 48 families; the species were identified using the Barcode of Life Data System. Phylogenetic analyses using COI sequences showed high levels of intraspecific genetic differentiation (Kimura 2-parameter distances >2%) in 20 of 115 species, suggesting many cryptic species or intraspecific genetic differentiation previously unknown in these species. We performed phylogenetic and population genetic analyses using multiple single-nucleotide polymorphism loci obtained by MIG-seq of 3 species that showed high levels of intraspecific genetic differentiation in COI sequences. The nuclear markers confirmed the genetic differentiation in all 3 species identified by the COI sequences. The high concordance between these different genetic markers indicates the effectiveness of DNA barcoding for identifying cryptic deep-sea species and characterizing genetic differentiation in these species.

KEY WORDS: Cryptic species · Biogeography · Distribution barrier · Species diversity · COI · MIG-seq

1. INTRODUCTION

The largest and least exploited fish stocks of the world's oceans inhabit the seabed and water column at depths below 200 m (Robison 2009, Priede 2017). Conservation efforts for deep-sea fish fauna are essential, especially when considering threats due to anthropogenic disturbances, such as seabed mining (Cuyvers et al. 2018) and deepwater fisheries (Smith 2007). It is important to obtain accurate estimates of deep-sea fish diversity (i.e. how many fish species live in each geographic region) to develop efficient

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management and conservation methods. However, morphological identification of deep-sea species is hindered by taxonomic confusion or poor diagnostic features (Kawaguchi & Shimizu 1978, Kenchington et al. 2017, Finucci et al. 2018). These major obstacles have resulted in the species diversity of deep-sea fish being much less well known than that of coastal fish species (Tanner et al. 2018, Miyazaki et al. 2019).

DNA barcoding has been developed into an efficient tool for identifying species and providing new perspectives on ecology, taxonomy, and biodiversity (Hebert et al. 2003, Krishnamurthy & Francis 2012, Gaither et al. 2016, Sachithanandam & Mohan 2018). Therefore, DNA barcoding should also offer an effective approach for identifying cryptic deep-sea fish species and characterizing genetic diversity in these species (Kenchington et al. 2017). The mitochondrial cytochrome *c* oxidase subunit I (COI) region is the standard mitochondrial DNA (mtDNA) marker used for DNA barcoding by the international community, and COI reference sequences of marine fish species have been collated (Ward et al. 2005, Hubert et al. 2012) and accumulated in the Barcode of Life Data System (BOLD; Ratnasingham & Hebert 2007) and the International Nucleotide Sequence Database Collaboration (Arita et al. 2021). The 2% Kimura 2-parameter (K2P) distance between a query sequence and a reference sequence was used as a criterion for fish barcode identification (Ward 2009); more robust methods, such as the barcode index number (BIN) system (Ratnasingham & Hebert 2013), have been devised and used recently. The accumulation of reference sequences from deep-sea fish species from around the world contributes to accurate species identification as well as aiding the discovery of cryptic species and characterizing intraspecific genetic differentiation of many widespread species (Priede 2017).

Although DNA barcoding has clear potential for exploring deep-sea fish species diversity, the barcoding method using mtDNA markers has some potential limitations to infer species boundaries; the pattern of genetic differentiation between species shown by mtDNA may differ from that shown by nuclear DNA due to the effect of ancestral polymorphism, sex-biased gene flow, selection of any mtDNA nucleotides, introgression following hybridization, etc. (Moritz & Cicero 2004). Therefore, intraspecific genetic differentiation in mtDNA sequences that suggests the existence of cryptic species needs to be confirmed by analyses of multiple nuclear genetic markers (Moritz & Cicero 2004); species diversity in various fish taxa has been explored by the combination of mtDNA barcoding and analysis of nuclear genetic markers (Monaghan et al. 2005, Raupach et al. 2010). To date, this approach for the study of deep-sea fish species has been limited; however, the combination of mtDNA barcoding with recently developed high-throughput sequencing methods, such as restriction site-associated DNA sequencing (RAD-seq) (Peterson et al. 2012), genotyping-in-thousands by sequencing (GT-seq) (Campbell et al. 2015), multiplexed intersimple sequence repeat (ISSR) genotyping by sequencing (MIG-seq) (Suyama & Matsuki 2015), and genotyping by random amplicon sequencing-direct (GRAS-Di) (Hosoya et al. 2019), offers a potentially rapid and reliable approach for analyses of the genomes of deep-sea fish species. Among these methods, MIG-seq is a method that amplifies anonymous genome-wide ISSR, which is widely used in the population genetics of fishes (e.g. Hirase et al. 2012, Ni et al. 2014, Kato et al. 2021), using multiplex PCR without prior genetic information. It has been shown to be cost effective and capable of identifying genetic diversity within and among species, even from small tissue samples and from relatively low-quality DNA from museum specimens (Iwasaki et al. 2019, Eguchi et al. 2020). Therefore, MIG-seq is considered optimal for DNA barcoding based on nuclear genomes (Suyama et al. 2022).

The northwestern Pacific Ocean has high species richness, which may be attributed to the high topographic complexity that includes large semi-enclosed seas, several islands, and deep-sea trenches (Fujikura et al. 2010, Brandt et al. 2019). Such topographic complexity is also expected to result in high species diversity and genetic variation in deep-sea fish species. However, only a limited number of large-scale DNA barcoding studies have been conducted on deep-sea fish species in the northwestern Pacific Ocean (Zhang & Hanner 2011, Wang et al. 2012). Here, we performed DNA barcoding of mesopelagic and demersal fish species on the continental shelf and upper slope in the seas around Japan and Taiwan; the analyzed specimens were obtained from fish landed at Japanese and Taiwanese fishing ports. All specimens used for DNA barcoding were identified using available monographs and taxonomic literature and deposited as accessible specimens in museum collections for future studies. Based on phylogenetic analyses using the obtained DNA barcoding data, we sought to identify cryptic species and to characterize the genetic diversity in deep-sea fish species that are distributed around the world. Our phylogenetic analyses indicated the oc-

currence of considerable intraspecific genetic differentiation in deep-sea fish species. To validate the effectiveness of DNA barcoding for identifying cryptic species and characterizing genetic diversity in deep-sea species, we focused on 3 northwestern Pacific Ocean deep-sea species and investigated whether the intraspecific genetic differentiation detected by COI sequences could also be detected using nuclear DNA markers obtained by MIG-seq.

2. MATERIALS AND METHODS

2.1. Specimens and sample collection

As many specimens as possible of mesopelagic and demersal fish species from continental shelves and slopes (n = 166) of Japanese and southern Taiwanese waters were collected from 2018 to 2020. The specimens were obtained from fishery catches landed at 10 ports: Ishinomaki (Miyagi Prefecture [Pref.], Japan), Kanaya (Chiba Pref., Japan), Hiratsuka (Kanagawa Pref., Japan), Numazu (Shizuoka Pref., Japan), Yui (Shizuoka Pref., Japan), Maisaka (Shizuoka Pref., Japan), Isshiki (Aichi Pref., Japan), Shin-Nagasaki (Nagasaki Pref., Japan), Kasasa (Kagoshima Pref., Japan), and Dong-gang (Pingtung County, Taiwan) (Fig. 1). After dissecting tissue samples for DNA analysis using a sterile blade and preserving in 99% ethanol, each specimen was fixed in 10% formalin. The specimens were deposited in the Kanagawa Prefectural Museum of Natural History (KPM-NI), Japan, and the National Museum of Marine Biology and Aquarium (NMMB-P), Taiwan. All specimens were identified based on morphological criteria described by Nakabo (2013) and other taxonomic references (e.g. Jordan & Snyder 1900, Kawaguchi & Shimizu 1978, Didier et al. 2012, Koeda & Ho 2019). The taxonomic system and scientific names were based on Motomura (2020). Information on the geographic distribution of each species is based on Nakabo (2013), Koeda & Ho (2019), FishBase (https://www.fishbase.se), and BOLD (www.boldsystems.org).

2.2. DNA extraction and COI sequencing

Genomic DNA was extracted using a Gentra Puregene Tissue Kit (Qiagen). PCR amplification of the mitochondrial COI gene was performed in a 10 µl mixture containing 10 to 50 ng of template DNA, 0.4 µmol of each forward and reverse primer (forward: FishF2 [5′-TCG ACT AAT CAT AAA GAT ATC GGC AC-3′] and reverse: FishR2 [5′-ACT TCA GGG TGA CCG AAG AAT CAG AA-3′], or forward: FishF1 [5′-TCA ACC AAC CAC AAA GAC ATT GGC AC-3′] and reverse: FishR1 [5′-TAT ACT TCG GGG TGG CCA AAG AAT CA-3′] [Ward et al. 2005]), 0.2 µl of Tks Gflex DNA Polymerase (Takara Bio), 5 µl of 2×

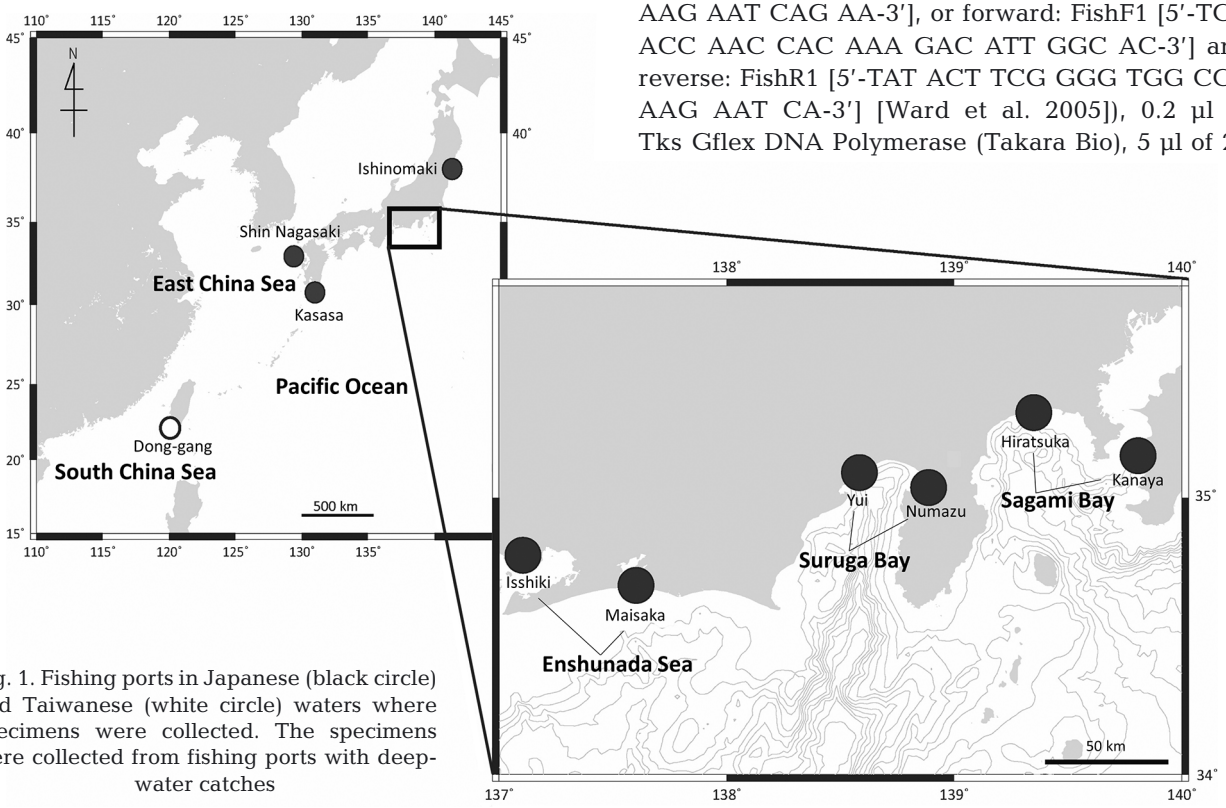


Fig. 1. Fishing ports in Japanese (black circle) and Taiwanese (white circle) waters where specimens were collected. The specimens were collected from fishing ports with deep-water catches

Tks Gflex Buffer (Takara Bio), and distilled water. The following thermal cycling program was used to amplify the DNA: 1 cycle at 95°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min; and, finally, a single extension step at 72°C for 10 min. PCR products were purified with ExoSAP-IT (Thermo Fisher Scientific) following the manufacturer's protocol. Purified PCR products were sequenced in the forward direction using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and an Applied Biosystems 3130 DNA Analyzer (Thermo Fisher Scientific). The obtained sequences were registered with the DNA Data Bank of Japan (DDBJ), BOLD, and the National Center for Biotechnology Information (NCBI). The project name for BOLD is 'DNA barcoding of deep-sea fishes around Japan' (code: TERA). Species identification for each obtained sequence was based on the BIN of BOLD (June 23, 2021). The BIN system is an online framework that clusters barcode sequences algorithmically; the BIN shows high concordance with species (Ratnasingham & Hebert 2013). The BIN system generally recognizes as the same species those with a 98% K2P distance or less and assigns sequences to the same BIN. However, if there is a possibility of misidentification or cryptic species, the BIN may not have been assigned correctly. Therefore, our study proposed that this framework classify species into 4 cases, as described below and in Table S1 in the Supplement at www.int-res.com/articles/suppl/m701p083_supp.pdf.

Case I, match to species: The top hit species (BIN) matches the morphological identification with >98% sequence similarity. The nearest neighbor shows ≤98% sequence similarity. Classification to this case indicates that morphological and molecular identification are consistent.

Case II, genetic differentiation: The top hit species (BIN) matches the morphological identification with >98% sequence similarity. The nearest neighbor shows ≤98% sequence similarity. However, 2 or more genetic lineages are found within the top hit species. This case suggests the existence of cryptic genetic differentiation or misidentification.

Case III, ambiguous match: Two or more species (BIN) show >98% sequence similarity. One of these species matches the morphological identification. This is caused by misidentification of the registered sequence or the existence of species that are genetically quite close.

Case IV, unmatched: No species match (no BIN is assigned to a query sequence, and the top hit sequence is less than 98%) because reference sequences are not registered in BOLD.

2.3. Phylogenetic analyses based on COI sequences

To identify potential cryptic deep-sea fish species from Japanese and Taiwanese waters, phylogenetic and population genetic analyses based on COI sequences and genome-wide single-nucleotide polymorphism (SNP) loci were performed for species for which sequences were obtained from multiple specimens (Tables 1 & 2). We downloaded COI sequences of each focal species from NCBI (https://www.ncbi.nlm.nih.gov) that matched the following criteria: at least 500 bp in length, clearly labeled with the species name, and labeled with the sampling location. We used these sequences for phylogenetic analyses with the newly obtained COI sequences. COI sequence alignment was performed using ClustalW (Thompson et al. 2002). The aligned DNA sequences were trimmed to the same lengths between samples using MEGA X (Kumar et al. 2018) and assigned into haplotypes using FaBox 1.5 (Villesen 2007). Neighbor-joining phylogenetic trees were constructed in MEGA X using the K2P substitution model with complete deletion options and 1000 bootstrap replicates.

2.4. Genome-wide SNP analysis by MIG-seq

Genome-wide SNP loci were used as nuclear genetic markers; these markers were obtained from each species using MIG-seq. MIG-seq, one of the reduced complexity methods for building next-generation sequencing libraries by PCR similar to GRAS-Di and GT-seq (Campbell et al. 2015), was developed to analyze ecological studies (Suyama & Matsuki 2015). As MIG-seq is a cost-effective method, available on non-model organisms without designing original primers, and optimized for low-quality DNA (Suyama & Matsuki 2015), it is suitable for use with relatively low-quality DNA from some specimens.

MIG-seq was performed for 3 species, *Chimaera phantasma* Jordan & Snyder, 1900 (n = 16), *Pyramodon ventralis* Smith & Radcliffe, 1913 (n = 6), and *Neoscopelus microchir* Matsubara, 1943 (n = 20), because mtDNA analyses showed cryptic differentiation within each of these species (see Results, Section 3). MIG-seq and quality control of the raw MIG-seq data were carried out as described by Suyama et

Table 1. Cosmopolitan deep-sea fishes that showed high intraspecific genetic diversity. K2P: Kimura 2-parameter

Species	Maximum interspecific K2P distance (%)	Reference data
Homostolus acer	2.1	Suruga Bay (this study), Taiwan (KU885675.1)
Peristedion orientale	2.9	Enshunada Sea (this study), Taiwan (KU892829.1)
Chimaera phantasma	3.8	South China Sea (this study), Taiwan (KU687932.1, KU687933.1), Kagoshima (this study), Nagasaki (this study), Enshunada Sea (this study), Suruga Bay (this study)
Diaphus garmani	3.5	Taiwan (this study, KU943110.1)
Benthosema pterotum	3.0	Sagami Bay (this study), Indonesia (HQ564294.1)
Deania calcea	5.4	Sagami Bay (this study), Chile (KU737834.1), Iberia (JN161153.1)
Etmopterus molleri	5.9	Enshunada Sea (this study), Okinawa (GU130715.1), Australia (HQ956341.1)
Dasyscopelus asper	8.7	Suruga Bay (this study), Gulf of Mexico (MG856572.1)
Dasyscopelus obtusirostris	2.0	South China Sea (this study), Gulf of Mexico (MG786362.1)
Neoscopelus microchir	11.8	Enshunada (this study), South China Sea (this study), Taiwan (KU943094.1, KU943097.1), South Africa (KF489667.1)
Synagrops japonicus	6.6	Enshunada Sea (this study), South China Sea (MH638809.1), Australia (JN313203.1)
Notacanthus chemnitzi	3.3	Ishinomaki (this study), Canada British Columbia (FJ164915.1), North Atlantic (EU148272.1), Greenland (LC163604.1)
Bathophilus longipinnis	16.8	Suruga Bay (this study), USA (MG856424.1)
Stomias affinis	2.8	Suruga Bay (this study), Gulf of Mexico (MG856779.1), Taiwan (LU943042.1)
Polymetme corythaeola	6.1	Suruga Bay (this study), South Africa (JF494214.1), Canada Nova Scotia (KY033938.1)
Chauliodus sloani	14.8	Suruga Bay (this study), Taiwan (KU943050.1), USA (MH378480.1)
Cyttopsis rosea	3.1	Enshunada Sea (this study), Taiwan (KU943302.1), USA (MH378494.1)
Antigonia capros	3.6	Suruga Bay (this study), South China Sea (KY371135.1)
Idiacanthus fasciola	6.0	Suruga Bay (this study), Gulf of Mexico (MT323722.1)
Photostomias guernei	17.4	Suruga Bay (this study), Canada Atlantic (KY033730.1)
Cryptopsaras couesii	6.1	Suruga Bay (this study), Taiwan (KU943197.1), East Pacific (GU440295.1), South East Pacific (HQ956180.1), Atlantic Ocean (MH033862.1)

Table 2. Cosmopolitan deep-sea fishes that showed low intraspecific genetic diversity. K2P: Kimura 2-parameter

Species	Interspecific K2P distance (%)	Reference data
Pseudotriakis microdon	0.013	Suruga Bay (this study), Atlantic Ocean (EU148299.1)
Dalatias licha	0.000	Suruga Bay (this study), Australia (HQ956264.1), Malta (KY909372.1), North Atlantic (GU130676.6)
Zameus squamulosus	0.005–0.009	Taiwan (this study), Australia (JN312348.1), India (KF899772.1)
Ruvettus pretiosus	0.000–0.002	Sagami Bay (this study), South Africa (HQ945992.1), Australia (JN313155.1), Mediterranean Sea (LN907524.1)
Simenchelys parasitica	0.002–0.007	Ishinoaki (this study), Canada (KY033948.1), New England (KF930446.1)
Epigonus denticulatus	0.000	Suruga Bay (this study), Africa (JF493429.1)

al. (2022), and 1632 to 7437 reads were obtained (Table S2). After the quality control was complete, the remaining reads were assembled using de novo map pipelines (ustacks, cstacks, sstacks) in Stacks v.1.48 (Catchen et al. 2011). Homologous sequences (loci) were assembled in each sample using ustacks with the following settings: minimum depth of coverage = 3, maximum distance allowed between stacks = 3, maximum distance allowed to align secondary reads to primary stacks = 4, and upper bound for epsilon

(error rate [bounded high]) = 0.01. A catalogue of consensus loci was built for each sample using ustacks to assemble the loci, allowing only 2 mismatches between sample loci. A list of loci was obtained with the following settings: minimum number of populations in a locus = 1, minimum percentage of samples in a population = 0.90, minimum minor allele frequency (-min-maf) = 0.05, and maximum observed heterozygosity (max-obs-het) = 0.7; data analysis was restricted to the first SNP per locus (-write-single-snp).

Maximum likelihood trees were constructed using RAxML with 1000 bootstrap replicates. Population genetic structure was assessed using STRUCTURE v.2.3.4 software (Pritchard et al. 2000). An admixture ancestry model with correlated allele frequencies was generated for a putative number of subpopulations (K) ranging from 2 to 5. Ten runs of 10 000 Markov chain Monte Carlo iterations after a burn-in period of 10 000 iterations were carried out for each K-value. The STRUCTURE output was analyzed in STRUCTURE HARVESTER (Earl 2012). The most likely number of clusters was identified by the ΔK method (Evanno et al. 2005).

3. RESULTS

3.1. DNA barcoding of northwestern Pacific Ocean deep-sea fish species

A total of 166 COI sequences were obtained from 115 species, 84 genera, 48 families, and 17 orders of fish. Since 468 deep-sea fish species (140 families; Shinohara & Matsuura 1997) have been recorded in Suruga Bay and 602 species (168 families; Shinohara et al. 2005) in the waters around the Ryukyu Islands, our samples are expected to cover more than 20% of deep-sea fish species in the waters around Japan and Taiwan. The sequence lengths ranged from 500 to 660 bp, and no stop codons, insertions, or deletions were observed in any of the sequences (Table S1). Genetic distances between species within genera were generally >6.0%, although there were a few exceptions (Fig. 2); for example, *Pentaceros japonicus* Steindachner, 1883 versus *Pentaceros wheeleri* (Hardy, 1983) (Pentacerotidae) showed low genetic differentiation (0.009%) despite the large morphological differences (Fig. S1). Intraspecific genetic distances were generally <1.0%.

Within the 115 species, 29 species were Case I (match to species), 13 species were Case II (genetic differentiation), and 44 species were Case III (ambiguous match); 19 species were assigned to Case IV (unmatched) (Fig. 3). Nine species were assigned to both Case II and Case III because 2 or more genetic lineages of the same species with ≤98% sequence similarity and different species with >98% sequence similarity were deposited in BOLD. Although 22 species (13 + 9) were assigned to Case II, 2 of these species, *Coelorinchus multispinulosus* Katayama, 1942 and *Coryphaenoides marginatus* Steindachner and Döderlein, 1887, might have been misidentified, as their morphological characteristics are very similar to the species *Coelorinchus kamoharai* Matsubara, 1943 and *Coryphaenoides microps* (Smith & Radcliffe, 1912), respectively. Thus, 20 of the Case II species showed evidence of intraspecific genetic differentiation. Overall, 53 species (44 + 9) were assigned to Case III, suggesting the existence of many misidentifications in deep-sea fish species. For example, 3 *Coryphaenoides* species (Macrouridae) were identified and sequenced in this study; however, some DNA database sequences appear to have been misidentified presumably due to the small morphological differences among these species (Fig. S2). For 35 species, including those assigned to Case IV, the COI sequences from Japanese waters were obtained for the first time in this study (Table S3).

3.2. Phylogenetic analyses of deep-sea fish species using COI sequences

We performed phylogenetic analyses using COI sequences obtained here and in previous studies of the 20 Case II species suspected of cryptic genetic differentiation (Table 1, Fig. 4). These species had mesopelagic and demersal ecotypes, and 2 major patterns were identified (Fig. 4). The first pattern was genetic differentiation between the western and eastern Pacific, in 9 species: *Bathophilus longipinnis* (Pappenheim, 1914) (Stomiidae); *Stomias affinis* Günther, 1887 (Stomiidae); *Chauliodus sloani* Bloch & Schneider, 1801 (Stomiidae); *Photostomias guernei* Collett, 1889 (Stomiidae); *Idiacanthus fasciola* Peters, 1877 (Stomiidae); *Dasyscopelus asper* (Richardson, 1845) (Myctophidae); *Dasyscopelus obtusirostris* (Tåning, 1928) (Myctophidae); *Cyttopsis rosea* (Lowe, 1843) (Parazenidae); and *Antigonia capros* (Lowe, 1843) (Caproidae). The second pattern was genetic differentiation between species in the South China Sea and Japanese waters in the northwestern Pacific Ocean, in 4 species: *Chimaera phantasma* Jordan & Snyder, 1900 (Chimaeridae); *Homostolus acer* Smith & Radcliffe, 1913 (Ophidiidae); *Benthosema ptero-*

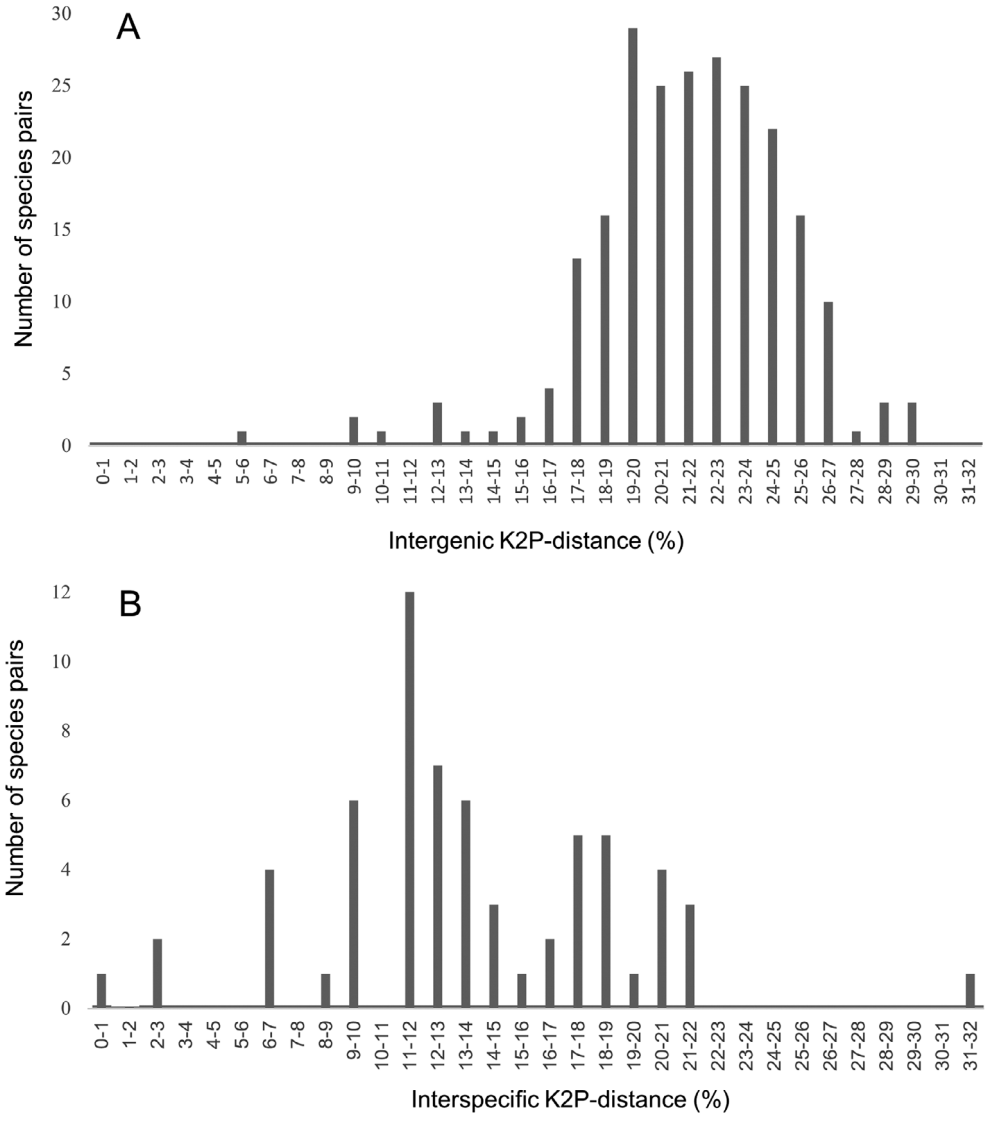


Fig. 2. Comparison and identification of DNA barcoding of deep-sea fishes from Japanese and Taiwanese waters. (A) Intergeneric Kimura 2-parameter (K2P) distance between species within the same family. (B) Interspecific K2P distance between species within the same genus

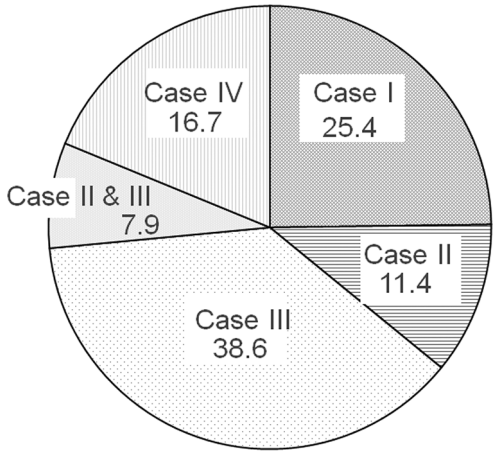


Fig. 3. Results of species identification based on the barcode index number of the Barcode of Life Data System. See Section 2.2 for definitions of cases

tum (Alcock, 1890) (Myctophidae); and *Peristedion orientale* Temminck & Schlegel, 1844 (Peristediidae).

In addition to the 2 major patterns described above, genetic differentiation was also found between the northwestern Pacific Ocean and Australia: *Etmopterus molleri* (Whitley, 1939) (Etmopteridae); between the Pacific Ocean and the North Atlantic: *Notacanthus chemnitzi* (Bloch, 1788) (Notacanthidae); between Australia and the northwestern Atlantic Ocean: *Cryptopsaras couesii* (Gill, 1883) (Ceratiidae); between the northwestern Atlantic Ocean and the Indian Ocean: *Synaphobranchus kaupii* Johnson, 1862 (Synaphobranchidae); and between the southeastern Pacific Ocean and the northeastern Atlantic Ocean: *Deania calcea* (Lowe, 1839) (Centrophoridae). *Diaphus garmani* Gilbert, 1906 (Myctophidae) had 2 clades that were distributed sympatri-

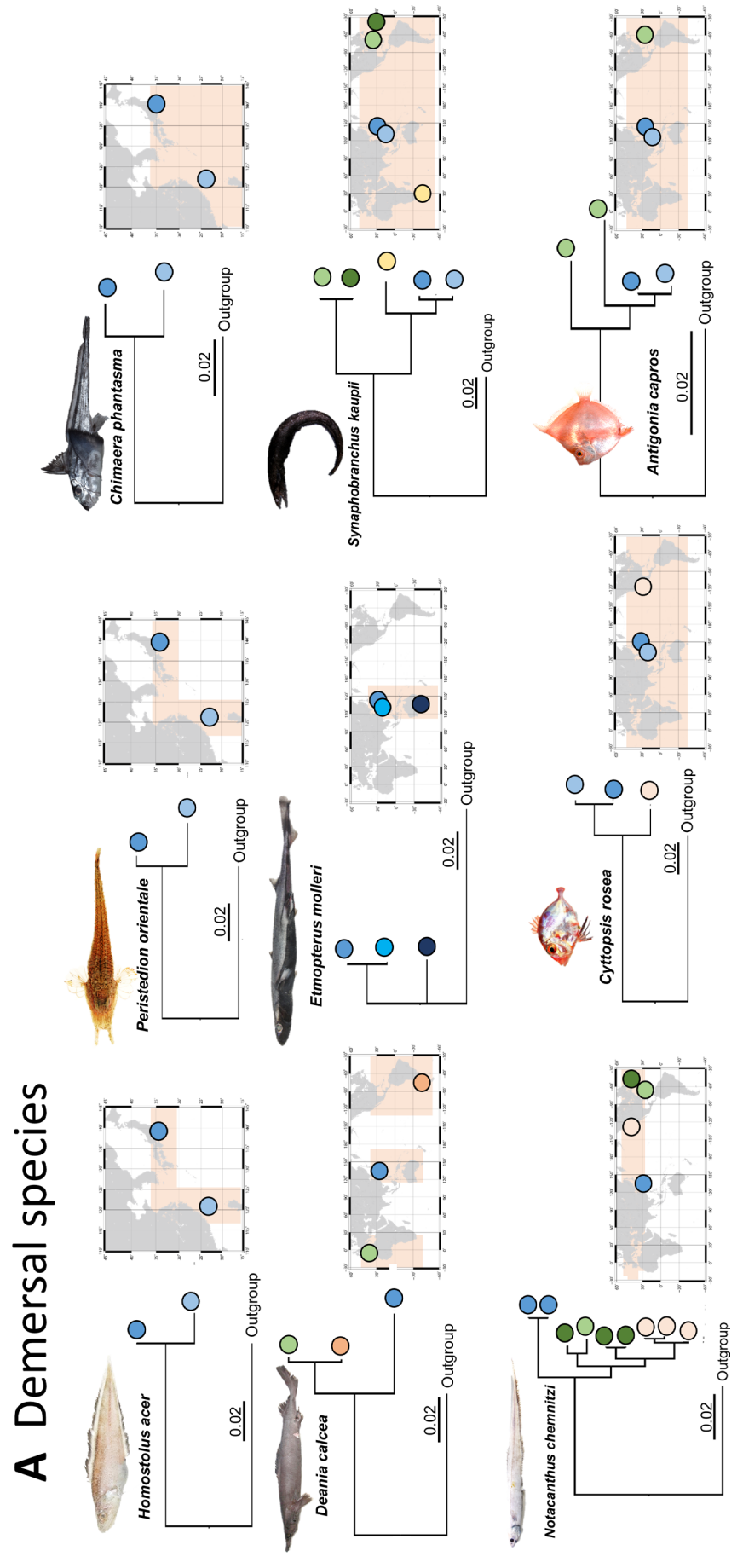
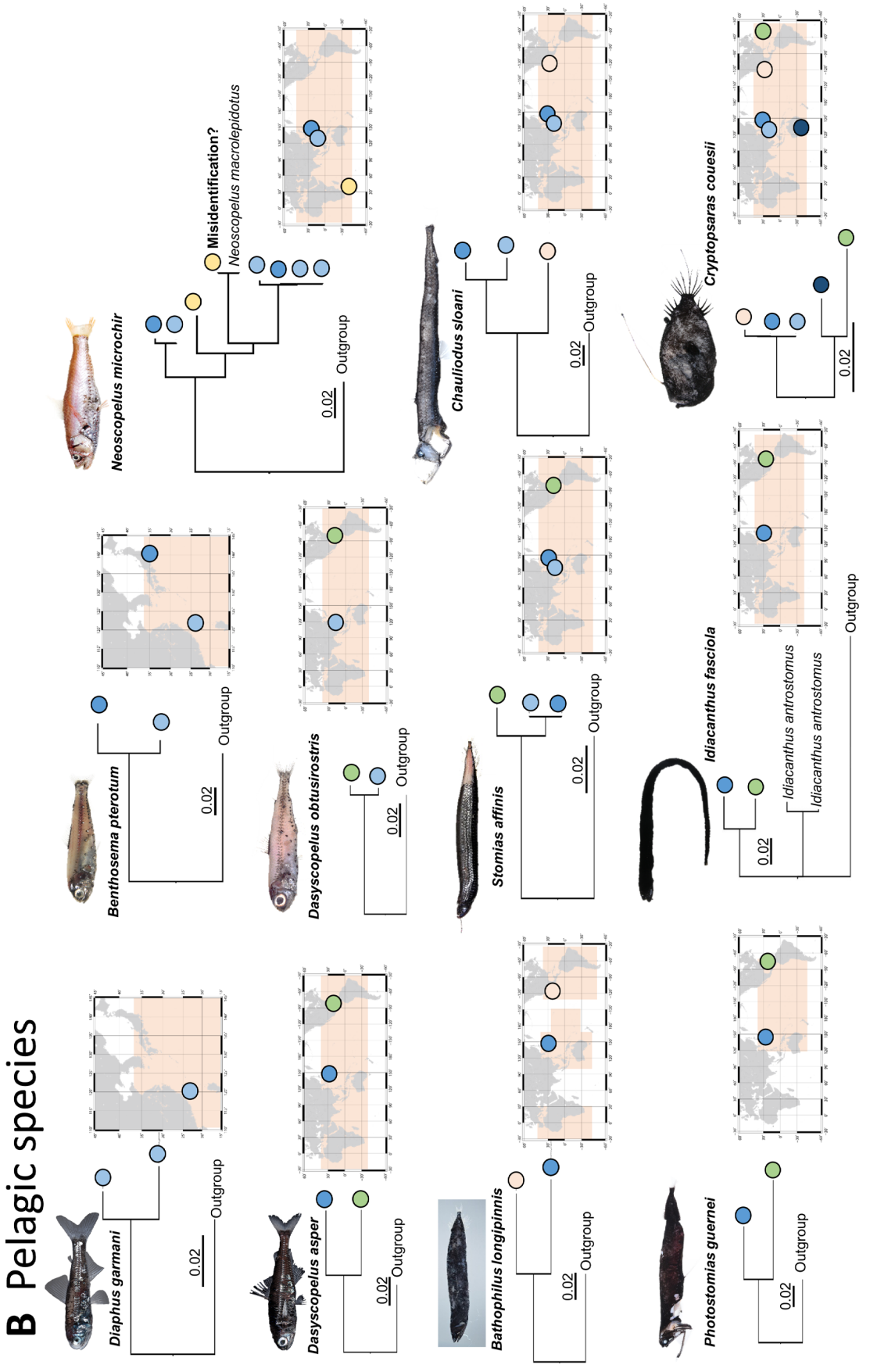


Fig. 4. Neighbor-joining trees of cytochrome *c* oxidase subunit I sequences from 20 species that possibly have cryptic genetic differentiation (belong to Case II; see Section 2.2 for definitions of cases). Intraspecific lineages that were assigned into a different barcode index number were used in each species. The sampling location of each sequence is indicated by a colored circle on the map. The light orange sea area on the map indicates the distribution of each species. Scale bars indicate 2% of the Kimura 2-parameter distance. Node supporting values lower than the 99% bootstrap value were collapsed. (A) Demersal species (n = 9); (B) pelagic species (n = 11)


B Pelagic species
Diaphus garmani
0.02
Outgroup
Benthosema pterotum
0.02
Outgroup
Neoscopelus microchir
Misidentification?
Neoscopelus macrolepidotus
0.02
Outgroup
Dasyscopelus asper
0.02
Outgroup
Dasyscopelus obtusirostris
0.02
Outgroup
Chauliodus sloani
0.02
Outgroup
Bathophilus longipinnis
0.02
Outgroup
Stomias affinis
0.02
Outgroup
Idiacanthus fasciola
0.02
Idiacanthus antrostomus
Idiacanthus antrostomus
Outgroup
Photostomias guernei
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Outgroup
Cryptopsaras couesii
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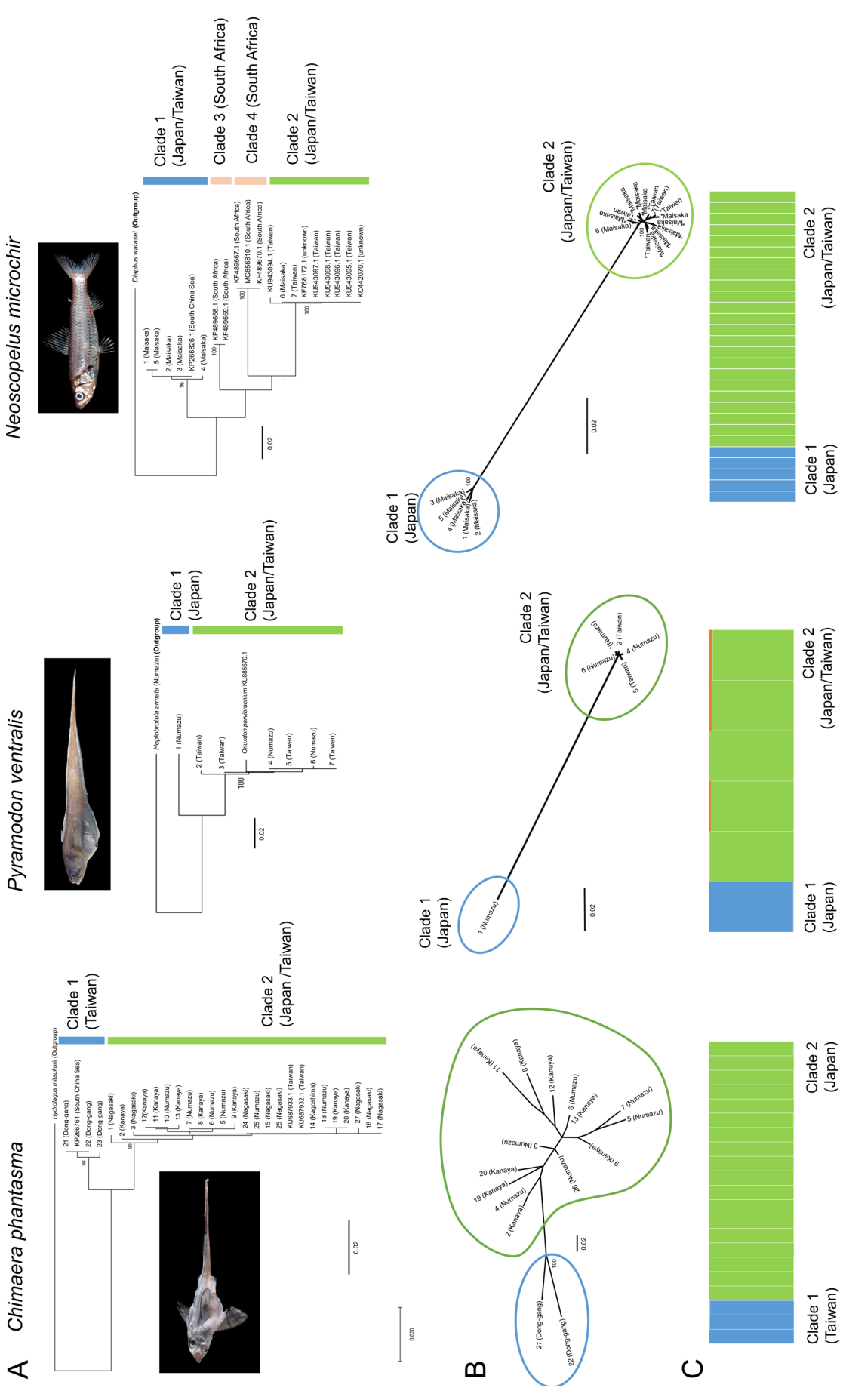

Fig. 5. Intraspecific genetic lineages in 3 deep-sea fishes. (A) Neighbor-joining tree of cytochrome *c* oxidase subunit I sequences based on Kimura 2-parameter (K2P) distance. Sequences from DNA databases are shown with their accession numbers. The scale bar indicates 2 % K2P distance. Boot strap values are also indicated. (B) RAxML trees based on single-nucleotide polymorphism (SNP) loci. The blue and green lines indicate 2 clades. The scale bar indicates the rate of nucleotide differences per sequence site. Node supporting values lower than the 99 % bootstrap value were collapsed. (C) Individual assignment probability bar plots from STRUCTURE for $K = 2$ (*C. phantasma* and *N. microchir*) or 3 (*P. ventralis*). Each vertical bar represents one individual, color-coded based on the results of STRUCTURE

cally around south Taiwan. *Neoscopelus microchir* Matsubara, 1943 (Neoscopelidae) was divided into 4 lineages; 2 of these lineages showed sympatric distribution in the northwestern Pacific Ocean and South Africa. By contrast, a BLAST search indicated that 1 lineage (1 individual) of *N. microchir* in South Africa was genetically close to *N. macrolepidotus* Johnson, 1863 (Neoscopelidae), suggesting that this individual might be *N. macrolepidotus* and was misidentified. Note that intraspecific genetic divergences in *C. phantasma* and *N. microchir* were supported here by the phylogenetic analyses using multiple samples from Japanese and Taiwanese waters (Fig. 5, Table S4).

Phylogenetic analyses based on COI sequences were also performed for 2 Case IV species, *Pyramodon ventralis* and *Hydrolagus mitsukurii* (Jordan & Snyder, 1904) (Fig. S3), for which sequences were obtained from multiple individuals. *P. ventralis* showed 2 intraspecific lineages with more than a 2% K2P genetic distance (*P. ventralis*: 7.4%, Table S4). The 2 *P. ventralis* lineages are distributed sympatrically in Japanese waters (Fig. 5A).

Thirty species were assigned to Case I. Six of these species showed less than a 1% intraspecific K2P distance among COI sequences despite the samples being obtained from the northwestern Pacific Ocean and other waters: *Pseudotriakis microdon* de Brito Capello, 1868 (Pseudotriakidae); *Dalatias licha* (Bonnaterre, 1788) (Dalatiidae); *Zameus squamulosus* (Günther, 1877) (Somniosidae); *Simenchelys parasitica* Gill, 1879 (Synaphobranchidae); *Epigonus denticulatus* Dieuzeide, 1950 (Epigonidae); and *Ruvettus pretiosus* Cocco, 1833 (Gempylidae) (Fig. 6, Table 2). *D. licha* had the same haplotype globally, while *R. pretiosus* had the same haplotype in the Pacific and Atlantic oceans.

3.3. Phylogenetic and population genetic analyses of three deep-sea species using SNP genotyping data

The DNA barcoding data suggested hidden genetic differentiation in 3 species from Japanese and Taiwanese waters that showed more than a 2% K2P genetic distance (Table 2, Table S4): *N. microchir* (10.0%), *C. phantasma* (3.8%), and *P. ventralis* (7.4%). We also confirmed that the sequences of these 3 species were assigned to 2 BINs: *C. phantasma*, BOLD ACV8569 and BOLD ADM5175; *N. microchir*, BOLD AAC6768 and BOLD ADC8835; *P. ventralis*, BOLD AAF4259 and an unregistered BIN.

We conducted phylogenetic and population genetic analyses of genome-wide SNP data obtained by MIG-seq to verify whether the results of COI sequences were supported by nuclear polymorphisms (Table S2; *C. phantasma*, 129 SNPs; *N. microchir*, 304 SNPs; *P. ventralis*, 416 SNPs). All 3 species showed 2 lineages in their COI sequences, and the RAxML trees based on SNPs also showed 2 lineages (Fig. 5A,B). The existence of 2 genetic clusters in each species was supported by the ΔK values from the STRUCTURE analyses (Fig. 5C); *P. ventralis* individuals were clearly divided into 2 major genetic clusters, but the optimal ΔK was for 3 clusters due to the existence of a minor cluster (Fig. 5C, Fig. S4).

4. DISCUSSION

COI sequences from 115 species from 48 families of deep-sea fish species were obtained from waters around Japan and southern Taiwan; many of these sequences were previously not available for the species nor for northwestern Pacific fish populations. Ward (2009) argued that pairs of COI sequences from fish species with a genetic distance greater than 2 to 3% are much more likely to be congeners than conspecifics. Subsequently, several studies used genetic distances (K2P) of 2 to 3% as a criterion for identifying differentiation at the species level (Zhang & Hanner 2011, Kenchington et al. 2017). Therefore, more than a 2% K2P distance shown in this study is considered to be an interspecific genetic difference. Although there are limits to the numbers of specimens per species due to the difficulty of sampling deep-sea fish populations (indeed, this study includes species for which only 1 individual was available to calculate interspecific and intergeneric genetic differences), our study showed that DNA barcoding is an effective tool for identifying deep-sea fish species and to resolve specimens that may be hard to identify due to damage to identification traits and the lack of taxonomic information at each life stage. For example, we were able to identify a damaged Myctophidae specimen as *Diaphus watasei* Jordan & Starks, 1904 (Fig. S4). On the other hand, even though *Pentaceros japonicus* and *P. wheeleri* have distinguishable morphological characters (Fig. S1; Kim 2012), their interspecific K2P distances were at intraspecific levels (0.9%). There are 3 possible explanations for this discordancy: hybridization between the 2 species (Bernatchez et al. 1995, Kwan et al. 2019), rapid morphological differentiation (Sistrom et al. 2012, Albarrán-Lara et al. 2019), and

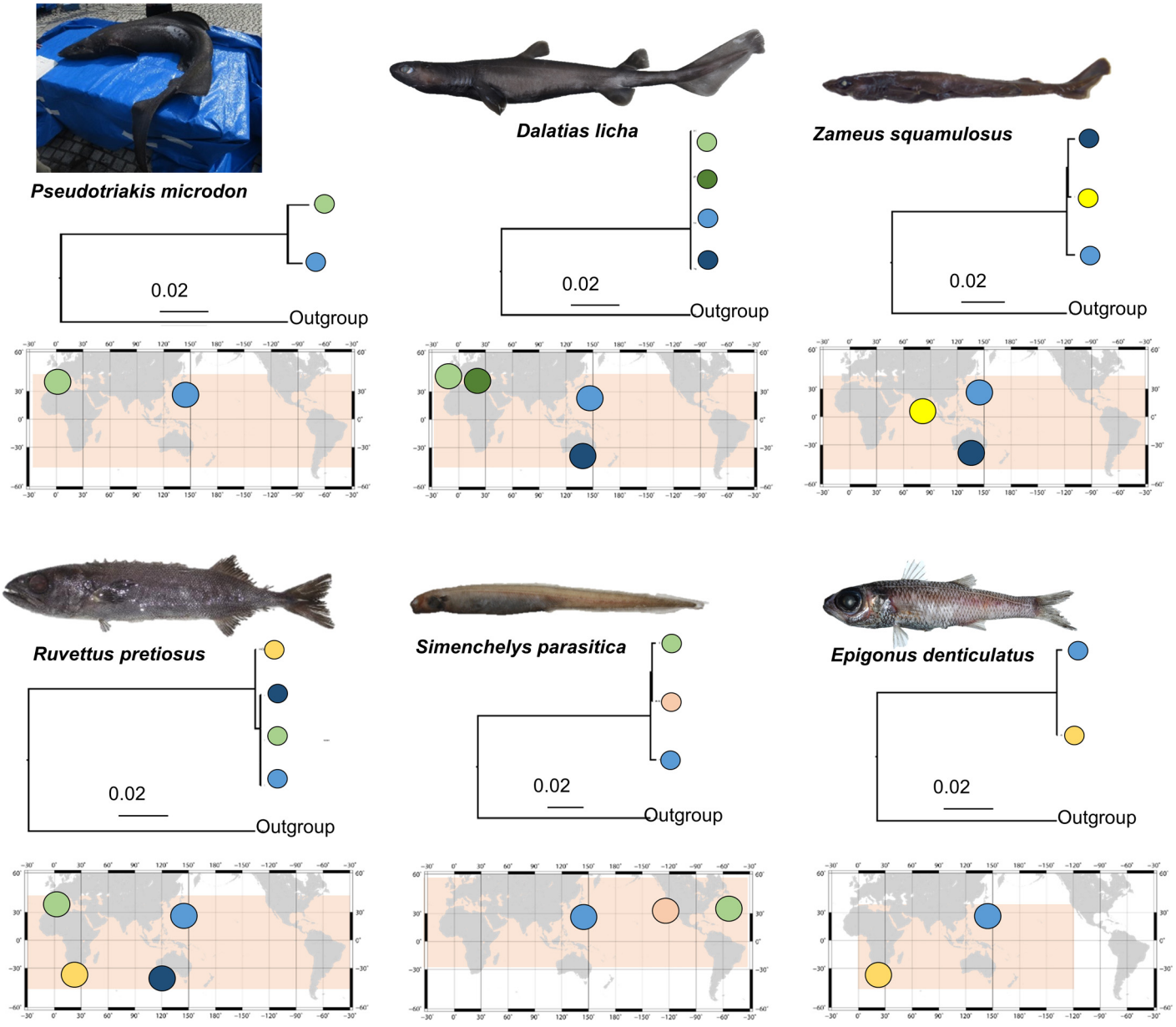

Fig. 6. Neighbor-joining trees of cytochrome *c* oxidase subunit I sequences from 6 widespread deep-sea fishes that belong to Case I (see Section 2.2 for definitions of cases). The light orange sea area on the map indicates the distribution of each species. Scale bar indicates 2% of the Kimura 2-parameter distance

slow COI (or mtDNA) mutation rates (Shearer et al. 2002, Lavinia et al. 2016). This suggests a degree of caution may be necessary for species identification using DNA barcoding data, and it is important to identify the species based on not only DNA but also the morphology of vouchered specimens.

Four deep-sea fish species that belonged to Case III may have been misidentified as closely related species in previous studies (*D. watasei* misidentified as *D. chrysorhynchus* Gilbert & Cramer, 1897; *Coryphaenoides marginatus* misidentified as *C. microps*; *Coelorinchus multispinulosus* misidentified as *C. kamoharai*; *Coelorinchus jordani* Smith & Pope, 1906 misidentified as *C. kishinouyei* Jordan & Snyder, 1900). For example, the downloaded COI sequences attributed to *C. marginatus* from BOLD were assigned to *C. microps* (Fig. S2). As these species pairs are very similar in morphology, misidentifications are highly probable. In the present study, at least 1 morphologically identifiable specimen of each species was used to obtain the sequences. Therefore, our findings emphasize the importance of careful

morphological identification of the specimens used for DNA barcoding of deep-sea fish.

The DNA barcoding data suggested intraspecific genetic differentiation in 20 deep-sea fish species (Fig. 4). Most of the genetic differentiation in these species occurs along well-known marine biogeographic boundaries such as between the northwestern and eastern Pacific Ocean, between the northwestern and southwestern Pacific Ocean, and/or between the northwestern Pacific and western Atlantic oceans (Bowen et al. 2016, Costello et al. 2017). Among the 20 species (Fig. 4), 11 were mesopelagic species. High intraspecific COI genetic differentiation in mesopelagic fish within and between oceans has been reported previously in several waters (Gordeeva 2014, Kenchington et al. 2017). Therefore, previous findings, as well as our own, suggest that many mesopelagic fish species that are characterized by a worldwide distribution have highly divided population structure or include several cryptic species. Nine other species were upper continental shelf demersal fish. Among them, multiple cryptic species in *Notacanthus chemnitzi* are also suggested by previous studies (Robertson et al. 2017, Poulsen et al. 2018). The other 8 species also showed 2 or more lineages with a high intraspecific genetic differentiation. Overall, our results suggest that upper continental shelf demersal fish species might contain several cryptic species or an undetermined population structure. Many pelagic and demersal species have a long floating larval stage (Merrett 1989, Baco et al. 2016, Priede 2017). Therefore, genetic differentiation in species with widespread populations is not expected (Smith 2007, Varela et al. 2012). However, 20 species in this study were found to have high genetic differentiation along biogeographic zones regardless of habitat ecotypes, indicating that there are unknown barriers for gene flow. Taken together, our results suggest that some deep-sea fish have cryptic species or population differentiation regardless of ecotypes, highlighting the need for DNA barcoding of widespread deep-sea species in an international framework.

In contrast to the 20 species with high genetic differentiation, 6 other species showed low intraspecific genetic differentiation, and they included 3 deep-sea sharks (Fig. 6). Low genetic differentiation in deep-sea sharks with worldwide distribution has been suggested to be caused by high migration ability (Catarino et al. 2015). Therefore, the 3 shark species are expected to have high swimming ability and may perform long-range migrations, although ecological studies of these species are limited. Future research should focus on taxonomical and ecological studies of mesopelagic and demersal fish species on the shelf-break zone.

As mentioned above, the combination of our and previous DNA barcoding data has suggested some possible hidden genetic differentiation in deep-sea fish species. However, genetic differentiation within a species needs to be verified by genetic analyses based on nuclear genetic markers that are informative for complex genetic structures, such as past hybridization events (Watanabe et al. 2020, Suyama et al. 2022). To illustrate how this might be accomplished, we tested whether the results of DNA barcoding were supported by analyses of nuclear SNP markers in 3 species: *Chimaera phantasma, Neoscopelus microchir*, and *Pyramodon ventralis*. Phylogenetic analyses of COI sequences of Japanese and Taiwanese populations of these 3 species indicated allopatric or sympatric genetic differentiation. This genetic differentiation was confirmed by phylogenetic and population genetic analyses of SNP markers (Fig. 5) even though these lineages could not be distinguished by the reported morphological diagnostic traits (Didier et al. 2012, Nakabo 2013). The high concordance between the 2 genetic markers (COI and SNP) shows the effectiveness of DNA barcoding in detecting hidden genetic differentiation. The result of *C. phantasma* showing significant genetic differentiation occurred between Japanese and Taiwanese populations. This allopatric differentiation between the 2 populations may have occurred because of geographic isolation during the last glacial period, as has been identified in some shallow-water species in the northwestern Pacific (Ni et al. 2014). Indeed, genetic differentiation in some deep-sea sharks has been suggested to be caused by sea-level changes during glacial–interglacial cycles (Catarino et al. 2015, Walter et al. 2017). Moreover, the fact that the predominant lineage in Japanese waters is also present in the waters of southern Taiwan may suggest secondary contact after geographic isolation (Fig. 5). In contrast, *P. ventralis* and *N. microchir* showed 2 lineages that were distributed sympatrically around Japanese waters. These findings strongly suggest that there may be other reproductively isolated cryptic species that have not been identified morphologically in Japanese waters.

In summary, our DNA barcoding data of deep-sea fish species from the northwestern Pacific Ocean suggested the occurrence of many cryptic species and the presence of previously unidentified intraspecific genetic differentiation. In addition, a comparison of the conclusions from phylogenetic and popula-

tion genetic analyses of COI sequences and SNPs in 3 species from Japanese and Taiwanese waters indicated the consistent identification of high levels of intraspecific genetic differentiation. The high concordance between these genetic markers indicates the effectiveness of DNA barcoding in searching for cryptic species and genetic differentiation in deep-sea fish.

Data accessibility. Data accessibility and benefit-sharing statement data sets supporting the results of this study are available in the DNA data bank (DDBJ Accession No. LC632353-LC632379, NCBI: ON398527-ON398697). MIG-seq data are available from DDBJ under Accession Nos. DRR408796-DRR408837 [BioProject: PRJDB14326 (PSUB018434)].

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