DNA barcoding of deep-sea fishes from the northwestern Pacific Ocean: a resource for identifying hidden genetic diversity in deep-sea fishes

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Abstract

COI-based DNA barcoding could be an efficient tool for species identification of deep-sea fishes and could lead to the discovery of cryptic species diversity. However, the availability of reference sequences of deep-sea fishes for DNA barcoding is limited, especially for fishes in the northwestern Pacific Ocean. In this study, we performed DNA barcoding of mesopelagic and demersal fish species on the continental shelf and upper slope, collected from deepwater fisheries around Japan and southern Taiwan, to accumulate the reference sequences of deep-sea fishes in the northwestern Pacific Ocean. Overall, we obtained the COI sequences of 123 species from 50 families. Genetic diversity within each species for which COI sequences were obtained from multiple specimens was examined, and we found that *Chimaera phantasma* (Chimaeridae), *Harpadon microchir* (Synodontidae), and *Pyramodon ventralis* (Carapidae) showed high intraspecific genetic differentiation of more than 2% Kimura two-parameter distance. Moreover, for 19 widespread deep-sea fishes, a comparison between our data and previously acquired COI sequence data suggested a high level (more than 2% Kimura two-parameter distance) of genetic differentiation between the northwestern Pacific Ocean and other oceans in each widespread species. These results suggest that many cryptic species or regional populations have not yet been discovered in deep-sea fishes. Alternatively, genetic differentiation was not found worldwide for six species. These results indicate that many taxonomic and biogeographical issues remain for deep-sea fishes, and our DNA barcoding data would provide better understanding of these issues.

Introduction

The largest and least exploited fish stocks of the world's oceans inhabit the seabed and water column at depths below 200 m (Robioson, 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 accrue accurate estimates of deep-sea fish diversity to develop efficient management and conservation methods. Nonetheless, morphological identification of deep-sea fishes is sometimes difficult because of taxonomic confusion or poor diagnostic features (Kenchington et al., 2017; e.g., Myctophidae: Kawaguchi & Shimizu, 1978; Chimaeridae: Finucci et al., 2018). As a result of these major obstacles to understanding fish species diversity in deep-sea areas, the species diversity of deep-sea fishes is much less well known than that of shallow coastal fishes (Tanner et al., 2018; Miyazaki et al., 2018).

DNA barcoding has been advanced as an efficient tool to develop species identification and new perspectives

in ecology, taxonomy, and biodiversity assessment (Krishnamurthy & Francis, 2012; Gaither et al., 2015; Sachithanandam & Mohan, 2018). Therefore, DNA barcoding could also be an effective tool for identifying deep-sea fishes. The mitochondrial cytochrome oxidase subunit 1 (COI) region is commonly used for fish DNA barcoding projects in the world, and COI reference sequences of marine fishes have been collected and accumulated in the Barcode of Life Database (BOLD: Ratnasingham & Hebert, 2007) or the International Nucleotide Sequence Database Collaboration (INSDC: Arita & Karsh-Mizrachi, 2020). The accumulation of reference sequences of deep-sea fishes from all over the world would contribute to their accurate identification as well as the discovery of cryptic species or intraspecific genetic differentiation that could include many widespread species (Priede, 2017).

The northwestern Pacific Ocean has high species richness that is considered to be attributed to its high topographic complexity, which includes large semienclosed 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 and genetic diversity in deep-sea fishes. However, only a limited number of large-scale DNA barcoding studies have been conducted on deep-sea fishes in the northwestern Pacific Ocean (Zhang & Hanner, 2011; Wang et al., 2012). Here, we performed COI-based DNA barcoding of mesopelagic and demersal fish species on the continental shelf and upper slope collected from deepwater fisheries around Japanese and southern Taiwanese waters. All specimens used for DNA barcoding were accurately identified using morphological characteristics and deposited as accessible specimens in museum collections for future studies. Furthermore, on the basis of the obtained sequence data, we estimated genetic diversity of deep-sea fishes in the northwestern Pacific Ocean from two perspectives. The first objective of present study is to examine deficiencies in current taxonomy and potential cryptic species in northwestern Pacific Ocean deep-sea fishes by calculating their interspecific and intraspecific genetic distances. The second objective is to examine worldwide genetic differentiation in widespread deep-sea fishes by calculating their intraspecific genetic distance based on comparisons between the northwestern Pacific Ocean and worldwide reference sequences obtained from the DNA database.

Materials and methods

Sample collection and specimens

Specimens of mesopelagic and demersal species of continental shelves and slopes (N = 165) were collected from Japanese and southern Taiwanese waters from 2018 to 2020. All of the specimens were collected from the fishery catches of 10 fishing ports: Ishinomaki (Miyagi 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), Nagasaki (Nagasaki Pref., Japan), Kasasa (Kagoshima Pref., Japan), and Dong-gang (Pingtung County, Taiwan) (Fig. 1). Each specimen was fixed with 10% formalin, and tissue samples for DNA analysis were dissected with a sterile blade and stored in 99% ethanol. The specimens were deposited in the Kanagawa Prefectural Museum of Natural History (KPM-NI), Japan, and the National Museum of Marine Biology & Aquarium (NMMB-P), Taiwan.

Taxonomic and biogeographic information

All specimens were identified based on the study by Nakabo (2013), with some additional taxonomic references (e.g., Jordan & Snyder, 1900; Kawaguchi and Shimizu, 1968; Dider, 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 the studies by Nakabo (2013) and Koeda & Ho (2019), Fishbase (https://www.fishbase.se), and BOLD (http://www.boldsystems.org).

DNA extraction and sequencing

Genomic DNA was extracted using the Gentra Puregene Tissue Kit (QIAGEN). PCR amplification of the mitochondrial COI gene was performed in a mixture with a final volume of 10 μL containing 10–50 ng of template DNA, 0.4 μmol of each forward and reverse primers [forward: FsihF2 (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: FISH F1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and reverse: FISH R1 (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 Inc.), 5 μL of 2× Tks Gflex Buffer (Takara Bio Inc.), and distilled water. Thermal cycling began with one cycle at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, 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 Inc., USA) following the manufacturer's protocol. Purified PCR products were sequenced in the forward direction using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc.) and the Applied Biosystems 3130 DNA Analyzer (Thermo Fisher Scientific Inc.).

Phylogenetic analyses

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 Kimura two-parameter (K2P) substitution model and complete deletion options, and 1,000 bootstrap replicates. We downloaded COI sequences from NCBI (*https://www.ncbi.nlm.nih.gov*), which were at least 500 bp in length, clearly labeled with the species name, labeled with the sampling location, and used for phylogenetic analyses with newly obtained COI sequences.

Results

DNA barcoding of northwestern Pacific Ocean deep-sea fishes

One hundred sixty-five COI sequences were obtained from 123 species, 89 genera, 50 families, and 17 orders of fishes. The sequence lengths ranged from 500 to 660 bp, and no stop codons, insertions, or deletions were observed in any of the sequences (Supplemental Table 1). The COI sequences of 23 species were new to the NCBI and BOLD databases (Table 1). For 35 species, COI sequences from Japanese individuals were obtained for the first time in this study (Supplemental Table 2).

The COI barcoding results were largely consistent with morphological identification, the genetic distance within genera was more than 6.0%, and intraspecies genetic distances were less than 1.0% for most species (Fig. 2). On the other hand, *Pentaceros japonicus* Steindachner, 1883/*Pseudopentaceros wheeleri* Hardy, 1983 (Pentacerotidae) (Fig. 3) had low genetic differentiation between species despite the large morphological differences. Moreover, *Chlorophthalmus* sp. 2 sensu Nakabo (2013) (Japanese name: Bake-aomeeso), which is treated as an undescribed species, formed a clade independent of the other species (Supplemental Fig. 1). Three *Coryphaenoides* species (Macrouridae) were identified and sequenced in this study, but some DNA database sequences may have been misidentified (Supplemental Fig. 2). Note that *Chimaera phantasma* (Chimaeridae), *Harpadon microchir* (Synodontidae), and *Pyramodon ventralis* (Carapidae) have more than 2.7% intraspecific genetic distance (Supplemental Table 3). Among them, *C. phantasma* and *H. microchir* showed two clades corresponding to individuals from Japanese and southern Taiwanese waters, and *P. ventralis* had two clades that are distributed sympatrically around Japanese waters (Fig. 4).

Comparison between our study results and previously acquired sequence data for 26 widespread deep-sea fishes

For 26 of the deep-sea fishes whose COI barcoding was conducted, COI sequences from waters outside the northwestern Pacific Ocean were registered in the DNA databases. We then compared COI sequences from the northwestern Pacific Ocean and other waters for such widespread deep-sea fishes. In this survey, to avoid COI sequences based on misidentification, we confirmed that the COI sequences of target species from waters outside the northwestern Pacific Ocean show no similarity to other species using BLAST. Collectively, 19 of the 26 widespread deep-sea fish species showed more than 2% intraspecific genetic distance among COI sequences, suggesting geographic differentiation in these species (Table 2 and Fig. 5).

Of them, 19 species suggested various patterns of geographic differentiation, but these patterns were classified into two major patterns (Fig. 5): genetic differentiation between the western and eastern Pacific [9

species: *Bathophilus longipinnis*(Pappenheim, 1914) (Stomiidae); *Stomias affinis* Günther, 1887 (Stomiidae); *Chauliodus sloani* Bloch & Schneider, 1801 (Stomiidae); *Photostomias guernei* Collett, 1889 (Stomiidae);*Idiacanthus antrostomus* Gilbert, 1890 (Stomiidae);*Dasyscopelus asper* (Richardson, 1845) (Myctophidae);*Dasyscopelus obtusirostris* (Tåning, 1928) (Myctophidae);*Cyttopsis rosea* (Lowe, 1843) (Parazenidae); and *Antigonia capros* (Lowe, 1843) (Caproidae)] and those between the South China Sea and Japanese waters in the northwestern Pacific Ocean [three species: *Homostolus acer* Smith & Radcliffe, 1913 (Ophidiidae);*Benthosema pterotum* (Alcock, 1890) (Myctophidae); and*Peristedion orientale* Temminck & Schlegel, 1844 (Peristediidae)]. Moreover, the genetic differentiation between the northwestern Pacific Ocean and other localities was as follows: Australia [*Etmopterus molleri* (Whitley, 1939) (Etomopteridae)], between the northeastern Pacific Ocean and North Atlantic [*Notacanthus chemnitzi* (Bloch, 1788) (Notacanthidae)], between Australia and the northwestern Atlantic Ocean [*Cryptopsaras couesii* (Gill, 1883) (Ceratiidae)], between the northwestern Atlantic and Indian Oceans [*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 two clades that were distributed sympatrically around south Taiwan. *Neoscopelus microchir* Matsubara, 1943 (Neoscopelidae) was divided into four lineages, and two lineages are distributed sympatrically in the northwestern Pacific Ocean and South Africa. By contrast, a BLAST search indicated that one lineage of *N. microchir* in South Africa was genetically close to *Neoscopelus macrolepidotus* Jhonson, 1863 (Neoscopelidae), suggesting that this might be due to misidentification.

Six of these 26 species showed less than 1% intraspecific K2P distances among COI sequences 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 and Table 3). Note that *D. licha* shared the same global haplotype and *R. pretiosus* also shared the same haplotype in the Pacific and Atlantic oceans.

Discussion

Availability of COI barcoding to identify deep-sea fishes

COI sequences from 123 species in 62 families of deep-sea fish species were obtained in waters around Japan and southern Taiwan, including these not available for speices or northwestern Pacific populations previously. Ward (2009) argued that pairs of COI sequences from fishes with a genetic distance greater than 2%–3% are much more likely to be congeners than conspecifics, and several studies used genetic distances (K2P) of 2%–3% as criteria for finding differentiation at a different species level (Zhang & Hanner, 2011; Kenchington et al., 2017). In the present study, genetic distances of more than 5% were calculated as almost species pairs. Therefore, DNA barcoding was confirmed to be an effective tool for identifying the majority of deep-sea fishes which resolve these specimens that are hard to identify (e.g., damaged identification traits and the lack of taxonomic information at each life stage). Indeed, we were able to identify a damaged Myctophidae specimen as *Diaphus watasei* (Supplemental Fig. 3).

Moreover, our dataset contributes to finding potential misidentifications in previous DNA barcoding studies of deep-sea fishes. For example, downloaded COI sequences of *Coryphaenoides marginatus* Steindachner & Döderlein, 1887 from the NCBI database were included in a Taiwanese linage *Coryphaenoides microps* (Smith & Radcliffe, 1912) in our dataset. These species are very similar morphologically, and misidentification is highly probable, but the possibility of hybridization and intraspecific polymorphism cannot be excluded. Such misidentified sequences are difficult to detect. In the future, it will be necessary to expand the number of reference sequences that can be easily reconfirmed by using voucher specimens and their pictures.

The COI sequences obtained in this study are a useful tool for identification and provide new research opportunities. First, DNA barcodes could provide information for more accurate descriptions of taxonomically problematic species. *Chlorophthalmus* sp. 2 (Chlorophthalmidae) sensu Nakabo (2013) (Japanese name: Bake-aomeeso) is undescribed species that has ambiguous morphologic features (Supplemental

Fig. 2). In this study, we compared the COI sequences of *Chlorophthalmus* sp. 2 with congener species, namely, *Chlorophthalmus albatrossis* Jordan & Starks, 1904, and *Chlorophthalmus nigromarginatus* Kamohara, 1953. As a result, *Chlorophthalmus* sp. 2 was found to be genetically distinct from congeners as suggested by Gomon et al. (2014). It can be difficult to obtain additional specimens of rare species such as *Chlorophthalmus* sp. 2, which hinders taxonomic studies. Therefore, DNA barcoding can provide new information for taxonomic problems that involve rare species. Second, our DNA barcoding could provide preliminary findings may lead to future issues related to evolutionary biology. Indeed, species in one genus have very differentiated morphological characteristics compared to genetic differentiation; Although *Pentaceros japonicus* and *Pentaceros wheeleri* have greatly different morphological characters (Fig. 3), their interspecific K2P distances were at intraspecific levels (0.9%). This result can be explained by one of three scenarios: hybridization between the two species (Bernatchez et al., 1995; Kwan et al., 2019), rapid morphological differentiation (Sistron et al., 2012; Albarrán-Lara et al., 2019), or slow COI (or mtDNA) mutation rates (Shearer et al., 2002; Lavinia et al., 2016).

Three potential cryptic deep-sea fishes in the northwestern Pacific Ocean

Chimaera phantasma , *Harpadon microchir* , and *Pyramodon ventralis* showed two intraspecific lineages with more than 2% of K2P distance (Fig. 4). These lineages might be cryptic species according to the criteria proposed by Ward (2009), although they cannot be distinguished by current references (Dider, 2012; Nakabo, 2013). The two lineages of *C. phantasma* and *H. microchir* almost correspond to waters in Japan and southern Taiwan. Such genetic differentiation has been reported for some shallow-water species, and these differentiations were inferred to arise because of geographic isolation during the last glacial period (Gang et al., 2013). Indeed, it has also been suggested that genetic differentiation in deep-sea sharks was caused by glacial-interglacial cycles (Catarino et al., 2015; Walter et al., 2017). By contrast, the fact that the lineage predominantly distributed in Japanese waters is also present in waters of southern Taiwan suggests secondary contact after geographic isolation (Fig. 4). Contrary to the two species, *P. ventralis* showed two lineages distributed sympatrically around Japanese waters, suggesting the existence of sympatric cryptic species in Japan. Further genetic and morphological analyses are required to reveal the presence of cryptic species in the three deep-sea fishes.

Comparison between our data and previously acquired sequence data for 26 widespread deep-sea fishes

We detected 19 widespread deep-sea fishes that showed high genetic differentiation worldwide (Fig. 5). Most of the genetic differentiation of these fishes occurs along well-known marine biogeographic boundaries such as between the northwestern Pacific Ocean and eastern Pacific, between the northwestern Pacific Ocean and southwestern Pacific, and between the northwestern Pacific Ocean and western Atlantic (Bowen et al., 2016; Costello et al., 2017). Therefore, deep-sea fishes, including these 19 fishes, might be genetically divided by marine biogeographic zones and could include multiple cryptic species.

These species have high intraspecific genetic differentiation can be classified into two ecotypes, namely, mesopelagic and demersal. Eleven of them were mesopelagic fishes. Such inter or/and outer oceanic genetic differentiation in mesopelagic fishes has already been observed (Gordeeva, 2013; Kenchington et al., 2017). Eight other species are upper continental shelf demersal fishes. Among them, we found intraspecific lineages in *N. chemnitzi* , and Poulsen et al. (2017) and Robertson et al. (2017) also suggested that this species has multiple cryptic species based on genetic analyses. The other seven species also showed two or more lineages with high intraspecific genetic split, and our results suggest that upper continental shelf demersal fishes might contain several cryptic species or an undetermined population structure. Many deep-sea fishes, both pelagic and demersal species, have a long floating larval stage (Merret, 1989; Baco et al., 2016; Priede, 2017). Therefore, genetic differentiation in widespread deep-sea fishes have not been expected (Smith, 2007; Varela et al., 2012). However, the 19 species obsrved in this study were found to have high genetic differentiation along biogeographic zones regardless of ecotype, indicating that there are unknown barriers for gene flow. Taken together, our results suggest that some deep-sea fishes have cryptic species or population differentiation regardless of these ecotypes, highlighting the need for DNA barcoding of widespread deep-sea fishes in an

international framework.

In contrast to these 19 species, 6 species showed low intraspecific genetic differentiation (Fig. 6). Such low genetic differentiation in deep-sea fishes worldwide has been suggested to be caused by their high migration ability or long lifespan (Smith, 2007; Varela et al., 2012; Catarino et al., 2015). Certainly, three sharks and *R. pretiosus*are most likely to have high swimming ability and may perform large migrations. Ecological studies of these widespread deep-sea fishes are limited. Therefore, DNA barcoding, as conducted in this study, might provide important ecological information that was previously unknown. Deep-sea fishes, which are distributed in waters shallower than 1000 m, are susceptible to the effects of human activities, yet there are very few studies on their genetic ecology. Future research should focus on taxonomical and ecological studies of mesopelagic fishes and demersal fishes on the shelf-break zone.

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Author contributions

T.A., K.Ki. and S.H. designed the research. T.A. and K.Ko. collected samples. T.A. performed research. T.A. and S.H. analysed the data. T.A. wrote the paper with S.H.. K.Ko., H.H and K.Ki edited the paper. S.H. supervised.

Conflict of interest

The authors have no conflicts of interest.

Data Accessibility and Benefit-Sharing Statement

Data sets supporting the results of this study are available in the DNA data bank (Accession No. DDBJ: @@-@@).

Figure captions

Fig. 1. Locations of fishing ports in Japanese (black circles) and Taiwanese (white circles) waters where specimens were collected. The specimens were collected from deep-sea fisheries targeting the offshore area of each fishing port.

Fig. 2. Interspecific Kimura two-parameter (K2P) distance (%) in the COI sequences of the deep-sea fishes from waters in Japan and southern Taiwan. **A.** K2P distances between species within the same family. **B** . K2P distances between species within the same genus.

Fig. 3. Neighbor-joining tree of COI sequences from*Pentaceros japonicus* and *P. wheeleri* (Pentacerotidae), which showed a low interspecific Kimura two-parameter distance (<2%). Sequences from DNA databases are shown with their accession numbers.

Fig. 4. Neighbor-joining trees of COI sequences from three deep-sea fish species, which showed a high intraspecific Kimura two-parameter (K2P) distance (>2%). Sequences are shown with the locations of the fishing ports where the samples were acquired. Sequences from DNA databases are shown with their accession numbers. Scale bars indicate 2% of the K2P distances.

Fig. 5. Neighbor-joining trees of COI sequences from 19 widespread deep-sea fishes, which showed a high intraspecific Kimura two-parameter (K2P) distance (>2%). The geographic origin of each sequence is indicated with color circles on the map. The colored sea area on the map indicates the distribution of each species. Scale bars indicate 2% of the K2P distances.

Fig. 6. Neighbor-joining trees of COI sequences from six widespread deep-sea fishes, which showed low intraspecific Kimura two-parameter (K2P) distances (>2%). The colored sea area on the map indicates the distribution of each species. Scale bar indicates 2% of the K2P distances.

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