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Molecular phylogenetic relationships of *Eptatretus* species in Japanese waters

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Abstract

The hagfish genus *Eptatretus* is the most specious group of the family Myxinidae, comprising 58 valid species worldwide. Because the species of the genus are difficult to distinguish morphologically, molecular approaches have become important. In this study, we conducted a molecular phylogenetic analysis of *Eptatretus* species from Japanese coastal waters using mitochondrial and nuclear genes, with an emphasis on clarifying the relationships between *E. walkeri* and *E. atami*, and including three Southern Hemisphere species for comparison. The present analysis revealed two major clusters corresponding to the Northern and Southern Hemisphere distributions. We also analyzed *COX1* of 123 specimens of the genus *Eptatretus* collected from nine localities in the coastal area of Japan and found that *E. burgeri* and *E. walkeri* exhibit broad distributions across Japanese coastal waters. These findings refine our understanding of *Eptatretus* distribution in Japan and highlight the importance of molecular data for studying deep-sea species.

Key words: *COX1*; nuclear gene; Myxini; Northwest Pacific; distribution

Introduction

Eptatretus is a genus of hagfish, of which five species are known to inhabit the coastal waters of Japan: *Eptatretus atami* (Dean, 1904), *Eptatretus burgeri* (Girard, 1855), *Eptatretus moki* (McMillan & Wisner, 2004), *Eptatretus okinoseanus* (Dean, 1904), and *Eptatretus walkeri* (McMillan & Wisner, 2004) (Motomura 2020).

Eptatretus atami was first described by Dean (1904) from a specimen collected in Sagami Bay on the Pacific coast of Honshu Island in Japan. Later, Honma (1962) expanded the known distribution of this species to include not only the Pacific coast of Honshu but also the Sea of Japan. However, Fernholm (1998) pointed out that *E. atami* has often been mistaken for one or two other undescribed species, leading to frequent misidentifications. McMillan and Wisner (2004) identified two new species, *E. moki* and *E. walkeri*, based on their distinct morphological characteristics. Specifically, *E. atami* had 3/3 fused cusps (anterior/posterior multicusps), whereas *E. moki* and *E. walkeri* exhibited 3/2 fused cusps. Additionally, *E. moki* can be distinguished by a well-developed ventral finfold with a pale margin, and its distribution is limited to Misaki, southern tip of the Miura Peninsula near facing Sagami Bay. In contrast, *E. walkeri* was found both off Choshi on the Pacific coast and along the northwest coast of the Sea of Japan, suggesting that earlier identifications of *E. atami* in the Sea of Japan were likely incorrect.

More recently, Kase et al. (2017) conducted genetic analyses using genes for the mitochondrial cytochrome oxidase subunit 1 (COX1) and three nuclear G protein-coupled receptors (GPRs). They revealed genetic differences between *E. atami* populations from the Pacific coast (Suruga Bay) and those from the Sea of Japan (off Akita), leading to the proposal that hagfish from the Sea of Japan may represent a distinct species, possibly *E. walkeri*.

Because the holotype of *E. walkeri* originated from Choshi on the Pacific coast (McMillan and Wisner, 2004), this suggests a potential genetic divergence between *E. walkeri* populations on the Pacific and Sea of Japan coasts. Owing to uncertainties in species identification, Kase et al. (2017) provisionally named the Sea of Japan population of *Eptatretus* sp. Akita.

Kitano et al. (2019) addressed this issue by collecting hagfish specimens from Choshi and conducting comprehensive morphological and molecular analyses. The results from both approaches strongly suggested that the hagfish species inhabiting the Sea of Japan was *E. walkeri*, rather than *E. atami*. Their study further revealed that *E. walkeri* is more widely distributed than previously recognized, occurring along both the Pacific coast of Honshu Island and in the Sea of Japan.

However, the genetic region used by Kitano et al. (2019) was limited to a part of the *COX1* in the mitochondrial DNA. Given the need to use multiple genes to clearly demonstrate that *E. walkeri* is distinct from *E. atami*, in the present study, we conducted a molecular phylogenetic analysis of the genus *Eptatretus* inhabiting the coastal waters of Japan, including *E. walkeri* and *E. atami*, using both mitochondrial and nuclear genes. Additionally, we expanded upon the work of Kitano et al. (2019) by including more sampling localities for the *COX1* analysis.

Materials and Methods

RNA-seq

A specimen of *E. walkeri* (sample ID: Kiku2) collected off Akita on September 11, 2013, was used for the total RNA extraction. Total RNA was isolated from the dorsal aorta using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and subsequently treated with DNase I (Nippon Gene, Tokyo, Japan) to eliminate any residual genomic DNA. To ensure the removal of DNase I proteins, the RNA samples were extracted using TRIzol LS Reagent (Thermo Fisher Scientific). The quality of the purified total RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the RNA integrity number (RIN) was determined to be 8.2.

Novogene (Beijing, China) carried out library construction and sequencing using the Illumina Novaseq6000 PE150 platform (Illumina, San Diego, CA, USA). Trimmomatic version 0.39 (Bolger et al. 2014) was used to eliminate adapter sequences using the following parameters: 2:30:10:2:keepBothReads LEADING:3 TRAILING:3 MINLEN:36. Further data filtering was conducted using the dynamictrim (with option -h 20) and lengthsort programs, which are part of SolexaQA++ version 3.1.7.1 (Cox et al. 2010).

The remaining clean reads were *de novo* assembled using the transabyss program with three different k values (32, 48, and 64) and merged using the transabyss-merge program implemented in Trans-ABySS version 2.0.1 (Robertson et al. 201). Benchmarking Universal Single-Copy Orthologs (BUSCO) version 5 (Simão et al. 2015) as implemented in gVolante (Nishimura et al. 2019) was used to check the quality of the transcriptome assembly (against the ortholog set for Metazoa).

For comparison, the FASTQ data deposited in the DDBJ Sequence Read Archive were assembled in the same was and included *E. atami* (DRR062842 and DRR062843, Suzuki et al. 2017), *E. burgeri* (DRR228568, Nishimura et al. 2022), *Eptatretus cirrhatus* (Forster, 1801) (SRR2146915, Lamb et al. 2016), *Eptatretus deani* (Evermann & Goldsborough, 1907) (SRR22512608, Zeng et al. 2023), *Eptatretus goslinei* Mincarone, Plachetzki, McCord, Winegard, Fernholm, Gonzalez & Fudge, 2021 (SRR22514584 and SRR22514586, Zeng et al. 2023), *Eptatretus mccoskeri* McMillan, 1999 (SRR22512605 and SRR22512606, Zeng et al. 2023), *E. okinoseanus* (SRR22514556, SRR22514557, and SRR22514558,

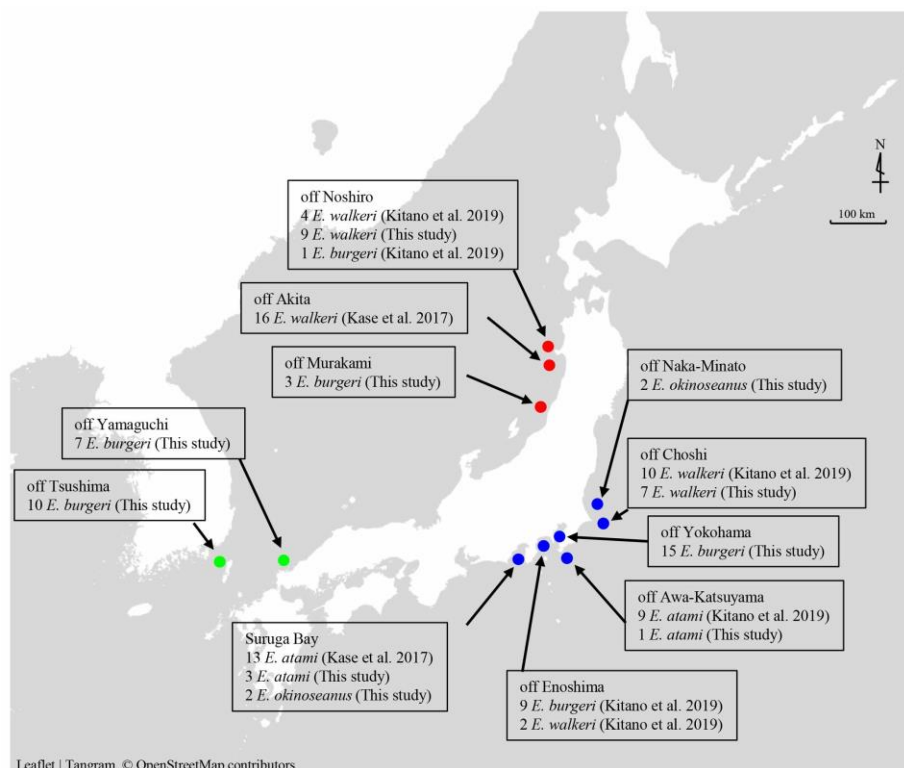

Fig. 1. Localities and numbers of specimens of *Eptatretus* used in this study. Data from Kase et al. (2017) and Kitano et al. (2019) are incorporated. The localities were tentatively grouped into three large areas: northern Sea of Japan coast (red), southern Sea of Japan coast (green), and central Pacific coast (blue).

Zeng et al. 2023), *Eptatretus stoutii* (Lockington, 1878) (SRR22512594 and SRR22512597, Zeng et al. 2023), and *Myxine glutinosa* Linnaeus, 1758 (SRR27189700, Fidler et al. 2017), the last being used as an outgroup.

To process the nuclear sequences, the coding sequences (CDS) and amino acid sequences were extracted using GeneMarkS-T version 5.1 (Tang et al. 2015). CD-HIT version 4.8.1 (Li and Godzik 2006) was used to eliminate redundant sequences from the data. OrthoFinder (Emms and Kelly 2019), an amino acid sequence–based orthology inference tool, was used to gather the orthologous sequences. The amino acid sequences of each gene were aligned using the MAFFT v7.450 program (Katoh and Standley 2013). TrimAl version 1.2 (Capella-Gutiérrez et al. 2009) was used to trim gap-containing sites and poorly aligned regions in the multiple sequence alignment, using the parameters "-gt 1 -st 0.001." The aligned amino acid sequences were converted back to their corresponding DNA sequences using PAL2NAL version 14 (Suyama et al. 2006). All gene sequences were concatenated into a single long sequence for phylogenetic analysis.

To compare the 13 mitochondrial CDS sequences, a blastn search was conducted using the BLAST 2.6.0+ package (Altschul et al. 1997). This search was performed on each assembled dataset using the 13 mitochondrial CDS sequences of *E. atami* (AP017471.1) as queries. These sequences were combined into a long sequence for subsequent phylogenetic analysis.

The *COX1* analysis

Specimens for the *COX1* analysis were collected from nine localities on the Pacific coast of Honshu Island and the Sea of Japan (Fig. 1). Given the limited number of localities and individuals in the data included in previous studies by Kase et al. (2017) and Kitano et al. (2019), in the present study,

we expanded the *COX1* dataset by incorporating specimens from five new localities (off Naka-Minato, off Yokohama, off Tsushima, off Yamaguchi, and off Murakami) and including more individuals from previously sampled localities (Fig. 1). The species were identified based on their cusps and gill pouches.

DNA extraction and PCR strategies followed the methods described by Kase et al. (2017) and Kitano et al. (2019), and multiple alignments were performed using the MUSCLE program (Edgar 2004).

Phylogenetic analysis

For phylogenetic trees reconstruction based on nuclear CDS sequences and 13 mitochondrial CDS sequences, ModelTest-NG 0.1.6 (Darriba et al. 2020) was used to select the most suitable models using the Akaike information criterion (AIC) (Akaike 1974). Phylogenetic trees were reconstructed using the maximum likelihood (ML) method (Felsenstein 1981) using RAxML-NG 1.0.1 (Kozlov et al. 2019). The reliability of the inferred tree was tested using the bootstrap method with 1,000 replications (Felsenstein 1985).

For phylogenetic tree reconstruction based on *COX1*, sequences from previous studies (Steinke et al. 2009; Zhang and Hanner 2011; Fernholm et al. 2013; Zintzen et al. 2015; Kase et al. 2017; Kitano et al. 2019) were also included, and *Myxine glutinosa* (Delarbre et al. 2001) was used as an outgroup (Supplementary Table S1). A phylogenetic tree of *COX1* sequences was reconstructed by the neighbor-joining method (Saitou and Nei 1987) using the Kimura two-parameter model (Kimura 1980) with 1,000 bootstrap replicates (Felsenstein 1985) implemented in MEGA7 (Kumar et al. 2016).

Results

Sequence determined data by RNA-seq

The total number of assembled nucleotide sequences of *E. walkeri* was 19,148. According to BUSCO analyses, 83.54 % of the genes were complete, and the combined percentage of complete and partial genes was 93.19 % (Supplementary Table S2). The FASTQ sequence data generated in this study were submitted to the DDBJ Sequence Read Archive under the accession number DRR626831. Additionally, the transcriptome shotgun assembly sequence data from

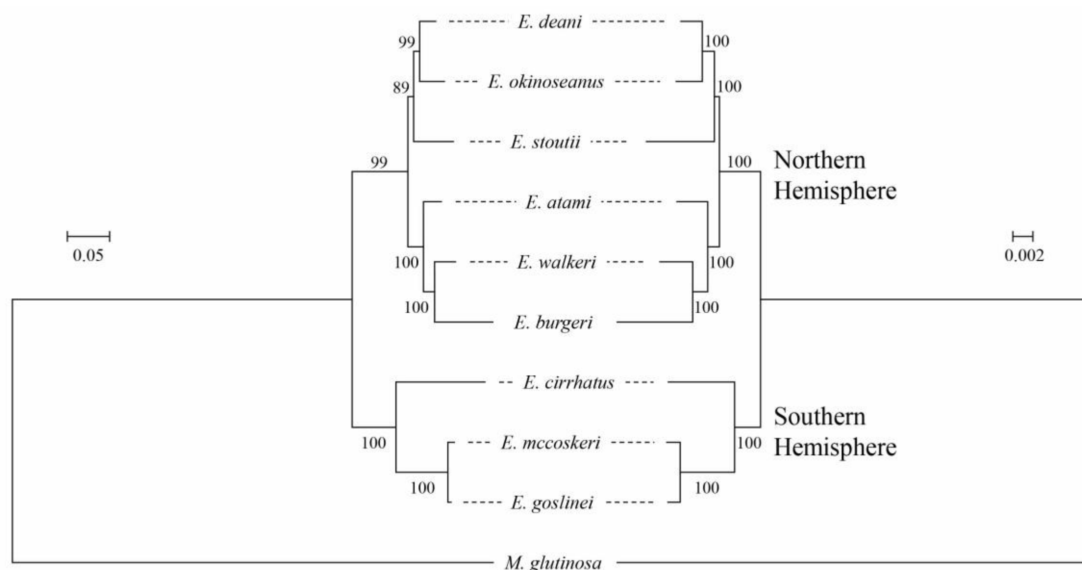

Fig. 2. Phylogenetic trees of *Eptatretus* species reconstructed using 13 mitochondrial CDS sequences (left) and 1,172 nuclear gene sequences (right). The trees were reconstructed using the maximum likelihood method with nucleotide sequence data. *Myxine glutinosa* is used as an outgroup. Each scale bar represents the number of nucleotide substitutions per site. Values near the nodes indicate the percentage of bootstrap probability.

this study have been deposited in the DNA databank with accession numbers ICWU01000001–ICWU 01019148 and ICWT01000001–ICWT01000015.

Phylogenetic tree

Phylogenetic trees reconstructed using 13 mitochondrial CDS sequences and 1,172 nuclear gene sequences are shown in Fig. 2. The substitution models selected by AIC for each nuclear orthologous sequence are listed in Supplementary Table S3, and those for the 13 mitochondrial CDS sequences are listed in Supplementary Table S4. Both phylogenetic trees displayed identical topologies with high bootstrap values. *Eptatretus walkeri* formed a cluster with *E. burgeri*, and together they formed a sister group to *E. atami*. *Eptatretus okinoseanus*, which is distributed in the Northwest Pacific (Taiwan and southern Japan), formed a cluster with the Eastern Pacific species *E. deani* and *E. stoutii*. Three Southern Hemisphere species, *E. cirrhatus*, *E. mccoskeri*, and *E. goslinei*, formed distinct clusters.

The *COX1* analysis

In addition to the sequences from previous studies by Kase et al. (2017) and Kitano et al. (2019), we sequenced 59 *COX1* sequences from nine localities, five of which were new (Fig. 1), and deposited them in a databank (accession numbers: LC858459-LC858517). We tentatively grouped these localities into three large areas: the northern Sea of Japan coast (red), southern Sea of Japan coast (green), and central Pacific coast (blue).

A phylogenetic tree is shown in Fig. 3, using 186 *COX1* sequences from the present and previous studies (Steinke et al. 2009; Zhang and Hanner 2011; Fernholm et al. 2013; Zintzen et al. 2015; Kase et al. 2017; Kitano et al. 2019) (Supplementary Table S1). Like the phylogenetic trees reconstructed using 13 mitochondrial CDS sequences and 1,172 nuclear gene sequences, this phylogenetic tree shows two major clusters corresponding to the Northern and Southern Hemisphere distributions, with the exception of *Eptatretus minor* Fernholm & Hubbs, 1981, which is included in the Southern Hemisphere cluster despite being distributed in the Gulf of Mexico. This phylogenetic tree also shows that *E. walkeri* clusters not with *E. burgeri* but with other Northern Hemisphere species. However, the bootstrap value supporting this cluster was low (24 %). *Eptatretus atami* was found only on the central Pacific coast, whereas *E. walkeri* was present on both the northern Sea of Japan coast and the central Pacific coast. In contrast, *E. burgeri* was found on the northern and southern Sea of Japan coasts and the central Pacific coast.

Discussion

Phylogeny of *Eptatretus* species

The phylogenetic tree obtained in this study (Fig. 2) broadly divided the species into two clusters: one for species distributed in the Northern Hemisphere and the other for those in the Southern Hemisphere. Although the datasets for the compared species did not match perfectly, the results were largely consistent with previous molecular phylogenetic studies on the genus *Eptatretus* (Chen et al. 2005; Kuo et al. 2010; Fernholm et al. 2013; Zintzen et al. 2015; Suzuki et al. 2017; Son and Kim 2020a; Son and Kim 2020b; Mincarone et al. 2021). Hagfish species inhabit deep-sea environments and are rarely observed, and their morphological differences are often subtle. Thus, past identification efforts have been limited to these species. However, it is essential to continue improving our understanding of molecular data.

***Eptatretus* species around Japan**

In this study, we analyzed the *COX1* sequences of 123 *Eptatretus* specimens collected from coastal waters around Japan. Such analyses are gradually clarifying the distribution patterns of *Eptatretus* species in these waters of Japan. *Eptatretus burgeri*

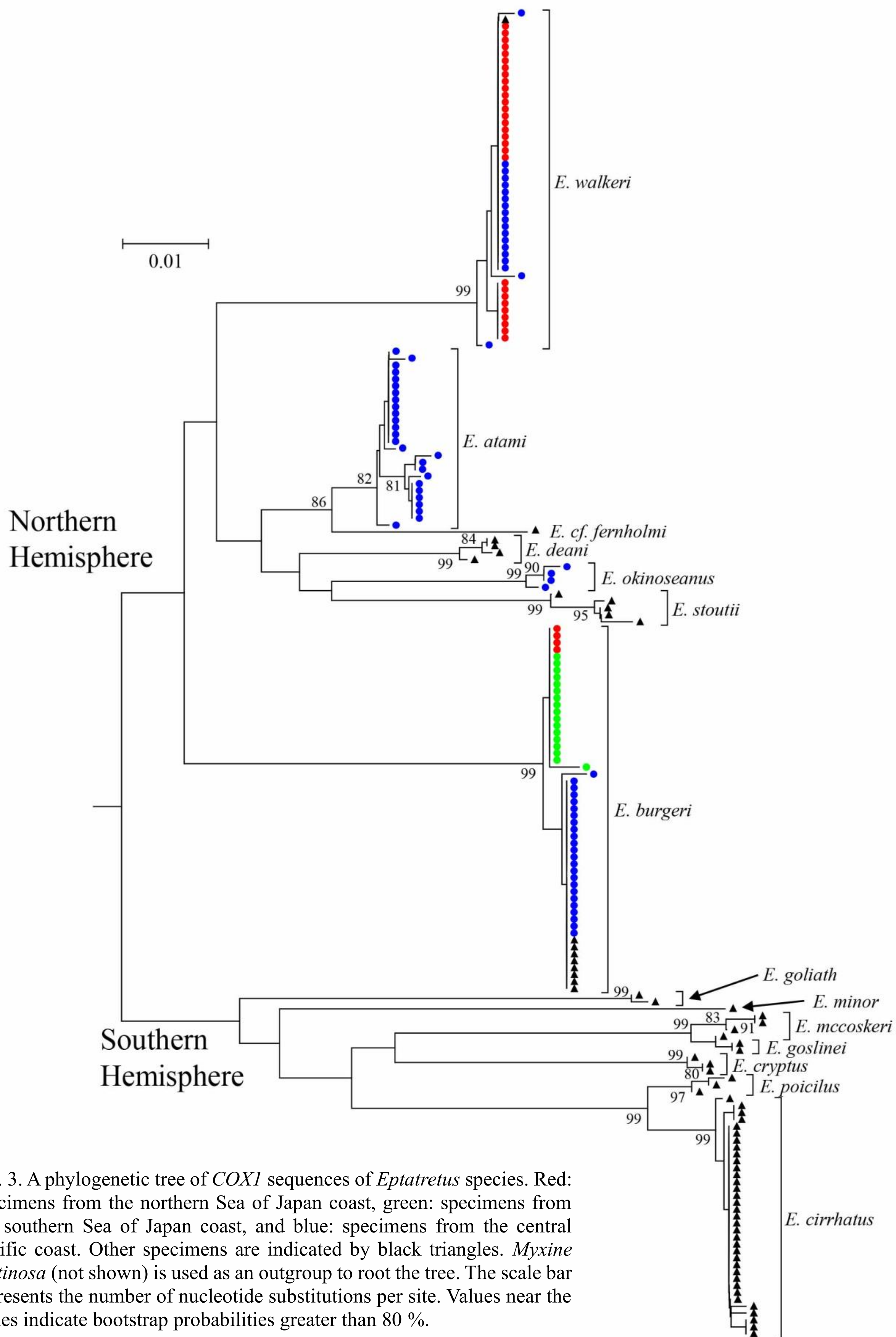

Fig. 3. A phylogenetic tree of *COX1* sequences of *Eptatretus* species. Red: specimens from the northern Sea of Japan coast, green: specimens from the southern Sea of Japan coast, and blue: specimens from the central Pacific coast. Other specimens are indicated by black triangles. *Myxine glutinosa* (not shown) is used as an outgroup to root the tree. The scale bar represents the number of nucleotide substitutions per site. Values near the nodes indicate bootstrap probabilities greater than 80 %.

is widely distributed from the Pacific coast to the Sea of Japan, similar to that of *E. walkeri*. However, as *E. walkeri* was not found on the southern coast of the Sea of Japan in this study, its presence there remains uncertain. In any case, *E. burgeri* and *E. walkeri* appear to have broad distributions in coastal waters around Japan, with some individuals inhabiting the same localities. In fact, *E. burgeri* and *E. walkeri* are occasionally captured together, such as off the coast of Noshiro and Enoshima (Kitano et al. 2019).

In contrast, *E. atami*, which inhabits deeper waters, appears to have a different distribution from *E. burgeri* and *E. walkeri*. According to McMillan and Wisner (2024), *E. burgeri* is typically captured at depths of 10–270 m, *E. walkeri* at 75–120 m, and *E. atami* at 300–536 m. In the deeper waters of Suruga Bay, *E. atami* is sometimes captured alongside *E. okinoseanus*, another deep-sea species with a depth range of 300–1,020 m (McMillan and Wisner 2024). In the samples analyzed in this study, *E. atami* and *E. okinoseanus* were collected at depths of 200–300 m, whereas *E. burgeri* and *E. walkeri* were obtained from shallower depths of 23–150 m (Supplementary Table S1). These observations are consistent with the depth ranges reported by McMillan and Wisner (2024). However, sampling remains limited in some regions and depth ranges. With additional sampling localities, future analyses could provide a more detailed understanding of the habitat and distribution of *Eptatretus* species in Japanese waters.

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