

No genetic deviation between two morphotypes of the snipefishes (Macroramphosidae: *Macroramphosus*) in Japanese waters

Taisuke Noguchi · Kay Sakuma · Tomo Kitahashi ·
Hajime Itoh · Yasunori Kano · Gento Shinohara ·
Jun Hashimoto · Shigeaki Kojima

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Abstract We examined morphological and molecular characteristics of individuals of *Macroramphosus* in Japanese waters (the East China Sea and the northwestern Pacific). Two morphotypes (*M. scolopax*-type and *M. gracilis*-type) that were differentiated based on 10 quantitative morphological characters were not supported by molecular analyses using nuclear and mitochondrial DNA markers, while a genetic deviation was observed between populations of *Macroramphosus* from the northwestern Pacific and the northeastern Atlantic. *Macroramphosus scolopax*-type and *M. gracilis*-type individuals are

thought to be intraspecific morphotypes adapted to plankton and benthos feeding, respectively.

Keywords *Macroramphosus* · Japanese waters · Morphotype · Molecular phylogeny

Introduction

Snipefishes of genus *Macroramphosus* Lacepède 1803 (Gasterosteiformes: Syngnathoidei: Macroramphosidae) are laterally compressed, streamlined fish with a long second spine on the dorsal fin and a tiny mouth at the tip of a greatly elongated snout. They are distributed at ocean depths less than 600 m in tropical and subtropical areas around the world (Ehrich 1976; Bilecenoglu 2006). Mohr (1937) suggested that only two of the 17 nominal species of *Macroramphosus* were valid: *Macroramphosus scolopax* (Linnaeus 1758), which is characterized by a deep body with a long second dorsal fin spine; and *Macroramphosus gracilis* (Lowe 1839), which has a slender body with a short dorsal fin spine. Based on Japanese specimens, Okada and Suzuki (1951) stated that this genus was monotypic based on large overlaps of morphological characters between them. Ehrich (1976) arrived at the same conclusion based on the existence of individuals with intermediate morphologies in the northeastern Atlantic and thought that *M. gracilis*-type individuals were juveniles of *M. scolopax*. However, Miyazaki et al. (2004) showed that they can be differentiated even at the larval stage based on specimens collected off Kochi in southern Japan. Presently, most researchers consider *M. scolopax* and *M. gracilis* as valid species (Bilecenoglu 2006).

Using nucleotide sequences of the mitochondrial control region and a nuclear DNA marker (the first intron of the S7

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T. Noguchi · H. Itoh · S. Kojima (✉)
Graduate School of Frontier Sciences, The University of Tokyo,
1-5-1 Kashiwanoha, Kashiwa, Chiba 277-8563, Japan
e-mail: kojima@aori.u-tokyo.ac.jp

K. Sakuma
National Research Institute of Far Seas Fisheries, Fisheries
Research Agency, 5-7-1 Orido, Shimizu, Shizuoka,
Shizuoka 424-0902, Japan

T. Kitahashi · Y. Kano
Atmosphere and Ocean Research Institute, The University of
Tokyo, 1-5-1 Kashiwanoha, Kashiwa, Chiba 277-8564, Japan

G. Shinohara
National Museum of Nature and Science, 4-1-1 Amakubo,
Tsukuba, Ibaraki 305-0005, Japan

J. Hashimoto
Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo,
Nagasaki, Nagasaki 852-8521, Japan

ribosomal protein gene), Robalo et al. (2009) analyzed the phylogenetic relationships among specimens of *M. scolopax*, *M. gracilis*, and specimens with intermediate morphologies collected off the coast of Portugal (northeastern Atlantic) and suggested that individuals of *Macroramphosus* in the northeastern Atlantic are monotypic. However, too few genetic variations were detected in the nuclear DNA region to determine the genetic differences between *M. scolopax*-type and *M. gracilis*-type individuals.

In this study, we conducted molecular phylogenetic analyses using mitochondrial and nuclear DNA markers with sufficient variability in Japanese individuals of *Macroramphosus* from the East China Sea and northwestern Pacific to evaluate the genetic differences between morphotypes (*M. scolopax*-type and *M. gracilis*-type). In addition, we analyzed the phylogenetic relationships among Japanese and Portuguese individuals of *Macroramphosus*.

Materials and methods

A total of 124 specimens of *Macroramphosus* were sampled at 11 sites (31°09.9'–32°29.8'N, 127°30.8'–129°09.6'E, 134 to 333 m deep) using an otter trawl or a beam trawl in the East China Sea during 10 cruises of the training vessel (T/V) *Nagasaki-maru* of Nagasaki University. Two specimens were collected using an otter trawl at two sites (36°29.9'N, 140°57.1'E, 150 m and 37°36.9'N, 141°35.8'E, 210 m) off the Pacific coast of northeastern Japan during two cruises of the research vessel (R/V) *Wakataka-maru* of Tohoku National Fisheries Research Institute, Fisheries Research Agency. Sixteen frozen specimens collected off Kochi and northwestern Pacific were provided by Prof. K. Sasaki, Kochi University. Information about the sampling sites is not available because these specimens were obtained from a fish market.

The specimens were classified based on the qualitative characters described by Miyazaki et al. (2004): *M. scolopax*-type having red-orange bodies and second dorsal fin spines with roughly serrated posterior margins, *M. gracilis*-type having dark or brownish bodies and second dorsal fin spines with smooth posterior margins, and intermediate-type individuals. A small piece of muscle tissue taken from each specimen was stored in a freezer (-30 °C) until use. The remaining parts were fixed in 10 % seawater formalin and preserved in 70 % ethanol.

Following Bilecenoglu (2006), the snout length, eye diameter, postocular head length, head length, postopercular body length, length of the dorsal spine, distance between the two dorsal fins, maximum body depth, length before the dorsal spine, length after the dorsal spine, and standard length of fixed specimens were measured using a

vernier caliper (0.05 mm accuracy). A non-metric multidimensional scaling (nMDS) analysis was performed using the Bray–Curtis similarity index based on the first 10 length values divided by standard length. Permutational multivariate analysis of variance (PERMANOVA) was used to examine the statistical significance of differences. All tests were conducted using R, version 3.1.0 (R Development Core Team 2008) and the package *vegan* (Oksanen et al. 2008).

Total DNA was extracted from frozen tissue using the DNeasy Tissue Extraction Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The mitochondrial DNA fragment, including the 5' part of the control region was amplified by polymerase chain reaction (PCR) using primers L-Pro1 (5'-ACT CTC ACC CCT AGC TCC CAA AG-3') and H-DL1 (5'-CCT GAA GTA GGA ACC AGA TGC CAG-3') (Ostellari et al. 1996). The PCR conditions were as follows: incubation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 40 s, 50 °C for 60 s, and 72 °C for 90 s. The nuclear DNA fragment, including the first intron of the S7 ribosomal protein gene, was amplified by PCR using primers S7RPEX1F (5'-TGG CCT CTT CCT TGG CCG TC-3') and S7RPEX2R (5'-AAC TCG TCT GGC TTT TCG CC-3') (Chow and Hazama 1998). The PCR conditions were as follows: incubation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. To degrade the remaining primers and nucleotides, 5 µl of PCR products was mixed with 1 µl of ExoSAP-IT (United States Biochemical, Cleveland, OH, USA) and incubated at 37 °C for 15 min followed by 80 °C for 15 min. Each purified PCR product was used in direct cycle sequencing reactions with corresponding primers and BigDye Terminator Cycle Sequencing Kit, version 3.0 (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were determined bi-directionally using an ABI 3130 automated DNA sequencer (Applied Biosystems). The determined nucleotide sequences were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers AB826127–826155 (control region) and AB856541–856547 (S7).

The sequences were aligned using Clustal W (Thompson et al. 1994) in the MEGA version 5.0 Beta software package (Tamura et al. 2007) under default settings. Additionally, the alignments were manually adjusted where needed. Phylogenetic trees were reconstructed using Bayesian inference, maximum likelihood (ML), and neighbor-joining (NJ) methods. The seahorse *Hippocampus kuda* Bleeker 1852 was used as the outgroup (GenBank Accession number NC010272). Bayesian inference was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). HKY (Hasegawa et al.

1985) + I + G model was selected through hierarchical likelihood ratio tests implemented in Modeltest 3.7 (Posada and Crandall 1998). Shape, proportion of invariant sites, state frequency, and substitution rate parameters were estimated empirically from the data in the Bayesian analysis. Two parallel runs were made for 1×10^7 generations (with a sample frequency of 1,000) and under the default value of four Markov chains. The first 5,000 trees for each run were discarded to ensure that the four chains reached stationarity by referring to the average standard deviation of split frequencies. The consensus tree and posterior probabilities were computed from the remaining 10,000 trees (5,000 trees \times two runs). Posterior probabilities $\geq 95\%$ were considered to be significant. The ML analysis was performed using RAX-ML 7.2.8 on the Black Box webserver (Stamatakis 2006; Stamatakis et al. 2008). Bootstrap runs consisted of 500 pseudoreplicates with the default GTR (Rodríguez et al. 1990) + G setting, following the software manual. The NJ analysis was performed using PAUP* 4.0b10 (Swofford 2002) based on HKY85 model with 1,000 bootstrap pseudoreplicates. Bootstrap probabilities $\geq 75\%$ were considered to be significant. Haplotype networks were constructed with the median-joining method using Network computer program, version 4.5.1.6 (Bandelt et al. 1999) based on differences in nucleotide sequences.

Results

Based on the qualitative characters shown in Miyazaki et al. (2004), the specimens of *Macroramphosus* collected in the East China Sea were classified into *M. scolopax*-type ($n = 78$), *M. gracilis*-type ($n = 42$), and intermediate-type ($n = 6$) individuals. Specimens collected off northeastern Japan ($n = 2$) were classified as *M. scolopax*-type and those off Kochi were classified as *M. scolopax*-type ($n = 9$), *M. gracilis*-type ($n = 1$), and intermediate-type ($n = 6$) individuals. One *M. gracilis*-type specimen collected off Kochi was badly damaged and could not be used for further morphological examination. Intermediate-type individuals showed various combinations of four diagnostic characteristics. The *M. scolopax*-type and *M. gracilis*-type individuals included both males and females.

In the nMDS ordination space based on 10 quantitative morphological characters, *M. scolopax*-type and *M. gracilis*-type individuals formed groups separated from each other, while the intermediate-type individuals were plotted between them (Fig. 1). No separation was observed between individuals of the same type from other sea areas. Differences in spatial distributions of the three types in the nMDS ordination space were significant (PERMANOVA, $P < 0.05$).

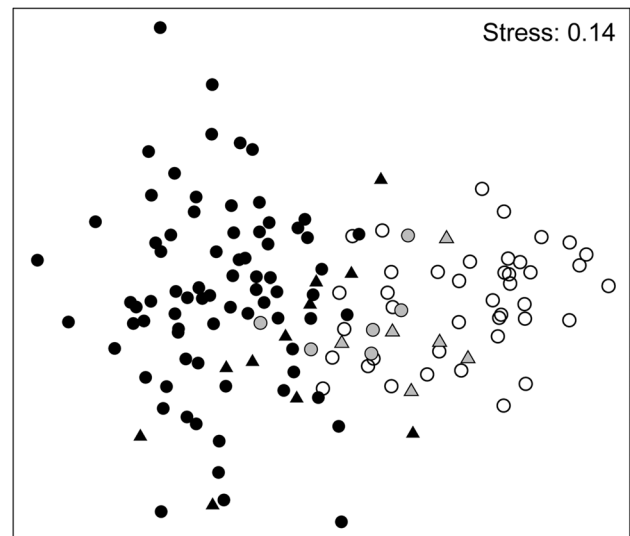
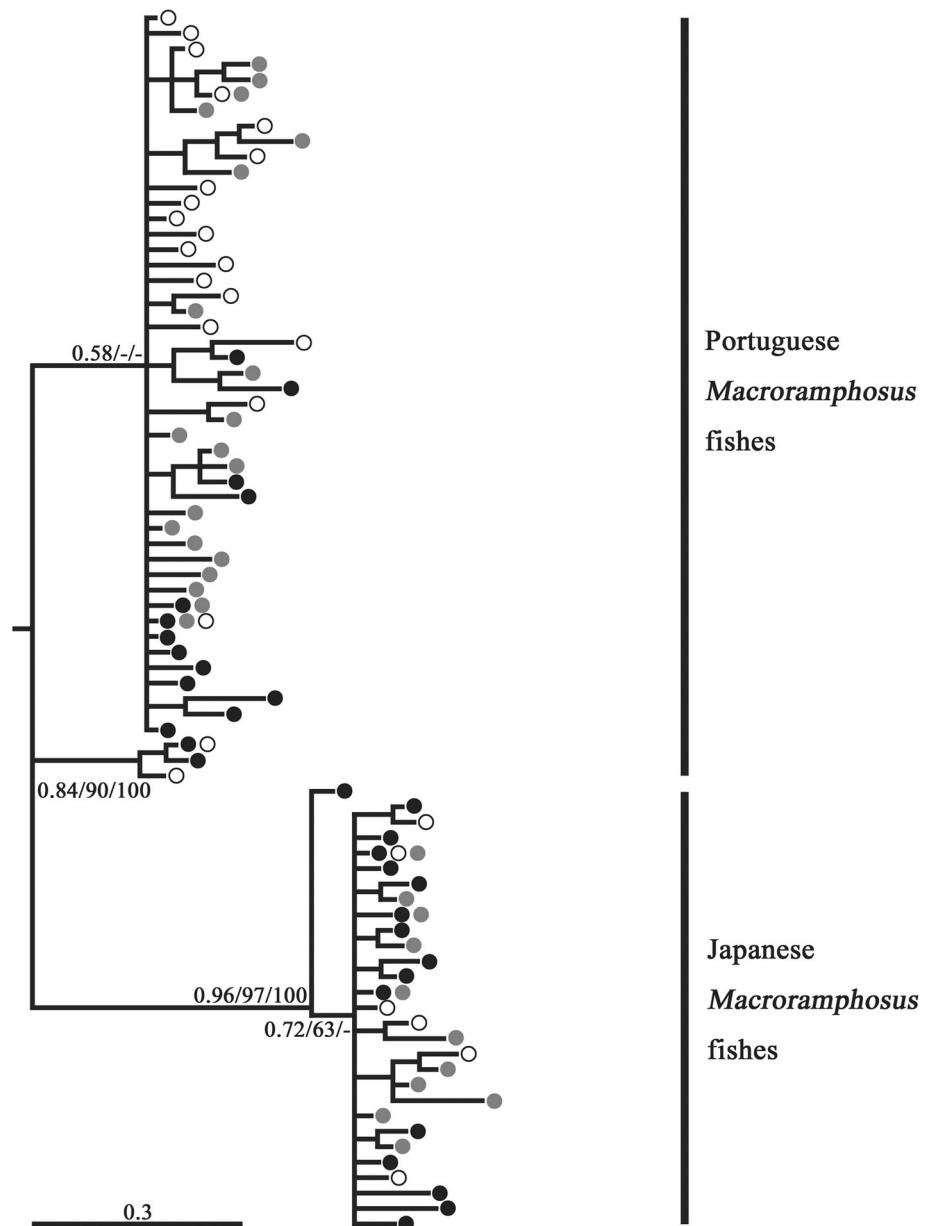


Fig. 1 The non-metric multi-dimensional scaling (nMDS) ordination of individuals of *Macroramphosus* from the East China Sea and northwestern Pacific based on 10 quantitative morphological characters. Circles and triangles indicate specimens from the East China Sea and the northwestern Pacific, respectively, and black, gray, and white symbols indicate *M. scolopax*-type, intermediate-type, and *M. gracilis*-type individuals, respectively

Nucleotide sequences of the 5' part of the mitochondrial control region (381 base pairs, bp) were determined for specimens of *Macroramphosus* from the East China Sea ($n = 26$), off northeastern Japan ($n = 2$), and off Kochi ($n = 15$). No indels were detected, and 29 types of sequences (haplotypes) were identified. The most dominant haplotype was obtained from individuals from all three geographic areas as well as from all three morphotypes.

Phylogenetic relationships among the Japanese and Portuguese individuals were analyzed based on 345-bp-long sequences of mitochondrial DNA, which were common to both the present and previous studies (Robalo et al. 2009). A single deletion was detected between one Portuguese haplotype and all other haplotypes. The Japanese specimens were retrieved as a robust clade with a Bayesian posterior probability of 0.96 and bootstrap values of 97% and 100% under ML and NJ criteria, respectively (Fig. 2). None of the *M. scolopax*-type, *M. gracilis*-type, or intermediate-type individuals formed subclades within the Japanese clade. The Portuguese fishes consisted of two genetically distinct lineages, as shown in Robalo et al. (2009). The relationship between the Japanese clade and Portuguese lineages was not resolved with a meaningful support value either in the Bayesian analysis, resulting in basal trichotomy, or in the ML or NJ analysis. The average genetic distance between Japanese and Portuguese fishes (0.090 under HKY85 model) was larger than that between the two Portuguese groups (0.058).

Fig. 2 Phylogenetic relationships among Japanese and Portuguese individuals of *Macroramphosus* inferred from Bayesian analyses based on partial (345 base pairs) nucleotide sequences of the mitochondrial control region. Black, gray, and white circles indicate haplotypes obtained from *M. scolopax*-type, intermediate-type, and *M. gracilis*-type individuals, respectively. Morphotypes of Portuguese individuals were determined by Robalo et al. (2009). The tree was rooted with a seahorse *Hippocampus kuda*. Support indices are shown on branches to major clades for Bayesian posterior probabilities above 0.5 (left) and bootstrap frequencies above 50 % under the likelihood and neighbor-joining criteria (middle and right)



Although we directly sequenced PCR products containing the first intron of the gene encoding the S7 ribosomal protein for 64 specimens of *Macroramphosus* from the East China Sea ($n = 46$), off northeastern Japan ($n = 2$), and off Kochi ($n = 16$), those from only 10 individuals (five *M. scolopax*-type, two *M. gracilis*-type, and two intermediate-type individuals from the East China Sea, and one *M. scolopax*-type individual from off Kochi) could be determined, because they showed one or two sequences that were differentiated by a single substitution. Most individuals in all three morphotypes had two heterozygous sequences of different lengths. Lengths of the obtained sequences were either 528 or 531 bp and an indel of three nucleotides was detected among them. The length of sequences from

individuals collected off the coast of Portugal (Robalo et al. 2009) corresponded to the length of our short sequences. However, more than five nucleotide substitutions were detected between Portuguese and Japanese individuals. Seven haplotypes were identified in Japanese individuals. Haplotypes obtained from neither *M. scolopax*-type, *M. gracilis*-type, nor intermediate-type individuals formed an exclusive cluster in a haplotype network (Fig. 3).

Discussion

As the present results showed no genetic deviation between the two morphotypes for either mitochondrial or

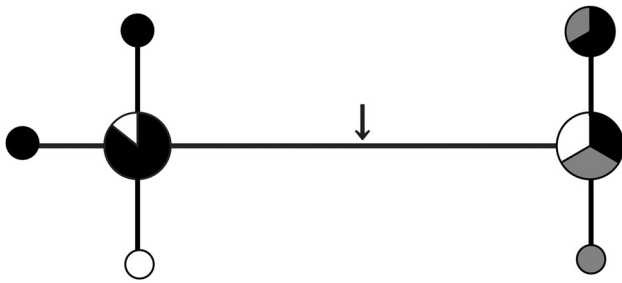


Fig. 3 Haplotype network of Japanese individuals of *Macroramphosus* based on nucleotide sequences containing the first intron of the S7 ribosomal protein gene (528 to 531 base pairs). The areas of the circles are proportional to the frequency of occurrence of the haplotypes. Black, gray, and white sectors indicate the relative frequencies of *M. scolopax*-type, intermediate-type, and *M. gracilis*-type individuals, respectively. The length of lines between haplotypes corresponds to the number of nucleotide substitutions estimated between them. The arrow denotes an indel of three nucleotides

nuclear markers, we conclude that Japanese individuals of *Macroramphosus* are monotypic in spite of significant morphological differences. Similar results were reported for species of *Macroramphosus* inhabiting the North Atlantic (Robalo et al. 2009). Genetic deviation found between Japanese and Portuguese populations (Fig. 2) and morphological differences between individuals from different sea areas (Kuranaga and Sasaki 2000; Clarke 1984; Noguchi et al., unpublished data) suggest that the genus *Macroramphosus* is not monotypic. However, molecular data have been obtained only from these two sea areas and morphological data of specimens used in Robalo et al. (2009) are not available. Close taxonomic examination using additional morphological and molecular data sets are necessary to judge whether this genus is monotypic or not.

Two morphotypes of *Macroramphosus* differ in depth distribution and food source: namely *M. gracilis*-type feeds on plankton within the water column and *M. scolopax*-type feeds on benthic organisms on the sea bottom (Ehrich and John 1973; Matthiessen et al. 2003; Miyazaki et al. 2004). In the East China Sea, the frequency of *M. gracilis*-type individuals was higher in samples collected using an otter trawl (49.1 %) than in those collected using a beam trawl (3.8 %) (Noguchi et al., unpublished data). Our beam trawl collects animals within about 1 m from the sea floor, while the sampling range of our otter trawl is much wider (0–4 m), suggesting that *M. gracilis*-type individuals are distributed at greater distances from the seafloor than *M. scolopax*-type individuals. Stable isotope analyses performed in our lab on the same samples (Seike et al., unpublished data) revealed significant differences in nitrogen stable isotope ratios between the morphotypes, which corroborates the differences in primary food sources.

Miyazaki et al. (2004) showed that morphotypes of individuals of *Macroramphosus* are determined at the larval stage, and that the body shape, body color, and development of scutes differ between the two morphotypes (Miyazaki et al. 2004; Bilecenoglu 2006). These results suggest that different sets of alleles determining the feeding habits are cooperatively expressed within each morphotype. Such trophic polymorphism has been shown in many freshwater fish species (Smith and Skúlason 1996), including charr (Anderson 2003), sticklebacks (Kristjánsson et al. 2002), perch (Olsson and Eklöv 2005), cichlids (Swanson et al. 2003), and whitefish (Whiteley 2007). Inland water bodies are often divided into separated regions and connected via tectonic movements and floods, which facilitates allopatric evolution of trophic polymorphism. In contrast, no clear dispersal barriers are recognized in the open ocean. To the best of our knowledge, trophic polymorphism has not been reported in other open sea fishes. Thus, species of *Macroramphosus* offer a rare case of trophic polymorphism in the open ocean.

Since the snipefishes of the genus *Macroramphosus* are dominant on the continental shelves around Japan and relatively easy to sample and breed (Kitajima, personal communication), they may be used as a model group in adaptive evolution studies in the open ocean. Therefore, further comprehensive studies on their morphology, molecular phylogeny, and ecology are necessary.

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