Genetic differentiation between two color morphs of *Apogon taeniophorus* from southern Japan

Kohji Mabuchi[1]✉, Noboru Okuda[2], and Mutsumi Nishida[1]

[1] *Ocean Research Institute, University of Tokyo, 1-15-1 Minamidai, Nakano-ku, Tokyo 164-8639, Japan*
(e-mail: mabuchi@ori.u-tokyo.ac.jp)
[2] *Center for Marine Environmental Studies, Ehime University, 2-5 Bunkyo-cho, Matsuyama 790-8577, Japan*

Received: October 6, 2003 / Revised: November 11, 2003 / Accepted: November 25, 2003

Ichthyological Research

Ichthyol Res (2004) 51: 180–183
DOI 10.1007/s10228-003-0201-7

Abstract The anterior half of the mitochondrial 16S rRNA gene (ca. 610 bp) was compared for two color morphs (spotted and lined types) of a dark-striped cardinalfish, previously identified as *Apogon taeniophorus*. Phylogenetic analyses using maximum-parsimony (MP) and neighbor-joining (NJ) methods, with haplotypes of *A. cookii* as an outgroup, showed that the haplotypes of each color-morph were reciprocally monophyletic with 100% bootstrap values. In addition, the degree of sequence difference between the two morphs was comparable to that existing between the other clearly distinct congeneric species. These results, together with the differences in coloration and overlapped geographical ranges, indicated that the two color morphs of *A. taeniophorus* represent two distinct species.

Key words *Apogon taeniophorus* · Mitochondrial DNA · 16S rRNA gene · Genetic differentiation

Apogonidae (cardinalfishes) is one of the largest families of perciform fishes, and the genus *Apogon* (the largest genus of the family) includes approximately 110 species (Nelson, 1994). Many of the Indo-Pacific species of *Apogon* have several dark stripes on the head and body, some being very similar in coloration. *Apogon taeniophorus* is one such dark-striped species, being widely distributed in the tropical regions of the Indo-West Pacific from the Red Sea eastward to the Line Islands (Randall and Lachner, 1986). From Japanese waters, it is hitherto known only from a single specimen washed up on a beach in Chichi-jima, Ogasawara Islands, being named "misuji-tenjikudai" in Japanese (Matsuura and Tachikawa, 1994). *Apogon taeniophorus* is particularly similar to *Apogon cookii*, also occurring widely in the Indo-West Pacific, differing from the latter by having 14 pectoral-fin rays (vs. usually 15 rays) and lacking a distinct dark spot in the midlateral stripe at the caudal-fin base (Randall and Lachner, 1986). According to Randall and Lachner (1986), the caudal-fin base spot of *A. taeniophorus* "if it can be distinguished at all, is elliptical, only slightly broader than the stripe, and at best slightly darker," which indicates that *A. taeniophorus* exhibits intraspecific variation in this character.

During the course of a molecular phylogenetic study of the genus *Apogon*, we found that the specimens identified as *A. taeniophorus*, based on Randall and Lachner (1986), included two genetically distinct color morphs that differed also in the state of the caudal-fin base spot. One of the two color morphs had an indistinct dark spot in the midlateral stripe at the caudal-fin base (Fig. 1, top: "spotted type"), while the other lacked a spot (Fig. 1, middle: "lined type"). The degree of genetic differentiation and phylogenetic relationship between the two color morphs of *A. taeniophorus* from Japanese waters is described here, based on mitochondrially encoded 16S rRNA gene sequences. The specimens used in this study are the second examples of *A. taeniophorus* from Japan, revealing its wider geographical range within the country. To evaluate taxonomic rank, the following criteria were used: if the two color morphs were each a well-established species, it was expected that (1) the levels of sequence divergence within each color morph would be much less than that between the two morphs; that (2) the sequence divergence between the two color morphs would be comparable to those among other distinct apogonid species; and (3) the haplotypes of each color morph would be respectively monophyletic.

Materials and Methods

Materials.—DNA sequences were determined for five and four specimens, respectively, of the spotted (Fig. 1, top) and lined types (Fig. 1, middle) of *Apogon taeniophorus* (sensu Randall and Lachner, 1986) from Japanese waters. Three and two specimens, respectively, of the spotted and lined types were collected from Kuchinoerabu Island (I.), Ryukyu Islands (Is.), with two specimens of the spotted type also being collected from Chichi-jima I., Ogasawara Is., and two specimens of the lined-type from Misho town, Ehime Pref. (Fig. 2). In addition, a single specimen of *Apogon cookii* (Fig. 1, bottom), collected from Kuchinoerabu I., was used as an outgroup. Species identifications were based on Randall and Lachner (1986). All specimens used in the present study have been deposited in the Faculty of Agricul-

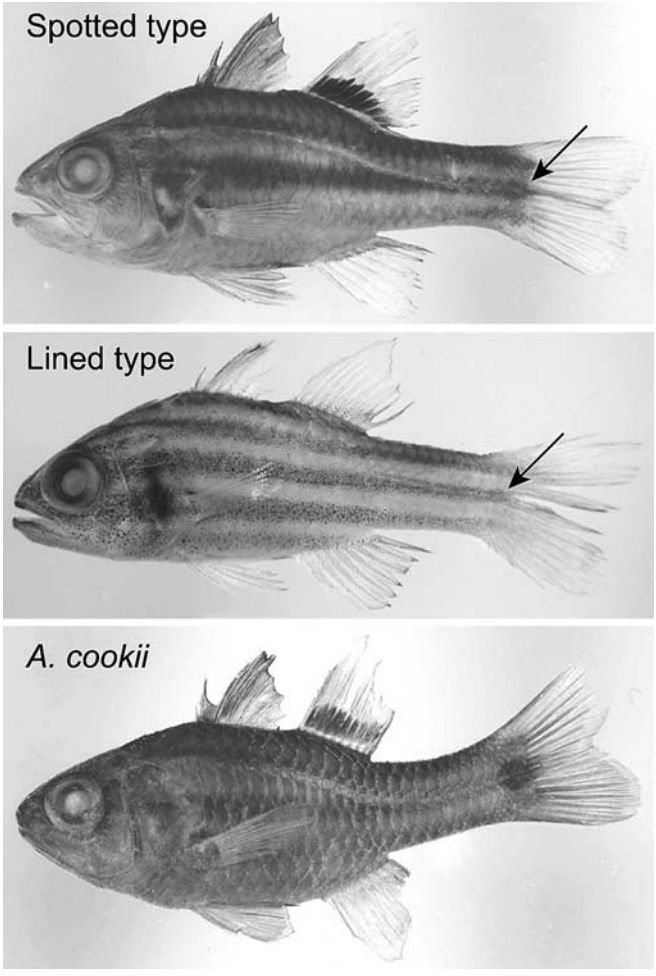

Fig. 1. Lateral view of two color morphs of *Apogon taeniophorus* and *A. cookii* (both sensu Randall and Lachner, 1986). Spotted type morph of *A. taeniophorus* (*top*), NSMT-P 62210, 84.2 mm SL, collected from Chichi-jima Island, Ogasawara Islands. Lined type morph of *A. taeniophorus* (*middle*), FAKU 73701, 50.5 mm SL, collected from Morode Beach, Misho town, Ehime Pref. *Apogon cookii* (*bottom*), FAKU 78676, 69.7 mm SL, collected from Kuchinoerabu Island, Ryukyu Islands. *Arrows* indicate presence (Spotted type) and absence (Lined type) of a dark spot

ture, Kyoto University (FAKU), except for two examples of the spotted type morph from Chichi-jima I., deposited in the Department of Zoology, National Science Museum (NSMT).

Apogon taeniophorus (spotted type): FAKU 78658, 78675, 78678, Kuchinoerabu I., Kagoshima Pref.; NSMT-P 62202, 62210, Chichi-jima I., Ogasawara Is. *Apogon taeniophorus* (lined type): FAKU 73701, 73709, Morode Beach, Misho town, Ehime Pref.; FAKU 78674, 78677, Kuchinoerabu I., Ryukyu Is. *Apogon cookii*: FAKU 78676, Kuchinoerabu I., Ryukyu Is.

DNA extruction and sequencing.—DNA was isolated by phenolchloroform extraction from muscle tissue preserved in 70–100% ethanol. The anterior half of the 16S rRNA gene region in mtDNA was amplified by means of the polymerase chain reaction (PCR) using Ex Taq polymerase

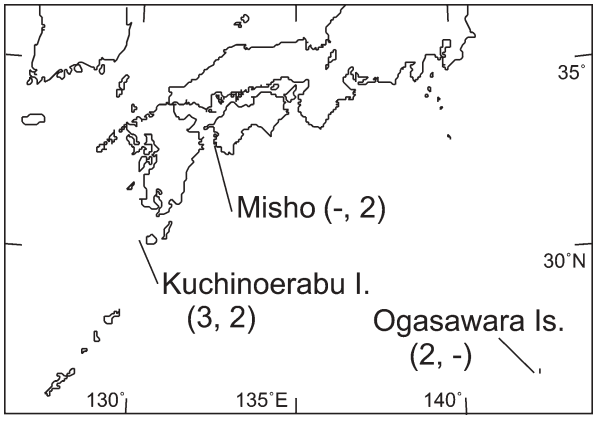

Fig. 2. Map of sampling localities for two color morphs of *Apogon taeniophorus* (sensu Randall and Lachner, 1986). Number of specimens of each type given in *parentheses* (spotted type, lined type)

(Takara) and the following primers: L1803-16S, 5′-AGTAC CGCAAGGGAAAGCTGAAA-3′; H2590-16S, 5′-ACAA GTGATTGCGCTACCTT-3′ (Miya and Nishida, 1999). PCR proceeded for 30 cycles on a model 9700 thermal cycler (Applied Biosystems), with denaturation at 94°C for 10 s, annealing at 45–51°C for 10 s and extension at 72°C for 30 s. The PCR products were electrophoresed on 1% L 03 agarose gel (Takara) and stained with ethidium bromide for band characterization via ultraviolet transillumination. Double-stranded PCR products, purified using a Pre-Sequencing Kit (USB), were sequenced with dye-labeled terminators (Applied Biosystems). Primers used were the same as those for PCR. All sequencing reactions were performed according to the manufacturer's instructions. Labeled fragments were analyzed on a model 3100 DNA sequencer (Applied Biosystems). To reduce sequencing errors, both the L- and H strands were sequenced and compared. All sequences are available from DDBJ, EMBL, and GenBank under accession numbers AB121138–121145.

Sequence analysis.—DNA sequences were edited and aligned with the computer programs EditView ver. 1.01 (Applied Biosystems), AutoAssembler ver. 2.1 (Applied Biosystems), and DNASIS ver. 3.2 (Hitachi). The number of fixed differences between the sequences and pairwise sequence distances were calculated using MEGA ver. 2.1 (Kumar et al., 2001). Evolutionary relationships among the haplotypes were inferred using maximum-parsimony (MP) (Cavalli-Sforza and Edwards, 1967) and neighbor-joining (NJ) (Saitou and Nei, 1987) methods. The MP analysis was performed using PAUP* ver. 4.0b8a (Swofford, 1998). The MP tree was sought using the heuristic search option with characters treated as unordered and equally weighted. The NJ analysis was also performed using PAUP* 4.0b8a, evolutionary distances being calculated using Kimura's two-parameter model (Kimura, 1980). Both the MP and NJ trees were rooted to *A. cookii*. For each analysis, bootstrap analysis (Felsenstein, 1985) using 500 pseudoreplicates was used to verify the robustness of the phylogenetic relationship among the haplotypes.

```
S1   ACGCGTCTCG TT-ATATCC- TCCGGCTTAA ATAT-GAATT CTTTATTTAA
S2   .......... ..-.....- .......... ....-..... ..........
S3   GT..A..... ..C.....TT .......... ..GA-A.... ..C..C...G
S4   GT..A..... ..C.....TT .......... ..GA-A.... ..C..C...G
L1   GTATACTATA C---GGCA.- CTTAATACCG GAG.GAGTCA AC.AT.CCG.
L2   GTATACTATA C---GGCA.- CTTAATACCG GAG.GAGTCA AC.AT.CCG.
L3   GTATACTATA C---GGCA.- CTTAATACCG GAG.GAGTCA AC.AT.CCG.

S1   CCCC-TTGCC TTTATCCATA CTTCCACGCC TAAA-AGAAC TGTA-AATCT CGCTCAG
S2   ....-..A.. ....C..... .......... ....-..... ....-..... ....A..
S3   ....C..AT. .C.T..T... A......A.. C..G-..... ..C.TG.C.. AC.CA..
S4   ....C..AT. .C.T..T... A......A.. C..G-T.... ..C.TG.C.. AC.CA..
L1   TTTTA-CT.T CCAC.T.CCT -ACATGTA-A .TG.G.TGGT CACC---C.C A.AAAGA
L2   TTTTA-CT.T CCAC.T.CCT -ACATGTA-A .TG.G.TGGT CACC---CAC A.AAT..
L3   TTTTA-CT.T CCAC.T.CCT -ACATGTA-A .TG.G.TGGT CACC---C.C A.AAA..
```

Fig. 3. Matrix of haplotypes derived from the aligned sequences of spotted (*S*) and lined (*L*) types of *Apogon taeniophorus* (sensu Randall and Lachner, 1986). Haplotypes are based on the 107 variable nucleotide positions

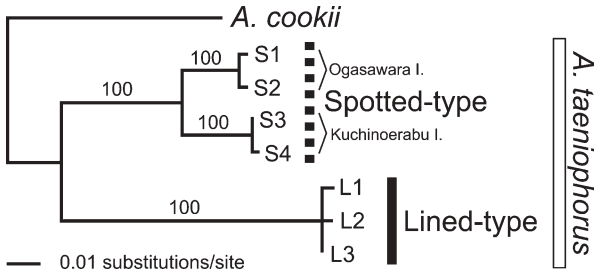

Fig. 4. Neighbor-joining tree for haplotypes of spotted (*S*) and lined (*L*) types of *Apogon taeniophorus* (sensu Randall and Lachner, 1986), with haplotypes of *A. cookii* as an outgroup. Distances corrected for multiple substitutions based on Kimura's (1980) two-parameter model. *Numbers above branches* indicate bootstrap values obtained from 500 pseudoreplications (only >90% values indicated). Haplotypes: *S1*, observed from NSMT-P 62202; *S2*, NSMT-P 62210; *S3*, shared by FAKU 78658, 78675; *S4*, observed from FAKU 78678; *L1*, FAKU 78677; *L2*, FAKU 73701; *L3*, shared by FAKU73709, 78677

Results and Discussion

Nucleotide sequence data of approximately 610 bp were collected for the anterior half of the mitochondrial 16S rRNA gene from nine specimens of *Apogon taeniophorus* and a single outgroup specimen of *Apogon cookii*. Among 615 aligned nucleotide positions, 137 were found to be variable, 91 being phylogenetically informative. The five specimens of the spotted type of *A. taeniophorus* exhibited four haplotypes, two of the three specimens from Kuchinoerabu I. indicating identical sequences. Although 0–3 substitutions were observed among sympatric specimens, moderate sequence differences (25–27 substitutions) were observed between Ogasawara Is. and Kuchinoerabu I. specimens. The two populations were distinguished by 19 transitions, 5 transversions, and 4 insertion/deletions (Fig. 3). On the other hand, four specimens of the lined type morph exhibited three haplotypes, differing from each other by 2 or 4 substitutions. Two individuals from Kuchinoerabu I. and Misho town showed identical sequences. These two color morphs were, however, distinguished by 44 transitions, 21 transversions, and 9 insertion/deletions (Fig. 3).

The two color morphs of *A. taeniophorus* were clearly genetically distinct, as follows. (1) The amount of pairwise sequence divergence within each of the two color morphs (0.0–4.4% in spotted type, 0.0–0.66% in lined type) was much less than that between the two (13.1–13.6%), and (2) the degree of sequence difference between the two morphs was comparable to that existing between each and *A. cookii* (13.0–15.6%) and that between the clearly distinct congeneric species, *Apogon cyanosoma* and *Apogon semilineatus* (11.7%: Mabuchi et al., 2003). Furthermore, (3) the haplotypes of each color morph were respectively monophyletic. NJ analysis resulted in a tree (Fig. 4) of identical topology to a single MP tree (tree length = 159, CI = 0.97, RI = 0.98). In both the MP and NJ trees, the haplotypes of the two color morphs were each monophyletic (100% bootstrap values). These results, together with their difference in coloration and overlapped geographical ranges, indicated that the two color morphs of *A. taeniophorus* represent two distinct species. The taxonomy of these fishes with detailed morphological comparisons will be discussed in a forthcoming revision (K. Mabuchi and N. Okuda, in preparation).

It is interesting that the two populations of the spotted type morph from Ogasawara Is. and Ryukyu Is. showed moderate genetic differentiation (4.1–4.4%), an amount comparable to that existing between *A. cyanosoma* and the lined type of *Apogon properuptus* (ca. 5.4%: Mabuchi et al., 2003). Detailed morphological study of a greater number of specimens may reveal further differences between the two populations.

Acknowledgments We are grateful to the following persons and institutions for their help in collecting specimens: K. Gushima and staff of Kuchinoerabu-jima Marine Biological Laboratory (Hiroshima University); and Y. Yanagisawa (Ehime University); Ogasawara Fisheries Center. We are also grateful to T. Nakabo and R. Doiuchi (The Kyoto University Museum) for their registration of specimens. We thank G.S. Hardy (Ngunguru, New Zealand), who read our initial manuscript. This study was supported in part by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (Tokyo, Japan).

Literature Cited

Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: models and estimation procedures. Am J Hum Genet 19:233–257

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2. Molecular evolutionary genetics analysis software. Bioinformatics 17:1224–1245

Mabuchi K, Okuda N, Kokita T, Nishida M (2003) Genetic comparison of two color-morphs of *Apogon properuptus* from southern Japan. Ichthyol Res 50:293–296

Matsuura K, Tachikawa H (1994) Fishes washed up on beaches in Chichi-jima, Ogasawara Islands. Bull Natl Sci Mus Tokyo Ser A 20:131–147

Miya M, Nishida M (1999) Organization of the mitochondrial genome of a deep-sea fish *Gonostoma gracile* (Teleostei: Stomiiformes): first example of transfer RNA gene rearrangements in bony fishes. Mar Biotechnol 1:416–426

Nelson JS (1994) Fishes of the world, 3rd edn. Wiley, New York

Randall JE, Lachner EA (1986) The status of the Indo-West Pacific cardinalfishes *Apogon aroubiensis* and *A. nigrofasciatus*. Proc Biol Soc Wash 99:110–120

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Swofford DL (1998) PAUP*. Phylogenetic analysis using parsimony (*and other methods), ver. 4.0. Sinauer, Sunderland, MA