Genetic comparison of two color-morphs of *Apogon properuptus* from southern Japan

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Abstract The anterior half of the mitochondrial 16S rRNA gene (ca. 650 bp) was compared for two color-morphs (dotted and lined types) of the orange-lined cardinalfish, previously identified as *Apogon properuptus*. Twelve and nine specimens, respectively, of dotted and lined types were collected from four and three localities along the coasts of southern Japan and the Ryukyu Islands, specimens of both color-morphs being collected from one of these localities on the southern coast of the Shikoku Island. Phylogenetic analyses using maximum parsimony (MP) and neighbor-joining (NJ) methods, with haplotypes of *A. semilineatus* and *A. cyanosoma* as an outgroup and comparative OTU, respectively, showed that the haplotypes of each color-morph were reciprocally monophyletic with 100% bootstrap values. These results, together with their distinct coloration and partly overlapping geographical ranges, indicated that the two color-morphs of *A. properuptus* from Japanese waters represent two distinct species.

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

Apogon properuptus is a member of the *Apogon cyanosoma* complex, which comprises five species [*A. cyanosoma*, *A. luteus*, *A. properupta* (=*A. properuptus*), *A. rubrimacula*, and *A. wassinki*] sharing several yellow-orange stripes and whitish interspaces on the body (Randall and Kulbicki, 1998). According to Hayashi (2002), *Apogon properuptus* occurs widely in rocky and coral reefs of the Indo-Pacific and Red Sea. Interestingly, two distinct color-morphs are recognized within the Japanese population (Hirata et al., 2001; Mabuchi, 2001). One of the two color-morphs ("dotted type") has a whitish interspace on the cheek and flank, comprising a series of white spots (Fig. 1, top), whereas the other ("lined type") has a whitish interspace on the cheek and flank as a narrow stripe (Fig. 1, bottom). The two color-morphs, however, occupy different but partly overlapping geographical ranges in Japanese waters as follows. The dotted type is distributed mainly from Izu Peninsula to northern coasts of Kyushu Island and the lined type mainly from Kochi Prefecture to Okinawa Prefecture, their ranges being overlapped along the Pacific coasts of Shikoku and Kyusyu Islands (Fig. 2) (Hirata et al., 2001). Their partly overlapped geographical ranges together with their distinct coloration indicate that they are two distinct species. In fact, in the CD-ROM pictorial book by Kuiter and Kozawa (1999), the two color-morphs were regarded as two distinct species; judging from the coloration and geographical ranges described, the Kuiter and Kozawa species, (1999) *Apogon* sp. 9 and *Apogon* sp. 12 correspond to the above dotted and lined types, respectively. However, some Japanese authors (Hayashi, 1997; Masuda and Kobayashi, 1994) have treated the two color-morphs as color variations of a single species. The taxonomic status of the two color-morphs is yet to be confirmed, pending further studies on morphological or genetic aspects.

In this study, a molecular genetic analysis was undertaken for the two color-morphs of *A. properuptus* (sensu Hayashi, 2002) from Japanese waters, by sequencing the mitochondrially encoded 16S rRNA gene. If the two color-morphs were each 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 color-morphs; (2) the sequence divergence between the two color-morphs would be comparable to those among other distinct apogonid species; and, moreover, (3) the haplotypes of each color-morph would be respectively monophyletic.

Materials and Methods

Materials.—DNA sequences were determined for 12 dotted-type and 9 lined-type specimens of *Apogon properuptus* (sensu Hayashi, 2002). Three individuals of each

Fig. 1. Lateral views of two color-morphs of *Apogon properuptus* (sensu Hayashi, 2002). Dotted type (*top*), FAKU 73704, 55.0 mm SL, collected from Morode Beach, Misho Town, Ehime Pref., Japan. Lined type (*bottom*), FAKU 73093, 55.9 mm SL, collected from Ajiro, Ohtsuki town, Kochi Pref., Japan

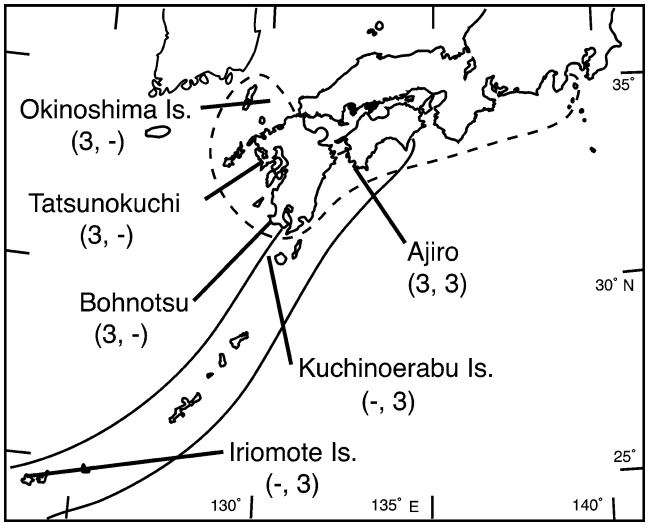

Fig. 2. Map of sampling localities with geographical ranges of the dotted (*broken line*) and lined (*solid line*) types of *Apogon properuptus* (sensu Hayashi, 2002). Numbers of specimens of each type are given in parentheses (dotted type, lined type)

color-morph were collected from each of four (dotted type) and three (lined type) localities in Japan (see Fig. 2). Specimens of both color-morphs were collected from Ajiro, Ohtsuki town, Kochi Prefecture (Pref.), with dotted-type specimens also being collected from Okinoshima Island (Is.), Fukuoka Pref., Tatsunokuchi, Koyaki town, Nagasaki Pref., and Bohnotsu town, Kagoshima Pref., and lined-type specimens from Kuchinoerabu Is., Kagoshima Pref. and Iriomote Is., Okinawa Pref. In addition, a single specimen of *A. cyanosoma* was used for comparison, as well as a single specimen of *A. semilineatus* as an outgroup. The former comparative species is morphologically very similar to *A. properuptus* (sensu Hayashi, 2002), the two species having been confused in the past (Hayashi, 2002). In this study, species identifications were based on Hayashi (2002), with discrimination of the two color-morphs of *A. properuptus* being based on Hirata et al. (2001). All specimens used in this study have been deposited in the Faculty of Agriculture, Kyoto University (FAKU), as below.

Apogon properuptus (dotted type): FAKU 77522-77524, Okinoshima Is., Fukuoka Pref.; FAKU 77512, 77514, 77515, Tatsunokuchi, Koyaki town, Nagasaki Pref.; FAKU 77734–77736, Bohnotsu town, Kagoshima Pref.; FAKU 77906–77908, Ajiro, Ohtsuki town, Kochi Pref. *A. properuptus* (lined type): FAKU 73084, 73092, 73093, Ajiro, Ohtsuki town, Kochi Pref.; FAKU 78659–78661, Yumugi, Kuchinoerabu Is., Kagoshima Pref.; FAKU 73160–73162, Amitori bay, Iriomote Is., Okinawa Pref. *A. cyanosoma*: FAKU 73152, Amitori bay, Iriomote Is., Okinawa Pref. *A. semilineatus*: FAKU 70779, Morode beach, Misho town, Ehime Pref.

DNA extraction and sequencing.—DNA was isolated by phenol–chloroform 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 (Takara) and the following primers: L1803-16S, 5′-AGTACCGCAAGGGAAAGCTGAAA-3′; H2590-16S, 5′-ACAAGTGATTGCGCTACCTT-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, stained with ethidium bromide for band characterization via ultraviolet transillumination, were electrophoresed on 1% L 03 agarose gel (Takara). 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 AB105107–105116.

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 Software Engineering). The number of fixed differences between the sequences and pairwise sequence distances was calculated

using MEGA ver. 2.1 (Kumar et al., 2001), and the haplotype and nucleotide diversities within each color-morph were calculated using ARLEQUIN ver. 2.000 (Schneider et al., 2000). 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. semilineatus*. For each analysis, bootstrap analysis (Felsenstein, 1985) using 1000 pseudoreplicates was used to verify the robustness of the phylogenetic relationship among the haplotypes.

Results and Discussion

Nucleotide sequence data of approximately 650 bp were collected for the anterior half of the mitochondrial 16S rRNA gene from 21 specimens of *Apogon properuptus* and single comparative and outgroup specimens. Among 649 aligned nucleotide positions, 121 were found to be variable, 77 being phylogenetically informative. The dotted-type specimens exhibited five haplotypes, differing from each other by one to four substitutions. Two of the five haplotypes, D1 and D2, were shared by seven and two individuals from three and two localities, respectively, while the remaining three haplotypes (D3–D5) were observed only once. Haplotype and nucleotide diversities for the dotted type were 0.67 ± 0.14 and 0.0018 ± 0.0014, respectively. On the other hand, the nine lined-type specimens exhibited three haplotypes, differing from each other by one or two substitutions. One of the three haplotypes, L1, was shared by seven individuals from all three localities, whereas the remaining two haplotypes (L2, L3) were each observed only once. Haplotype and nucleotide diversities for the lined type were 0.42 ± 0.19 and 0.00069 ± 0.00076, respectively.

The two color-morphs of *A. properuptus* were clearly genetically distinct as follows: (1) the amount of mean pairwise sequence divergence within each of the two color-morphs (0.18% in dotted type, 0.069% in lined type) was much less than that between the two (11.6%), and (2) the degree of sequence difference between the two morphs (11.3–11.9%) was comparable to that existing between the obviously distinct congeneric species, *A. cyanosoma* and *A. semilineatus* (11.7%). Furthermore, (3) the haplotypes of each color-morph were respectively monophyletic. NJ analysis resulted in the tree shown in Fig. 3, which differed from each of four equally parsimonious MP trees (tree length = 145, CI = 0.95, RI = 0.97) only in the positions of haplotypes within each of the dotted-type and lined-type clades. In both the MP and NJ trees, the haplotypes of the two color-morphs were each monophyletic (100% bootstrap values). Interestingly, the lined-type clade had a sister-relationship not to the dotted-type clade but to *A. cyanosoma*. It is noteworthy that the monophyletic *A. cyanosoma* and the lined type of *A. properuptus* share a lined whitish interspace on cheek and flank.

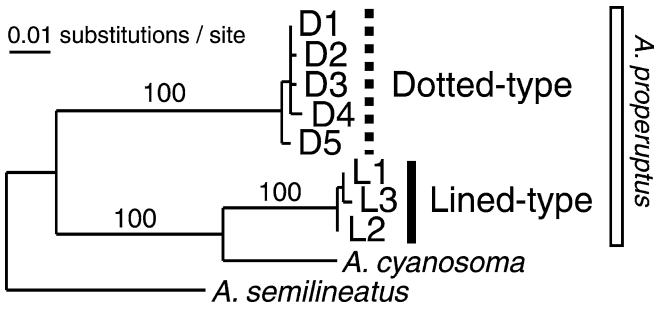

Fig. 3. Neighbor-joining tree for haplotypes of dotted (*D*) and lined (*L*) types of *Apogon properuptus* (sensu Hayashi, 2002), with haplotypes of *A. semilineatus* and *A. cyanosoma* as an outgroup and comparative OTU, respectively. Distances corrected for multiple substitutions based on Kimura's (1980) two-parameter model. *Numbers above branches* indicate bootstrap values obtained from 1000 pseudoreplications (only >90% values are indicated). Haplotypes: *D1*, shared by FAKU 77523, 77524, 77734–77736, 77906, 77908; *D2*, FAKU 77512, 77907; *D3*, observed from FAKU 77522; *D4*, FAKU 77514; *D5*, FAKU 77515; *L1*, shared by FAKU 73084, 73092, 73093, 73160, 73162, 78660, 78661; *L2*, observed from FAKU 78659; *L3*, FAKU 73161

These results, together with their distinct coloration and partly overlapping geographical ranges, indicated that the two color-morphs of *A. properuptus* from Japanese waters represent two distinct species. Accordingly, they should be taxonomically reviewed. The name *A. properuptus*, however, has a taxonomic problem as follows. Some authors (Kuiter, 1993, 1996; Randall et al., 1997) restricted the name to the "population" off the east coast of Australia and in the Coral Sea, the latter including Frederick Reef (type locality), considering the possibility that the Australian "population" was a species distinct from other "populations," including that in Japan. If this is the case, the name *A. properuptus* cannot be applied to each of the two Japanese species recognized here. To resolve this taxonomic problem, extensive morphological and genetic studies must be conducted for specimens from both hemispheres, together with the type specimen.

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