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Molecular phylogeny and stripe pattern evolution in the cardinalfish genus *Apogon*

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Abstract

Cardinalfishes of the genus *Apogon* (Apogonidae) are one of the most speciose (>200 species) and numerically dominant fishes in coral reefs. Although the genus is divided into 10 subgenera, more than 70% of the species are included in the subgenus *Ostorhinchus*, most having either horizontal or vertical lines on the body. The phylogenetic relationship among 32 species of subgenus *Ostorhinchus* and 11 species of four other subgenera of *Apogon*, based on mitochondrially encoded 12S and 16S ribosomal genes and intervening $tRNA^{Val}$ gene, were investigated, using two species of the apogonid genus *Fowleria* as outgroups. The analyses demonstrated that *Ostorhinchus* (the most speciose subgenus) was polyphyletic, comprising at least three lineages, *Ostorhinchus* I, II, and III. *Ostorhinchus* I included two species, *A.* (*O.*) *amboinensis* and *A.* (*O.*) *sangiensis*, being a sister group to subgenus *Zoramia*. *Ostorhinchus* II and III included species with horizontal and vertical lines on the body, respectively. The respective monophylies of the latter two groups, together with a molecular clock calibration, indicated that in the evolutionary history of the genus, basic stripe patterns evolved first (more than 20 million years BP), with subsequent pattern diversification and modification.

Keywords: *Apogon*; Stripe pattern; Mitochondrial DNA; Phylogenetic niche conservatism

1. Introduction

Coral reef fishes in tropical marine waters and cichlid fishes in the African Great Lakes represent some of the most diverse vertebrate assemblages on earth. Most members of each assemblage are brightly and boldly colored, with many closely related species being distinguishable only by color. Marine parrotfish and Lake Malawi rock-dwelling cichlids are typical representatives of such fishes, recent phylogenetic studies (parrotfish: Streelman et al., 2002; cichlids: Albertson et al., 1999, 2003) having indicated that sexual selection of nuptial coloration played a central role in their rapid diversification.

However, nuptial coloration is only one aspect of body coloration, and the coloration of fishes has diverse functions and often is under strong natural selection (Endler, 1980, 1983, 1986). Fishes have many visual predators (Endler, 1978, 1991; Milinski, 1993) and communicate both intra- and interspecifically using visual signals. Stripes (including both horizontal and vertical patterns), which frequently occur on the side of the body, are thought to serve simultaneously as antipredator markings and social signals (Barlow, 1972). A phylogenetic study of East African cichlid fishes by Seehausen et al. (1999) demonstrated that their melanic striped patterns are constrained ecologically rather than phylogenetically: the evolution of vertically lined patterns being associated with structurally complex habitats, such as

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rocky substrates and vegetation, compared with horizontal lines which are associated with shoaling behavior and/or a piscivorous feeding mode. The diversity of stripe patterns found in the lacustrine cichlids was explained as having resulted from their radiation into a variety of ecological niches (Seehausen et al., 1999).

The question remains: Do the weak historical constraints on stripe pattern in East African cichlids represent a general phenomenon in stripe pattern evolution in fishes? Stripe pattern evolution in East African cichlids is one aspect of rapid evolution occurring in a unique environment. Cichlids are known for their fast evolutionary responses to changing ecological demands (summarized by Galis and Metz, 1998). Furthermore, Seehausen et al.'s (1999) analysis demonstrated that the evolution of melanic stripe patterns in East African cichlids was historically just as constrained as other ecological adaptations. Among the African lacustrine cichlids, haplochromines underwent several independent radiations after they colonized lakes Malawi and Victoria over the past 1–2 million years (Meyer et al., 1990; Nishida, 1991; Verheyen et al., 2003). Such radiations also included independent evolutionary changes in stripe patterns, such as from vertical to horizontal lines (Seehausen et al., 1999). Although such rapid evolution of stripe patterns has been well studied, the "general" example for fish stripe-pattern evolution based on a reliable phylogeny has at no time been given. In the course of a phylogenetic study of a coral reef fish genus *Apogon*, we found strong historical conservatism in basic stripe-pattern.

Cardinalfishes (Apogonidae) are one of the most numerically dominant reef fish families (Bellwood, 1996), with the genus *Apogon* being the most speciose in the family. The majority of the species (>75%) occur in the tropical Indo-West Pacific (Kuiter and Kozawa, 1999), the species' richness of the genus largely owing to one of 10 subgenera defined by Fraser (1972). Based on Fraser and Lachner (1985), Gon (1995), and Fraser (1998), the genus *Apogon* includes at least 174 species, nearly 70% (at least 117 species) belonging to the subgenus, *Ostorhinchus* [referred to as *Nectamia* in Fraser (1972)]. Of the remaining subgenera, *Apogon* includes 35, *Lepidamia* four, *Zoramia* four, *Pristiapogon* three, *Brephamia* two or three, *Pristicon* two, *Zapogon* two, whilst *Paroncheilus* and *Yarica* are monotypic. Although the name "cardinalfish" refers to the red coloration of some early discovered species, most *Apogon* species are drab in color and have melanic stripe (horizontal and vertical) patterns on the body (see Fig. 2). They are mostly nocturnal and feed on plankton, such as small crustaceans, fish larvae and eggs. They occur singly, in pairs or in shoals, some being extremely secretive, only venturing into the water column at night to feed. All species appear to have mouth-breeding males without distinct nuptial coloration. The Tethyan distribution of the subgenus *Apogon* (*Apogon*) seems to indicate that the minimum age of the genus dates back to isolation of the Mediterranean, beginning in the Oligocene and Miocene (Fraser, 1972).

In this article, the evolution of the typical marine reef fish genus *Apogon* is outlined, with special reference to stripe-pattern evolution. To determine the evolutionary history, we constructed phylogenetic trees based on partial mitochondrial DNA sequences of ca. 1500 bp. Stripe pattern evolution in *Apogon* was also compared with that of African Great Lake cichlids previously reported, to characterize marine fish evolutionary radiation.

2. Materials and methods

2.1. Materials examined

Fishes used in this study came from a number of sources, including collection by hand- and gill-nets, SCUBA diving, angling, and from museum collections. All specimens used herein were preserved in alcohol. Tissue samples were obtained from specimens representing five of the 10 recognized subgenera of the genus *Apogon* (Fraser, 1972) (Table 1). A single individual was sampled for each species investigated. The subgenera *Brephamia*, *Lepidamia*, *Paroncheilus*, *Yarica*, and *Zapogon* were not examined here. Two species of an apogonid genus *Fowleria* were used as outgroups. Species' identifications were based on Hayashi (2002), except for *Apogon guamensis* and two colormorphs of both *A. properuptus* and *A. taeniophorus*, the first species being based on Myers (1999) and the four colormorphs on Mabuchi et al. (2003, 2004). A single specimen of subgenus *Ostorhinchus*, which could not be identified, was referred to as *Apogon* sp. in this study. All of the species examined here were collected from Japanese waters, although most are widely distributed in the Indo-West Pacific (Hayashi, 2002).

2.2. DNA amplification and sequencing

Genomic DNA was extracted from muscle tissue preserved in 99.5% ethanol, using the Qiagen DNeasy tissue kit. An approximately 1500 bp fragment of the mitochondrial genome, including the posterior half of 12S rRNA, entire $tRNA^{Val}$ and anterior half of the 16Sr RNA gene, was amplified using the following two sets of primers: L1083-12S (ACA AAC TGG GAT TAG ATA C) + H1903-16S (GTA GCT CGT YTA GTT TCG GG) and L1803-16S (AGT ACC GCA AGG GAA AGC TGA AA) + H2590-16S (ACA AGT GAT TGC GCT ACC TT) (Miya and Nishida, 2000). The two primer-sets amplified two different fragments partially overlapping each other. PCR proceeded for 30 cycles on a model 9700 thermal cycler (Applied Biosystems), with denaturation at 94 °C for 10 s, annealing at 45 °C for 10 s and

Table 1
Specimens used in this study with catalog numbers, DDBJ/GenBank/EMBL accession numbers and collection localities

Taxon	Catalog No.	Accession No.	Collection localities
Family Apogonidae			
Genus *Fowleria*			
F. isostigma	YCM-P 41602	AB206125	Kabira Bay, Ishigaki Island, Ryukyu Islands, Japan
F. variegata	FAKU 73055	AB206126	Morode Beach, Ainan Town, Ehime Prefecture, Japan
Genus *Apogon*			
Subgenus *Apogon*			
A. (*A.*) *crassiceps*	FAKU 73056	AB206127	Kashiwa-jima Island, Kochi Prefecture, Japan
A. (*A.*) *erythrinus*	NSMT-P 66577	AB206128	Aquarium shop
A. (*A.*) *fuscus*	FAKU 73060	AB206129	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*A.*) *semiornatus*	NSMT-P 66576	AB206130	Aquarium shop
Subgenus *Pristiapogon*			
A. (*Pristia.*) *exostigma*	FAKU 73132	AB206131	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*Pristia.*) *fraenatus*	FAKU 73175	AB206132	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*Pristia.*) *kallopterus*	FAKU 73163	AB206133	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
Subgenus *Pristicon*			
A. (*Pristic.*) *rhodopterus*	FAKU 73146	AB206134	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*Pristic.*) *trimaculatus*	FAKU 73180	AB206135	Ishigaki Port, Ishigaki Island, Ryukyu Islands, Japan
Subgenus *Zoramia*			
A. (*Z.*) *leptacanthus*	FAKU 73114	AB206136	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*Z.*) *gilberti*	FAKU 73139	AB206137	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
Subgenus *Ostorhinchus*[a]			
A. (*O.*) *amboinensis*	NSMT-P 68198	AB206138	Shirahana, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *angustatus*	FAKU 78681	AB206139	Yumugi, Kuchinoerabu-jima Island, Ryukyu Islands, Japan
A. (*O.*) *apogonides*	FAKU 73085	AB206140	Ajiro, Otsuki Town, Kochi Prefecture, Japan
A. (*O.*) *aureus*	FAKU 78665	AB206141	Iwayadomari, Kuchinoerabu-jima Island, Ryukyu Islands, Japan
A. (*O.*) *carinatus*	FAKU 73706	AB206142	Irino Fishery Port, Kochi Prefecture, Japan
A. (*O.*) *cathetogramma*	FAKU 70752	AB206143	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) *compressus*	FAKU 73108	AB206144	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *cookii*	FAKU 78676	AB206145	Mukaehama, Kuchinoerabu-jima Island, Ryukyu Islands, Japan
A. (*O.*) *cyanosoma*	FAKU 73152	AB206146	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *doederleini*	FAKU 70744	AB206147	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) *ellioti*	FAKU 73386	AB206148	Iburi, Tosashimizu City, Kochi Prefecture
A. (*O.*) *endekataenia*	FAKU 70759	AB206149	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) *guamensis*	FAKU 72023	AB206150	Kume-jima Island, Ryukyu Islands, Japan
A. (*O.*) *ishigakiensis*	FAKU 73137	AB206151	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *kiensis*	FAKU 73707	AB206152	Irino Fishery Port, Kochi Prefecture, Japan
A. (*O.*) *lineatus*	FAKU 77532	AB206153	Hiroshima Bay, Seto Inland Sea, Japan
A. (*O.*) *melas*	FAKU73129	AB206154	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *niger*	FAKU 70753	AB206155	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) *nigrofasciatus*	FAKU 73166	AB206156	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *notatus*	FAKU 70738	AB206157	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) *novemfasciatus*	NSMT-P 68199	AB206158	Hoshisuna Beach, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *properuptus* (dotted type)	FAKU 73708	AB206159	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) *properuptus* (lined type)	FAKU 73093	AB206160	Ajiro, Otsuki Town, Kochi Prefecture, Japan
A. (*O.*) *quadrifasciatus*	YCM-P 41584	AB206161	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *sangiensis*	FAKU 73133	AB206162	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *savayensis*	FAKU 73141	AB206163	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *selas*	FAKU 73150	AB206164	Amitori Bay, Iriomote-jima Island, Ryukyu Islands, Japan
A. (*O.*) *semilineatus*	FAKU 70779	AB206165	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) sp.	FAKU 73710	AB206166	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) *taeniophorus* (lined type)	FAKU 73709	AB206167	Morode Beach, Ainan Town, Ehime Prefecture, Japan
A. (*O.*) *taeniophorus* (spotted type)	NSMT-P 62210	AB206168	Chichi-jima Island, Ogasawara Islands, Japan
A. (*O.*) *ventrifasciatus*	FAKU 73700	AB206169	Morode Beach, Ainan Town, Ehime Prefecture, Japan

Subgeneric classifications of the genus *Apogon* follow Fraser (1972). Institutional abbreviations given with catalog numbers are as follows: FAKU, Faculty of Agriculture, Kyoto University, NSMT, National Science Museum, Tokyo, YCM, Yokosuka City Museum.

[a] Subgeneric name *Nectamia* in Fraser (1972) later replaced with *Ostorhinchus* by Gon (1987).

extension at 72 °C for 30 s, the final cycle being followed by an extension at 72 °C for 5 min. The PCR products were electrophoresed on a 1% agarose gel, purified using a Pre-Sequencing Kit (USB) and then sequenced with dye-labeled terminators (Applied Biosystems). Primers used were the same as those for PCR, each of the two partially overlapping fragments being sequenced for both strands. All sequencing reactions were performed

according to the manufacturer's instructions. Labeled fragments were analyzed on a model 3100 DNA sequencer (Applied Biosystems). All sequences are available from DDBJ, EMBL, and GenBank under the accession numbers shown in Table 1.

2.3. DNA alignment

DNA sequences were edited using the computer programs EditView ver. 1.01 (Applied Biosystems), AutoAssembler ver. 2.1 (Applied Biosystems) and DNASIS ver. 3.2 (Hitachi Software Engineering). The accuracy of base determination was checked by comparison of light and heavy complementary DNA strands. The two rRNA and single tRNA genes, together with their secondary structures, were identified by homology to other vertebrates (for rRNA, European ribosomal RNA database: http://oberon.fvms.ugent.be:8080/rRNA/index.html; for tRNA, Kumazawa and Nishida, 1993). Initial alignment of the sequences for all taxa was made using the Clustal X (Thompson et al., 1997) with default settings. Loop and stem regions of the secondary structure were identified as separate categories of data for the analyses. The stem regions were checked for base pair complementarity and the alignments in the loop region were manually adjusted (Kjer et al., 1994). Bulges (bases within stem regions not involved in base pair complementarity) were included in the stem category in the analyses. The number of variable and parsimony-informative sites, and frequencies of transitions and transversions were calculated separately for stem and loop regions using PAUP* 4.0b8a (Swofford et al., 2001). Kimura's (1980) two parameter corrected genetic distances were calculated for all pairwise comparisons among taxa for total aligned sequences. Site saturation in loop and stem regions was assessed by plotting the corrected distances against the total number of transitions and transversions for each pairwise comparison.

2.4. Phylogenetic analyses

A total of 202 nucleotides from sequenced and aligned fragments were removed from all of the analyses because the homology of those characters could not be determined. All phylogenetic analyses were performed on the remaining 1287 bp. Maximum parsimony (MP) analyses were performed using the heuristic search option, 1000 random sequence addition replicates, and the tree bisection and reconnection algorithm in PAUP* 4.0b8a (Swofford et al., 2001). Two apogonid species of genus *Fowleria* were used as outgroups to root the trees. All characters were equally weighted. Parsimony trees were evaluated using summary statistics reported by PAUP* (e.g., tree length, ensemble consistency index). Support for nodes was evaluated by calculating decay indices (Bremer, 1988, 1994) and nonparametric bootstrap values (Felsenstein, 1985) using PAUP*, the latter employing a heuristic search and 1000 bootstrap pseudoreplications.

Modeltest ver. 3.06 (Posada and Crandall, 1998) was used to determine a model of sequence evolution closely approximating that which best fitted the dataset. The hierarchical likelihood-ratio tests implemented in Modeltest selected the GTR + I + Γ model (proportion of invariable sites = 0.3949; $\alpha = 0.5757$; empirical base frequencies: A = 0.3542; C = 0.2496; G = 0.1938; substitution rates: A-C = 2.8211; A-G = 9.7206; A-T = 2.6451; C-G = 0.4220; C-T = 17.6721) as the best model for the data. Under these parameter settings, maximum-likelihood (ML) analysis was conducted using PAUP*.

A Bayesian inference (BI) of *Apogon* phylogeny was performed with Mr. Bayes v3.0b4 (Huelsenbeck and Ronquist, 2001). Monte Carlo Markov chains under the GTR + I + Γ model were run for 1,000,000 generations, trees being sampled every 100 generations where the Markov chain reached stationarity, leaving 9850 trees for analysis. The posterior probabilities of trees and tree parameters were estimated from this distribution.

Alternative phylogenetic hypotheses were tested using the Shimodaira–Hasegawa test (SH; Shimodaira and Hasegawa, 1999) implemented in PAUP*. To elucidate the relative timing of major cladogenetic events in the genus *Apogon*, ultrametric trees were constructed using the nonparametric rate smoothing (NPRS) method (Sanderson, 1997). Before the method was conducted, a likelihood-ratio test (Huelsenbeck and Crandall, 1997) was performed on ML trees with and without a molecular clock constraint, to establish if a molecular clock can be accepted. Because the test rejected the molecular clock hypothesis, a NPRS tree was constructed using TreeEdit version 1.0 (Rambaut and Charleston, 2001). To roughly calibrate the tree, age of the subgenus *Apogon* was calculated, applying a molecular clock [the Tv rate for 12S and 16S rRNA genes of 0.14% per million years (Hanel et al., 2002; Ritchie et al., 1996)] to the average Tv divergence between the subgenus *Apogon* and its sister clade including subgenera *Zoramia* and *Ostorhinchus* I.

3. Results

Mitochondrial DNA sequence data were obtained for a total 1489 aligned bases, including 449 bp of the 12S rRNA gene, 73 bp of the $tRNA^{Val}$ gene and 967 bp of the 16S rRNA gene, for 45 taxa. A total of 1287 positions were analyzed, of which 709 were invariant and 162 phylogenetically informative under the parsimony criterion. Plotting the number of differences between pairs of taxa against corrected distance revealed no discernable evidence of site saturation, even in the least conservative class of mutations, transitions in loop regions (Fig. 1).

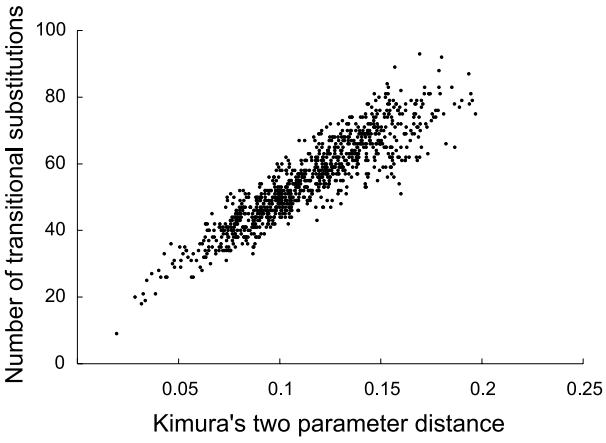

Fig. 1. Scatter-plot of number of transition substitutions versus corrected distance using Kimura's (1980) two parameter model in pairwise comparisons in loop regions.

The maximum-parsimony analysis yielded 25 most-parsimonious trees, each with a total length of 2551 steps (CI = 0.35, RI = 0.44, RC = 0.15). The strict consensus of these trees is presented in Fig. 2. The 43 species of the genus *Apogon* analyzed here were monophyletic with a bootstrap value of 100 and a decay index of 37. The subgenus *Apogon* (four of the 35 extant species were analyzed here) was also monophyletic, being the sister group to all other species of the genus *Apogon* analyzed here. The remaining taxa were divided into five monophyletic clades. Two of the five clades corresponded to the subgenera *Pristiapogon* and *Pristicon*, respectively, while the remaining three clades (*Zoramia* + *Ostorhinchus* I clade, *Ostorhinchus* II clade and *Ostorhinchus* III clade) did not correspond to accepted subgeneric delimitations. The *Zoramia* + *Ostorhinchus* I clade included two sub-clades, *Zoramia* (including *Apogon* (*Zoramia*) *gilberti* and *A.* (*Z.*) *leptacanthus*) and *Ostorhinchus* I sub-clades (including *Apogon* (*Ostorhinchus*) *amboinensis* and *A.* (*O.*) *sangiensis*), the monophyly of the two clades being supported by a bootstrap value of 99 and a decay index of 11. The *Ostorhinchus* II clade, however, included 10 species of *Ostorhinchus*, *A.* (*O.*) *carinatus*, *A.* (*O.*) *cathetogramma*, *A.* (*O.*) *ellioti*, *A.* (*O.*) *guamensis*, *A.* (*O.*) *ishigakiensis*, *A.* (*O.*) *lineatus*, *A.* (*O.*) *melas*, *A.* (*O.*) *niger*, *A.* (*O.*) *savayensis* and *A.* (*O.*) sp., while the *Ostorhinchus* III clade included the remaining 20 species of the subgenus examined herein. Although the monophyly of each of the *Ostorhinchus* II and III clades was supported by only low values (bootstrap values <50 and decay indices of 1), the members of each clade shared a common basic color pattern on the body, members of *Ostorhinchus* II shared some vertical lines on the body and an oblique dark bar on the cheek (some species lacked one of the two patterns), while those of *Ostorhinchus* III shared horizontal line(s) on the head and body (some species on the head only).

The trees reconstructed by maximum-likelihood and Bayesian phylogenetic analyses are shown in Figs. 3A and B, respectively. In both trees, the six clades (subgenus *Apogon*, *Pristiapogon*, *Pristicon*, *Zoramia* + *Ostorhinchus* I, *Ostorhinchus* II, *Ostorhinchus* III) recognized in the MP consensus tree were recovered, although their phylogenetic relationships varied according to the analyses. In the BI tree (Fig. 3B), the respective monophylies of subgenera *Apogon*, *Pristiapogon*, *Pristicon*, and *Zoramia* were supported by 100% posterior probabilities. The monophyly of *Zoramia* and *Ostorhinchus* I was also supported by 100% posterior probability, and those of *Ostorhinchus* II and III by 96 and 91% posterior probabilities, respectively.

A likelihood-ratio test, with ($-\ln L = 13374.65$) and without ($-\ln L = 13102.26$) the molecular clock enforced, rejected overall constancy of evolutionary rate in the genus *Apogon* ($\delta = 544.78$, df = 43, $P \ll 0.001$). In the absence of rate constancy, we used the NPRS method to construct an ultrametric tree (Fig. 3C), based on best of three ML trees (Fig. 3A). The results of the statistical comparisons between the ML tree and alternative tree topologies among the species of the genus *Apogon* are discussed below.

4. Discussion

4.1. Taxonomic implications

Fraser (1972) divided the genus *Apogon* into 10 subgenera, based on the presence or absence of some osteological characters, and has been followed by most subsequent authors (Fraser and Lachner, 1985; Gon, 1986; Myers, 1999). Five of the 10 subgenera, including the most speciose subgenus *Ostorhinchus*, were examined in the present study, four of them (*Apogon*, *Pristiapogon*, *Pristicon*, and *Zoramia*) being recovered as monophyletic groups in all of the analyses performed. However, the subgenus *Ostorhinchus* was polyphyletic and separated into Clades I, II, and III. Although the phylogenetic relationships among the three *Ostorhinchus* clades and four other subgenera varied according to the analyses, a sister relationship between *Ostorhinchus* I and *Zoramia* was recovered in all analyses. Moreover, while the monophyly of all three *Ostorhinchus* clades was rejected by the SH test ($P < 0.01$), that of *Ostorhinchus* II and III was not rejected ($P = 0.132$). These results indicate that the allocation of *Ostorhinchus*-I species [*A.* (*O.*) *amboinensis* and *A.* (*O.*) *savayensis*] to the subgenus *Ostorhinchus* requires review. It was interesting that these two species share six first dorsal spines and a semi-transparent body with their sister *Zoramia* species whereas most *Ostorhinchus* species usually have seven first dorsal spines (Fraser, 1998).

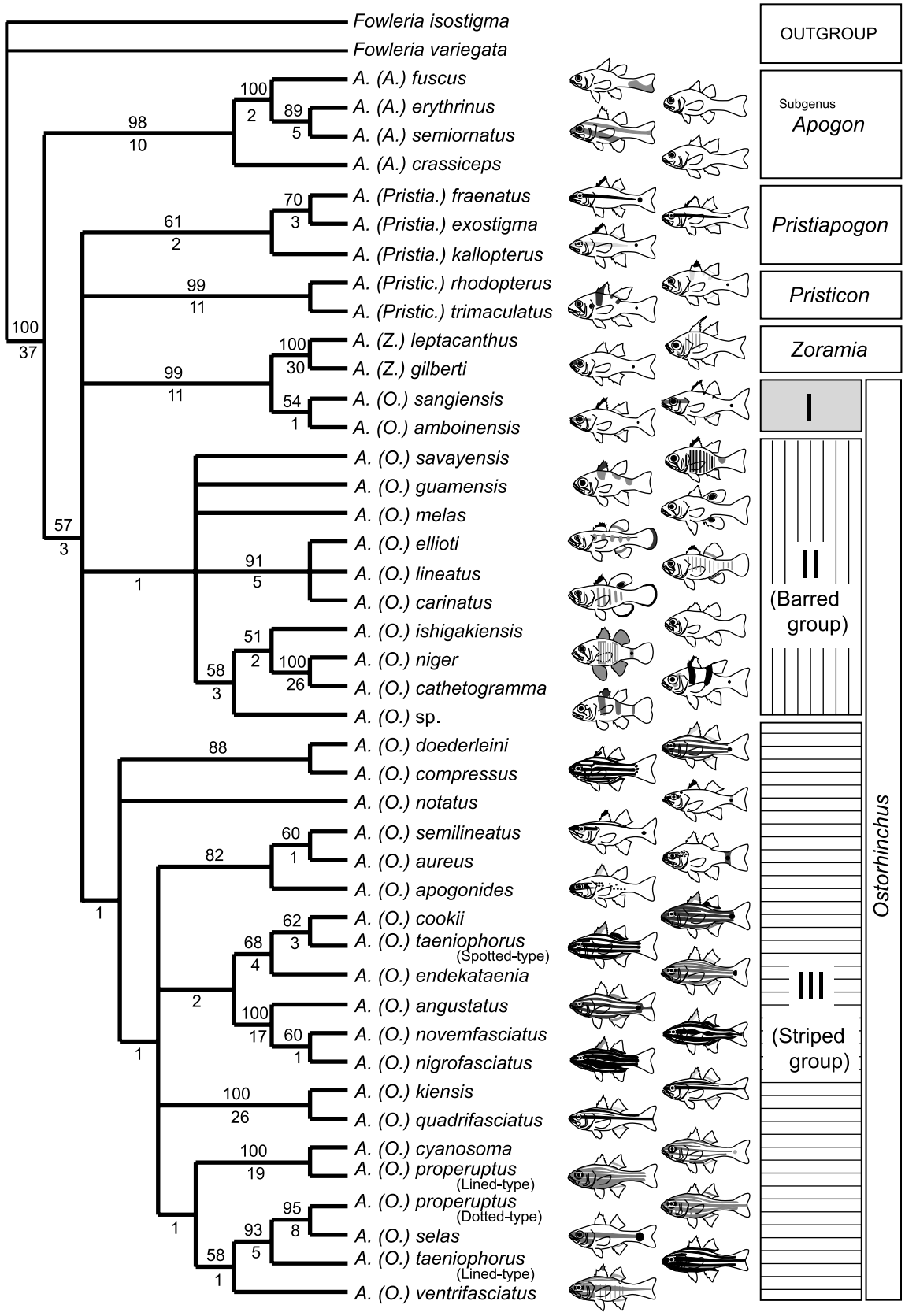

Fig. 2. Strict consensus of 25 most-parsimonious trees, total length = 2551, CI = 0.35, RI = 0.44, RC = 0.15 (values for each most-parsimonious tree). Bootstrap values for 1000 replicates (above) and decay index support (below) indicated at each node (bootstrap values below 50% omitted).

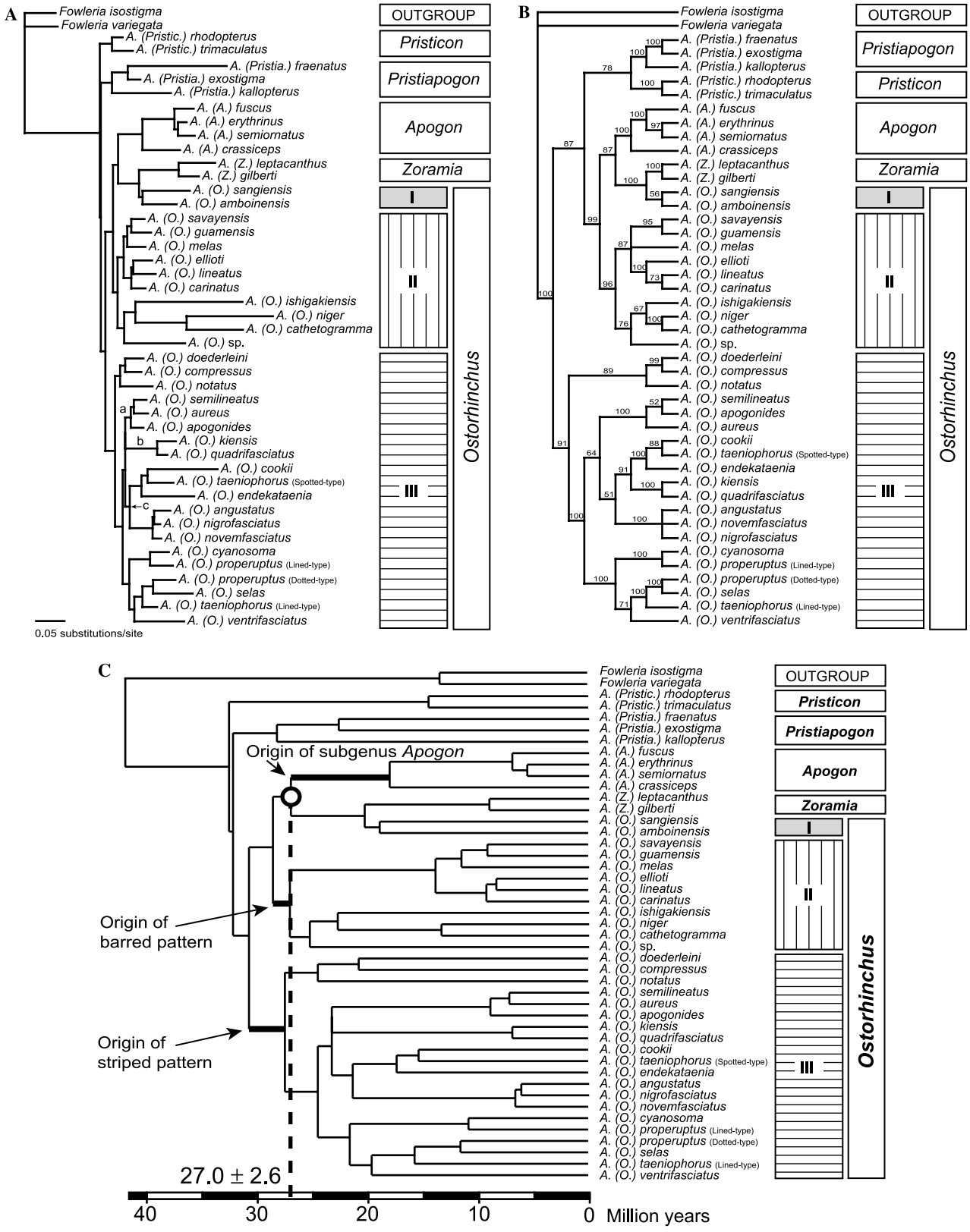

Fig. 3. (A) Best maximum-likelihood tree based on the GTR + I + Γ evolutionary model (−ln L = 13102.26150). Three ML trees were retained in the analysis. They differs from each other in a relationship among sub-clades a, b, and c. (B) Fifty percent majority rule tree produced by Bayesian analysis under the GTR + I + Γ evolutionary model. Posterior probabilities from 9850 trees indicated on branches. (C) Ultrametric tree based on non-parametric rate smoothing (NPRS) analysis of the ML tree. Scale bar shows time scale resulting from calibration of the molecular clock [Tv rate of 0.14% per million years (Hanel et al., 2002; Ritchie et al., 1996)] based on the circled node.

4.2. Stripe-pattern evolution in the genus Apogon

The subgenus *Ostorhinchus*, which includes >100 species, can be phenotypically classified into two large subgroups based on stripe patterns on the head and body. Species of one subgroup are characterized by vertical lines on the body and an oblique dark bar on the cheek, and those of the other subgroup by horizontal line(s) on the head and body. The present phylogenetic analyses demonstrated that the subgenus, excepting two of the 32 species, comprises two large lineages corresponding to their basic stripe-patterns. Although the study included <30% of *Ostorhinchus* species, the clear monophylies of the vertically and horizontally lined species appear to indicate a major evolutionary trend within the genus: early evolution of basic stripe patterns followed by subsequent diversification within each group.

Such an evolutionary trend also applies to the subgenus *Apogon*, including 35 species. These species lack a striped pattern on the body and head [except *A.* (*A.*) *semiornatus* which has two oblique lines on the body]. Because the monophyly of subgenus *Apogon* was recovered by all of the analyses conducted here, the evolution of a "no-stripe" pattern appears to date back to at least the origin of the subgenus.

Using a molecular clock calibration with a Tv rate of 0.14% per million years (Hanel et al., 2002; Ritchie et al., 1996), the origin of the subgenus *Apogon* dates back to 27 ± 2.6 million years, which does not conflict with the present Tethyan distribution of the subgenus. Based on the NPRS tree (Fig. 3C), the evolution of the two major stripe patterns occurred at a similar time to the non-striped subgenus, more than 20 million years ago.

4.3. Evolutionary conservativeness of basic stripe patterns in the genus Apogon

It is biologically interesting to compare basic stripe-pattern evolution of *Apogon*, as revealed here, with that of the African Great Lake cichlids previously reported. In this study, we have shown that the basic stripe patterns shown in the genus *Apogon* have diversified more than 20 million years ago into three major forms: non-striped, striped and barred. However, the stripes of the African Great Lake cichlids are not confined to specific clades in molecular phylogeny (Seehausen et al., 1999), indicating rapid evolutionary switching of basic stripe patterns, such as from vertically to horizontally lined patterns, during the past 1–2 million years in Lakes Malawi and Victoria (Meyer et al., 1990; Verheyen et al., 2003). This remarkable contrast in constraints in stripe evolution suggests that the ecological factors have played an important role in stripe-pattern evolution, as described below.

Relatively higher persistence of the stripe pattern in the genus *Apogon* may be due to either phylogenetic constraint (sensu Brooks and McLennan, 1994) or phylogenetic niche conservatism (Harvey and Pagel, 1991). Phylogenetic constraint involves the presence of lineage-specific barriers to evolutionary change, such as a lack of additive genetic variation for a particular trait, or genetic burden imposed by coadaptation among traits (Westoby et al., 1995a,b). Phylogenetic niche conservatism, in contrast, involves the tendency of lineages to retain similar ecological niches over evolutionary time scales (Peterson et al., 1999; Ricklefs and Latham, 1992; Webb et al., 2002). In other words, an intrinsic capacity to evolve may exist in a lineage, but a shift to other niches may not occur owing to the presence of competitors already well adapted to those niches (Harvey and Pagel, 1991).

Although some genes affecting pigmentation pattern on the body have been cloned and characterized in zebrafish (Haffter et al., 1996; Johnson et al., 1995; Kawakami et al., 2000) and cichlids (Sugie et al., 2004; Terai et al., 2002, 2003), the genetic control of pigmentation patterns in fishes remains largely unknown. This lack of knowledge regarding genetic constraints on stripe pattern prevents us from fully differentiating between the above two scenarios (Lord et al., 1995). It is, however, noteworthy that the evolutionary environment differs between African cichlids and *Apogon*, the former inhabiting relatively closed young lakes and the latter, a continuous old marine environment. This suggests that phylogenetic niche conservatism has played an important role in maintaining basic stripe patterns in *Apogon*.

In the African Great Lake cichlids, basic stripe patterns were constrained ecologically (for example, vertical lines are associated with structural habitat and horizontal lines with shoaling behavior and/or a piscivorous feeding mode), the stripe-pattern diversity resulting from their radiation into a variety of ecological niches (Seehausen et al., 1999). Such evolutionary radiations are supposedly facilitated by the colonization of novel habitats (newly created lakes) without competitors (Schluter, 2000). The basic stripe patterns in *Apogon* also appear to be constrained ecologically, species of the vertically lined (and non-striped) lineage tending to be cryptic, occurring solitarily or in pairs within structural habitats all their life, while those of the horizontally lined lineage occur in intra- or interspecific shoals above substrates, especially as juveniles. *Apogon* species, however, appear not to have had the opportunity to colonize novel habitats without competitors. Whereas the African Great Lake niches are occupied by a single family, Cichlidae (Snoeks, 2000), those in coral reefs are occupied by diverse families, such as Labridae, Pomacentridae and Serranidae, each being specialized to particular niches as long-time members of a 50 million year old marine community (Bellwood, 1996). In other words, Apogonidae (and the genus *Apogon*) has coexisted with diverse families over a long evolutionary time. Such an ecological

factor, together with the continuity of marine habitat, may have prevented *Apogon* from occupying alternative niches other than as nocturnal planktivores. The NPRS tree (Fig. 3C) indicates that the three alternative pattern lineages, widespread in the Indo-West Pacific, evolved almost simultaneously. Furthermore, the long coexistence of the three major lineages may have prevented each lineage from invading the other's niche and thereby becoming subject to selective pressures to retain the basic stripe pattern. Marine fish evolution may, in fact, be characterized by phylogenetic niche conservatism caused by their historically stable and spatially continuous environment.

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