Influence of different substrates on the evolution of morphology and life-history traits of azooxanthellate solitary corals (Scleractinia: Flabellidae)

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Sessile organisms are influenced considerably by their substrate conditions, and their adaptive strategies are key to understanding their morphologic evolution and traits of life history. The family Flabellidae (Cnidaria: Scleractinia) is composed of the representative azooxanthellate solitary corals that live on both soft and hard substrates using various adaptive strategies. We reconstructed the phylogenetic tree and ancestral character states of this family from the mitochondrial 16S and nuclear 28S ribosomal DNA sequences of ten flabellids aiming to infer the evolution of their adaptive strategies. The *Javania* lineage branched off first and adapted to hard substrates by using a tectura-reinforced base. The extant free-living flabellids, including *Flabellum* and *Truncatoflabellum*, invaded soft substrates and acquired the flabellate corallum morphology of their common ancestor, followed by a remarkable radiation with the exploitation of adaptive strategies, such as external soft tissue [e.g. *Flabellum* (*Ulocyathus*)], thecal edge spine, and transverse division (e.g. *Placotrochus* and *Truncatoflabellum*). Subsequently, the free-living ancestors of two genera (*Rhizotrochus* and *Monomyces*) invaded hard substrates independently by exploiting distinct attachment apparatuses such as tube-like and massive rootlets, respectively. In conclusion, flabellids developed various morphology and life-history traits according to the differences in substrate conditions during the course of their evolution. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **101**, 184–192.

ADDITIONAL KEYWORDS: adaptive strategy – attached – Cnidaria – diversification – free-living – mode of life – molecular phylogeny – rootlet – spine.

INTRODUCTION

The adaptive strategies of sessile organisms largely depend on their substrate conditions. Therefore, a habitat shift during the course of evolution would be closely related to changes in morphology and life-history traits (Bromley & Heinberg, 2006). However, little attention has been paid to this issue in the case of solitary scleractinian corals.

The Family Flabellidae Bourne, 1905 (Cnidaria: Scleractinia) comprises ten genera with 98 species, which are exclusively solitary and azooxanthellate (Cairns, 1999). These corals are widely distributed over depths in the range 0–3200 m and exhibit specific modes of life and morphologies. Six genera including two subgenera [*Flabellum* (*Flabellum*), *Flabellum* (*Ulocyathus*), *Truncatoflabellum*, *Blastotrochus*, *Placotrochus*, *Placotrochides*, and *Falcatoflabellum*] show free-living modes of life whereas four genera (*Rhizotrochus*, *Javania*, *Monomyces*, and *Polymyces*) have developed an attached mode of life, at least during their post-larval stages (Table 1).

The free-living flabellids show distinct morphological characters, such as variously flattened flabellate

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Table 1. Morphology and life history traits of flabellids (the family Flabellidae) used in the present study

Taxa	Mode of life	Corallum morphology	External soft tissue	Thecal edge spine	Transverse division	Fixation*
Flabellum (*Flabellum*)	Free-living	Flabellate	Absent	Absent	Absent	Simple
Flabellum (*Ulocyathus*)	Free-living	Flabellate	Present	Absent	Absent	Simple
Truncatoflabellum	Free-living	Flabellate	Absent	Present	Present	Simple
Placotrochus	Free-living	Flabellate	Absent	Present	Present	Simple
Rhizotrochus	Attached	Nonflabellate†	Absent	Absent	Absent	Simple and hollow rootlet
Javania	Attached	Nonflabellate‡	Present	Absent	Absent	Tectura-reinforced
Monomyces	Attached	Flabellate	Absent	Absent	Absent	Simple and massive rootlet

*Simple fixation indicates nonreinforced small pedicel.
†Turbinate.
‡Ceratoid to trochoid.

corallum shapes with or without ornamentation. All of them live by lying on either flattened side of the corallum on soft substrates (Hoeksema, 1993; Buhl-Mortensen *et al.*, 2007). Five free-living genera, namely *Truncatoflabellum*, *Placotrochus*, *Placotrochides*, *Blastotrochus*, and *Falcatoflabellum* (Cairns, 1989a, b, 1995), reproduce asexually (by transverse division and anthoblast production). The genera exhibiting a flabellate corallum shape and asexual reproduction (i.e. *Truncatoflabellum*, *Placotrochus*, and *Blastotrochus*) typically have one or more pairs of thecal edge spines (Fig. 1). Although each free-living genus or subgenus exhibits characteristic distribution ranges (Cairns, 1989a), *Flabellum* (*Ulocyathus*) occupies particularly deeper-water environments. *Javania* has a characteristic ceratoid to trochoid corallum and attaches to hard substrates by secreting concentric layers of sclerenchyme (i.e. tectura reinforced; Cairns, 1989a, 2004). By contrast, *Rhizotrochus*, *Monomyces*, and *Polymyces* attach to hard substrates by using rootlets, and not a tectura-reinforced base (Cairns, 1989a). It is worth noting that the rootlets of *Rhizotrochus* and *Monomyces* are different in shape and structure: the former forms tube-like rootlets around the corallum, whereas the latter has massive rootlets only on the thecal edge (Scheer & Pillai, 1983; Cairns, 1989a).

In the present study, we investigated the phylogenetic relationships of flabellids at the molecular level by using mitochondrial 16S and nuclear 28S ribosomal DNA (rDNA) sequences to infer the evolution of their morphologies and modes of life (i.e. adaptive strategies), for which we used the maximum parsimony and likelihood reconstruction methods. The findings obtained study reveal how flabellids developed various adaptive strategies according to the differences in their substrate conditions during the course of evolution.

MATERIAL AND METHODS

SPECIMENS

We newly-collected seven flabellid species in Japan. Details of the specimens used in the present study are provided in Table 2.

DNA EXTRACTION AND SEQUENCING

DNAs were extracted from the ethanol-preserved tissues by using DNAzol (Invitrogen) in accordance with the manufacturer's instructions and then dissolved in water. RNAs in the samples were digested with Ribonuclease A (Affymetrix).

Fragments of the 16S and 28S rDNAs were obtained by the polymerase chain reaction (PCR). The amplification was performed in a final concentration

Flabellum (*Flabellum*) *magnificum*

Flabellum (*Flabellum*) *pavoninum*

Flabellum (*Ulocyathus*) *deludens*

Flabellum (*Ulocyathus*) *japonicum*

Spine �

Rootlet ➤

Tectura-reinforced base ↘

Truncatoflabellum spheniscus

Rhizotrochus typus

Javania insignis

Figure 1. External morphology of the flabellids used in this study. Scale bars = 1 cm.

of $1 \times$ PCR buffer with MgSO$_4$ (Roche), 0.2 mM of dNTP, 0.6 μM of each primer, and 0.05 U μL^{-1} of Pwo DNA polymerase (Roche). The primers used are detailed in the Supporting information (Table S1). The annealing temperature was set at 50 °C or 55 °C.

The PCR products were run on 1.5% agarose gel, extracted from the gel, and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The fragments were directly sequenced on a 3130 Genetic Analyzer (Applied Biosystems) with a BigDye Terminator Cycle Sequence kit, version 1.1 or 3.1 (Applied Biosystems). Each sequence was determined from at least three independent cycle sequencing reactions. The DDBJ/

Table 2. Experimental species, collection localities, and accession nos. of 16S and 28S rDNAs

SUBORDER Family Species	Specimen	Collection locality*	16S rDNA Accession number	28S rDNA Accession number	Reference
CARYOPHYLLIINA					
Flabellidae					
Flabellum (*Flabellum*) *magnificum* Marenzeller, 1904		a	AB510167	AB510179	Present study
Flabellum (*Flabellum*) *pavoninum* Lesson, 1831		b	AB510168	AB510180	Present study
Flabellum (*Flabellum*) *impensum* Squires, 1962			AF265582	AF265649	Romano & Cairns (2000)
Flabellum (*Ulocyathus*) *japonicum* Moseley, 1881	A	a	AB510169	AB510181	Present study
	B	a	AB510178	AB510182	Present study
Flabellum (*Ulocyathus*) *deludens* Marenzeller, 1904	A	a	AB510170	AB510183	Present study
	B	a	AB510171	AB510184	Present study
Truncatoflabellum spheniscus (Dana, 1846)	A	b	AB510172	AB510185	Present study
	B	d	AB510173	AB510186	Present study
Placotrochus laevis Milne Edwards & Haime, 1848			AF265604	AF265650	Romano & Cairns (2000)
Rhizotrochus typus Milne Edwards & Haime, 1848	A	b	AB510175	AB510188	Present study
	B	e	AB510176	AB510189	Present study
	C	c	AB510177	AB510190	Present study
Javania insignis Duncan, 1876		c	AB510174	AB510187	Present study
Monomyces pygmaea (Risso, 1826)			AF265583	AF265651	Romano & Cairns (2000)
Turbinoliidae					
Notocyathus sp.			AF265584	AF265648	Romano & Cairns (2000)
Guyniidae					
Guynia annulata Duncan, 1872			AF265580	AF549233	Romano & Cairns (2000), Cuif *et al*. (2003)
DENDROPHYLLIINA					
Dendrophylliidae					
Dendrophyllia gracilis Milne Edwards & Haime, 1848			AF265588	AF265627	Romano & Cairns (2000)
Tubastraea coccinea Lesson, 1829			L76022	AF265625	Romano & Cairns (2000)
FUNGIINA					
Poritidae					
Porites compressa Dana, 1846			L76020	AF265630	Romano & Cairns (2000)

*a, 31°08′N, 130°24′E, Off Makurazaki, Kagoshima Prefecture, Japan (approximately 300 m depth). b, 33°50′N, 134°58′E, Off Hinomisaki, Wakayama Prefecture, Japan (approximately 100 m depth). c, 34°14′N, 136°32′E, Off Minamiise, Mie Prefecture, Japan (approximately 50 m depth). d, off Saga Prefecture, Japan, exact locality unknown. e, off Japan, exact locality unknown.

GenBank/EMBL accession numbers of the sequences are given in Table 2.

PHYLOGENETIC INFERENCES

The nucleotide sequences were aligned using CLUST-ALX, version 1.83 (Thompson *et al*., 1997), with manual modification. DDBJ/GenBank/EMBL accession numbers or the sequence used in the present study are shown in Table 2.

Bayesian methods coupled with Markov chain Monte Carlo (BMCMC) inference as implemented in MRBAYES, version 3.1.2 (Huelsenbeck & Ron-quist, 2001; Ronquist & Huelsenbeck, 2003), were used for the phylogenetic inferences. The best-fit model was selected by hLRT in MRMODELTEST, version 2.3 (Nylander, 2004). Two independent BMCMC analyses were run, with each consisting of four chains. Trees were saved in every 100 generations. Graphical inspection of tree log-likelihood in the sample revealed that stationarity was reached within 5.0×10^5 generations, and the average standard deviation of split frequencies became lower than 0.01. Thus, we discarded the first quarter generations as burn-in and used the remaining generations.

Notocyathus sp., *Guynia annulata*, *Porites compressa*, *Dendrophyllia gracilis*, and *Tubastraea coccinea* were used as outgroups in all the cases. In the alignment of the 28S rDNA sequences with 250 sites including alignment gaps, 41 (16.4%) and 32 (12.8%) sites varied when the outgroups were included and excluded, respectively. In the alignment of the 16S rDNA sequences with 330 sites including alignment gaps, 57 (17.2%) and 39 (11.8%) sites varied when the outgroups were included and excluded, respectively. GTR + G and HKY + G were selected as the best-fit models for the 28S and 16S rDNAs, respectively. The incongruence length difference (ILD) test (Mickevich & Farris, 1981; Farris *et al.*, 1994, 1995) was performed to assess the incongruence between the rDNAs with PAUP, version 4.0b4a (Swofford, 2000). One thousand permutations were executed to generate a null distribution of tree length differences. The ILD test revealed that the 16S rDNA data did not show significant incongruence with the 28S rDNA data ($P > 0.05$); therefore, we constructed a combined data set. GTR + G was recovered as the best-fit model.

The Kishino–Hasegawa test (Kishino & Hasegawa, 1989) was conducted using PAUP to evaluate the phylogenetic hypotheses.

ANCESTRAL CHARACTER STATE RECONSTRUCTION

Information on the mode of life, corallum shape, external soft tissue, thecal edge spines, and asexual reproduction by transverse division was obtained from previous studies (Cairns, 1982, 1989a, 1994, 1995; Hoeksema, 1993; Stolarski, 2000) (Table 1). Character state evolution was reconstructed by the maximum parsimony and likelihood methods, which were based on the AsymmMk and Mk1 models of the combined data set tree, using MESQUITE (Maddison & Maddison, 2007) under default settings. The proportional likelihood (PL) values by the AsymmMk model were almost identical to those by the MK1 model. The modes of life were scored as attached or free-living. The corallum shapes were scored as flabellate or nonflabellate; the latter contains corallum morphologies of ceratoid, trochoid, and turbinate. The external soft tissue, thecal edge spine, and asexual reproduction by transverse division were scored as present or absent.

RESULTS

PHYLOGENETIC RELATIONSHIPS

The ILD test did not show significant incongruence between the 16S and 28S rDNA data sets, and therefore we combined the data and constructed the phylogenetic tree based on the combined data set

(Fig. 2). In the tree, monophyly of Flabellidae was highly supported. In the Flabellidae lineage, *Javania insignis* branched off first, followed by (1) *Flabellum* (*Flabellum*) *pavoninum*, *Flabellum* (*Flabellum*) *magnificum*, and *Monomyces pygmaea*; (2) *Flabellum* (*Ulocyathus*) *deludens*, *Flabellum* (*Ulocyathus*) *japonicum*, and *Flabellum* (*Flabellum*) *impensum*; and (3) *Placotrochus laevis*, *Truncatoflabellum spheniscus*, and *Rhizotrochus typus*. Trees based on each of the 16S and 28S rDNA data set also produced almost identical topology to the tree constructed by the combined data set (see Supporting Information, Fig. S1). However, resolution was not high and the relationships among some species/clusters were not well-resolved in these trees.

The phylogenetic hypotheses shown in Figure 2 (see also Supporting information, Fig. S1) were analyzed with the Kishino–Hasegawa test (Kishino & Hasegawa, 1989). The results of this test revealed that the tree reconstructed from the combined data set (Fig. 2) was the best for all the data sets (see Supporting Information, Table S2). The Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999), one-sided Kishino–Hasegawa test based on pairwise Shimodaira–Hasegawa tests (Kishino & Hasegawa, 1989; Shimodaira & Hasegawa, 1999; Goldman, Anderson & Rodrigo, 2000), and expected likelihood weighting test (Strimmer & Rambaut, 2002) also supported this result (data not shown).

CHARACTER STATE EVOLUTION

Parsimony and likelihood analyses could not resolve the mode of life of the common ancestor of the turbinoliid and the flabellids (Fig. 3A, node 1) or that of the common ancestor of all the flabellids (Fig. 3A, node 2), although they revealed that the common ancestor of all the flabellids other than *Javania* (Fig. 3A, node 3) followed the free-living mode of life. The most recent common ancestors of *Monomyces* and *Rhizotrochus* (Fig. 3A, nodes 4 and 9) were expected to be free-living. Thus, the free-living mode of life arose once during the evolution of flabellids, and the attached mode of life arose at least twice independently in the attached genera *Monomyces* and *Rhizotrochus*.

Phylogenetic estimations of the ancestral corallum morphology revealed that: (1) the common ancestor of the turbinoliid and the flabellids (Fig. 3B, node 1) was nonflabellate; (2) the common ancestor of all the flabellids (Fig. 3B, node 2) was nonflabellate in the parsimony analysis, although the likelihood analysis provided only weak support for this; (3) the common ancestor of all the flabellids other than *Javania* (Fig. 3B, node 3) was flabellate; and (4) the nonflabellate corallum appeared in the ancestor of *Rhizotro-*
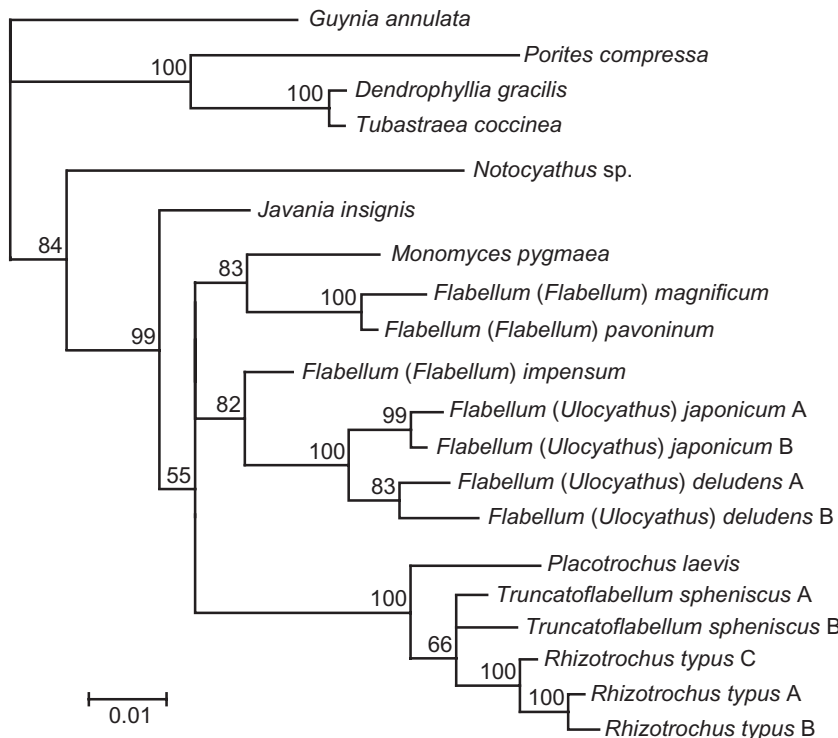
Figure 2. Bayesian phylogenetic tree based on the combined data set (16S rDNA + 28S rDNA). Bayesian posterior probabilities are shown at the nodes. Branch lengths are proportional to the scale provided in substitutions per site.

chus. Thus, the flabellate corallum arose once during the flabellids' evolution and was lost in the ancestor of *Rhizotrochus*.

The external soft tissue of the common ancestor of the turbinoliid and the flabellids (Fig. 3C, node 1) was shown as present; that of the common ancestor of all the flabellids (Fig. 3C, node 2) was also inferred to be present by the parsimony analysis, although the likelihood analysis did not support this result; and that of the common ancestor of all the flabellids other than *Javania* (Fig. 3A, node 3) was reconstructed as absent with high probability. The common ancestor of *Flabellum* (*Ulocyathus*) (Fig. 3C, node 7) was shown to have external soft tissue. Thus, external soft tissue arose once in the *Flabellum* (*Ulocyathus*) lineage.

The ancestor of *Placotrochus*, *Truncatoflabellum*, and *Rhizotrochus* (Fig. 3D, node 8) was revealed to have a thecal edge spine, whereas the recent ancestor of *Rhizotrochus* (Fig. 3D, node 10) did not have the spine. Similarly, asexual reproduction by transverse division arose in the *Placotrochus–Truncatoflabellum–Rhizotrochus* lineage, although *Rhizotrochus* itself did not inherit the character and altered its reproductive system from both asexual and sexual to sexual only.

DISCUSSION

Although Romano & Cairns (2000) and Le Goff-Vitry, Rogers & Baglow (2004) investigated the molecular phylogenetic relationships of scleractinians, the evolution of flabellids has not yet been clarified. The analysis based on the combined data set of the 16S and 28S rDNA sequences revealed that all flabellids are monophyletic, and *Javania* branched off first in Flabellidae next to *Notocyathus* sp. (Turbinoliidae), followed by the branching of the clades of *Monomyces*–*F.* (*F.*) *magnificum*–*F.* (*F.*) *pavoninum*, *F.* (*F.*) *impensum*–*Flabellum* (*Ulocyathus*), and *Placotrochus*–*Truncatoflabellum*–*Rhizotrochus*. The result of the phylogenetic analysis is almost identical with the fossil evidence obtained for the flabellids (Cairns, 1989a, 1994, 1995). According to the topology of this tree, we reconstructed the character state evolution of flabellids.

Javania branched off first in the flabellid lineages and adapted to hard substrates by using a tectura-reinforced base. However, it is still uncertain whether the mode of life is plesiomorphic or apomorphic. The ancestor of all the flabellids other than *Javania* (Fig. 3, node 3) is suggested to have exhibited the free-living mode of life and flabellate corallum morphology
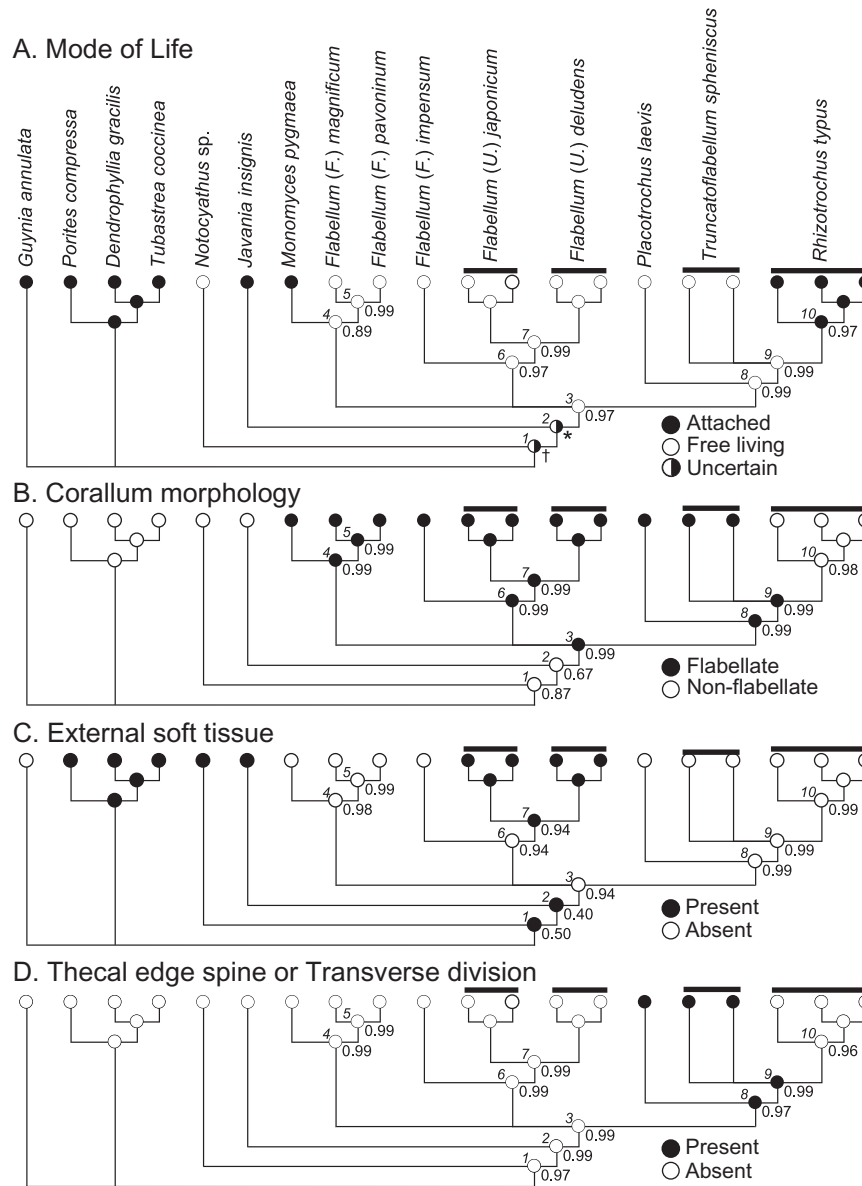
Figure 3. Ancestral character state reconstructions of mode of life (A), corallum morphology (B), external soft tissue (C), and thecal edge spine or transverse division (D), inferred from the maximum parsimony method. The results of thecal edge spine and transverse division were identical. Ancestral and extant states are shown at the nodes or tips of the tree. The topology of the Bayesian phylogenetic tree based on the combined data set (16S rDNA + 28S rDNA) shown in Fig. 2 was adopted in these analyses. The numbers shown to the lower right of the nodes are the proportional likelihood (PL) values by the AsymmMk model. At the node indicated with an asterisk, the ancestral states of attached and free-living modes of life were supported by the PL values of 0.37 and 0.63, respectively. At the node indicated with a dagger, the PL values for the attached and free-living modes of life were both 0.50. For the node numbers (i.e. 1–10) to the upper left of the nodes.

without external soft tissue, thecal edge spine, and transverse division. The broadly-flattened flabellate corallum surface is considered to help prevent individual corals from sinking into soft substrates, even if they increase their size and weight (i.e. snowshoe effect; Thayer, 1975). The flabellate corallum with a

free-living life mode (Fig. 3, node 3) clearly indicates that the ancestor lived on soft substrates and phyletic diversification of free-living flabellids occurred on these substrates. *Flabellum* (*Flabellum*) inherited all the characters from the ancestor (Fig. 3, node 3). *Flabellum* (*Ulocyathus*) similarly inherited these

characters but with the additional acquisition of external soft tissues. This subgenus generally lives in deeper water (up to 3200 m deep) than the other free-living flabellids (Cairns, 1989a; Cairns & Keller, 1993). Roniewicz & Stolarski (1999) suggested that the development of external soft tissues is indispensable for deep-water corals aimed to protect them from chemical corrosion of their calcareous skeleton in these environments.

In the case of the ancestor of *Placotrochus*, *Truncatoflabellum*, and *Rhizotrochus* (Fig. 3, node 8), asexual reproduction by transverse division uniquely appeared with the development of thecal edge spines. After transverse division in *Truncatoflabellum* and *Placotrochus*, cloned offspring reduce the flattened surface area of the flabellate coralla, making the flabellate coralla disadvantageous for their stability of life position on soft substrates. However, the presence of thecal edge spines could help stabilize their life position (Carter, 1972).

The attached corals *Rhizotrochus* and *Monomyces* appeared again from distinct free-living coral lineages with different attachment apparatuses. *Monomyces* has massive rootlets formed from thecal edges, whereas *Rhizotrochus* has tube-like rootlets around the corallum. *Monomyces* has inherited the flabellate corallum from the free-living ancestor, probably because the corallum shape is indispensable to develop the massive rootlets from the thecal edges (Squire, 1963; Zibrowius, 1980: Pl. 80G). On the other hand, *Rhizotrochus* has lost both the flabellate corallum and thecal edge spines, and reproduces only sexually, probably as a result of the acquisition of the attached life mode. The nonflabellate corallum of *Rhizotrochus* is advantageous for attachment with the aid of numerous tube-like rootlets around the corallum instead of the thecal edge spines. The thecal edge spines of *Truncatoflabellum* and *Placotrochus* were originally hollow tube-like structures (Cairns, 1989a), from which *Rhizotrochus* developed the special attachment apparatus that originated in the ancestor of this genus (Fig. 3, node 10).

The present study thus suggests that flabellids developed various adaptive strategies according to the differences in their substrate conditions during the course of evolution. For sessile organisms, a habitat shift between hard and soft substrates must have been a great driving force in the evolution of morphology and life-history traits.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bayesian phylogenetic trees based on the 16S rDNA (A) and 28S rDNA (B) sequences. Bayesian posterior probabilities are shown at the nodes. Branch lengths are proportional to the scale provided in substitutions per site.
Table S1. Primer sequence.
Table S2. The log-likelihood of the phylogenetic trees and results of the Kishino–Hasegawa test at the 5% confidence limit.

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