Phenotypic plasticity revealed by molecular studies on reef corals of *Fungia* (*Cycloseris*) spp. (Scleractinia: Fungiidae) near river outlets

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Abstract

On a patch reef off Makassar, Sulawesi, Indonesia, corals identified as *Fungia* (*Cycloseris*) *costulata*, *Fungia* (*Cycloseris*) *tenuis* and *Fungia* (*Cycloseris*) cf *costulata* were collected down to a maximum depth of 10 m. The corals lived sympatrically. Mushroom coral clones resulting from fragmentation can be recognized by their equal coloration and close proximity. Therefore, to ensure that no clones were collected, corals of dissimilar colors were selected at a mutual distance of 5 m. The corals were kept alive in two 30 liter sea-water aquariums with an air-pump. They were photographed in detail. Using allozyme electrophoresis in a laboratory close to the field area, it was tested whether the separate coral morphs should be considered three species. Eventually it was concluded that there are only two species, i.e. *F.* (*C.*) *costulata* and *F.* (*C.*) *tenuis*, of which *F.* (*C.*) *costulata* has two distinct morphs, one of which may be an eco-phenotype occurring on reefs off river outlets or inside estuaries.

Contents

Introduction

Intraspecific variation in scleractinian corals is a classic problem in their taxonomy, both in regard to recent and fossil species (Best *et al.*, 1999; Knowlton and Budd, 2001). Habitat-induced variability has been observed in coral species distributed along depth ranges, in which the specimens from the deeper sites used to be flatter than those from shallower places. The former ones exposing more surface area in order to compensate for less light penetration at greater depths (Wijsman-Best, 1972, 1974; Hoeksema, 1993). Other environmental factors of importance in coral shape plasticity may be sedimentation, salinity and water temperature, turbulence and flow; in addition to coral morphology also the pigmentation in the soft tissue may be affected (Bruno and Edmunds, 1997; Todd *et al.*, 2002a, 2002b, 2004b, 2004c). Coral damage and subsequent regeneration also have specific effects on shape, which may impede taxonomy and easy identification (Hoeksema, 1989, 1991b, 1993; Oren *et al.*, 1997; Nagelkerken and Bak, 1998).

For no obvious reason, most of the research on intraspecific coral variation has been performed on massive faviids, both in the Atlantic (Dustan, 1975; Foster, 1977, 1979; Lasker, 1981; Graus and Macintyre, 1982; Dodge, 1992; Beltran-Torres and Carricart-Gavinet, 1993; Amaral, 1994; Manica and Carter, 2000) and the Indo-Pacific (Wijsman-Best, 1972, 1974; Miller, 1994; Oren *et al.*, 1997; Todd *et al.*, 2001, 2002a, 2002b, 2004a, 2004b, 2004c). Mushroom corals (Fungiidae), which are endemic to the Indo-Pacific, have mainly been studied in relation with light penetration, sedimentation, and traumatic damage (Hoeksema, 1989, 1991b, 1993; Hoeksema and Moka, 1989). A physiological difference with regard to vulnerability to elevated temperature has also been shown among individuals of the same mushroom coral species within close range of each other (Hoeksema, 1991a).
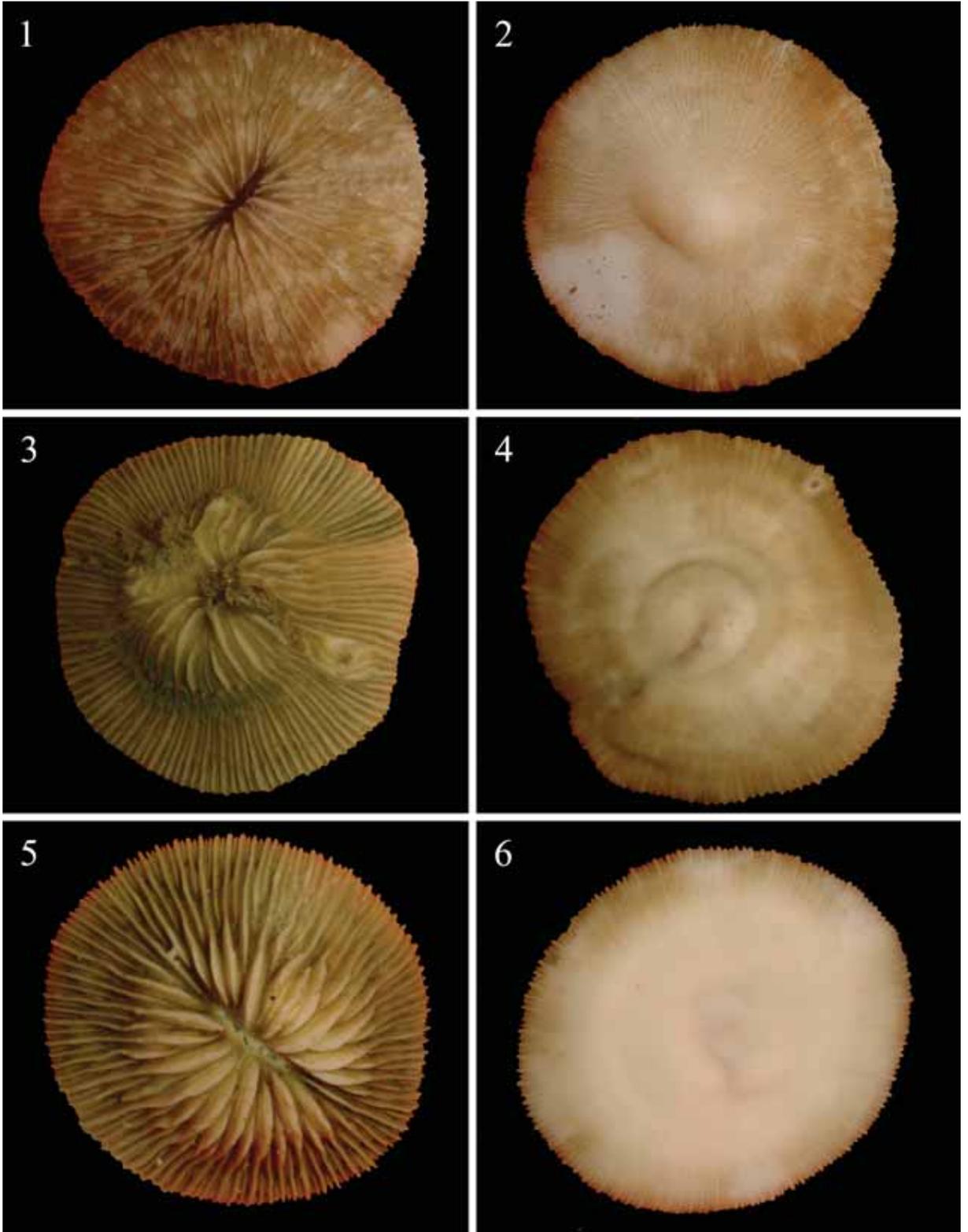
Figs. 1-6. Upper and lower surfaces of mushroom corals. 1-2, *Fungia* (*Cycloseris*) *costulata*. 3-6, *Fungia* (*Cycloseris*) cf *costulata*. Scale = 1:1.

During earlier taxonomic and morphological studies on mushroom corals three different morphs of *Fungia* (*Cycloseris*) spp. were distinguished (Hoeksema, 1989; Hoeksema and Moka, 1989). Two of these were considered separate species, viz. *Fungia* (*Cycloseris*) *costulata* Ortmann, 1889 (figs. 1-2), and *F.* (*C.*) *tenuis* Dana, 1864 (figs. 7-8); the third one was seen as a morph of the first (figs. 3-6; Hoeksema and Moka, 1989: fig. 12). *Fungia* (*C.*) *tenuis* has much rougher costae and usually a slightly different coloration (dark brown stomatal ends) as compared to *F.* (*C.*) *costulata*. The two are usually observed on the same reefs. However, *F.* (*C.*) *costulata* may also be present on reefs that are more nearshore, in more sediment-rich water, and they may occur deeper when found on the same reef as *F.* (*C.*) *tenuis*. The alleged separate morph of *F.* (*C.*) *costulata* is thinner and shows an evenly brown-olive green color (figs. 3-6). It occurs on nearshore reefs, either on patch reefs near river outlets or inside deep bays and estuaries. The three morphs cannot always be distinguished easily. Therefore, in order to investigate the taxonomic implications of the morphological differences, several allozymes of the morphs were compared. Allozyme electrophoresis has successfully been used to solve similar taxonomical problems (Gittenberger *et al.*, 2001;

Sanjuan *et al.*, 1997). The three morphs will be referred to as morphs A, B and C for respectively *Fungia* (*Cycloseris*) *costulata*, *F.* (*C.*) *tenuis* and *F.* (*C.*) cf *costulata*.

Material and methods

Sampling

Specimens of morphs A, B and C were found sympatrically at Bone Baku reef, off Makassar, Sulawesi, Indonesia. Twelve specimens of each morph were collected within an area of about 200 m^2 at depths between 2 and 12 meters. They were coded A1-12, B1-12 and C1-12. To make sure that no clones were included, only individuals that differed in polyp coloration were selected. While diving, the specimens were individually put into separate plastic bags and transported to the laboratory in a bucket with seawater. They were kept alive in two 30 liter aquariums with two air-pumps each. To reduce the pollution in the aquariums, the water was taken a few kilometers off the coast and filtered through a coffee filter. Before the allozyme electrophoresis, all corals were digitally photographed on both sides with a Fujifilm MX-2700 camera.
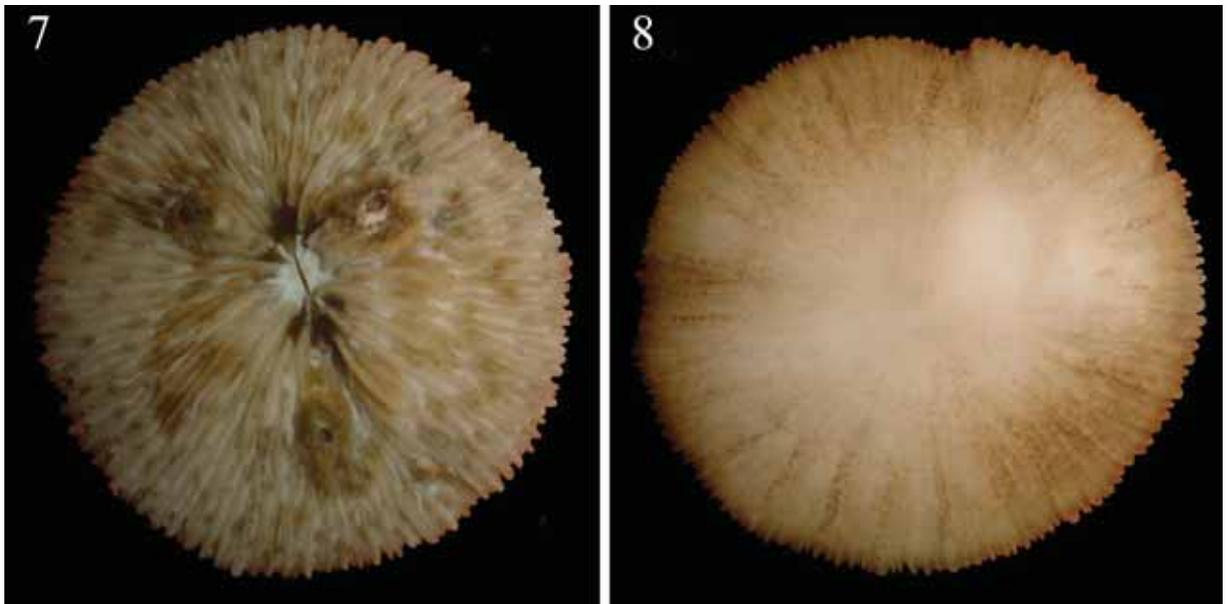
Figs. 7-8. Upper and lower surface of *Fungia* (*Cycloseris*) *tenuis*. Scale = 1:1.

Allozyme electrophoresis

Each coral was taken out of the aquarium. After that half a 1.5 ml test-tube of coral tissue mixed with small pieces of skeleton was rasped of the septae with a scalpel, and 0.050 ml of homogenizing buffer (0.01M Tris, 0.001 M NaEDTA, 0.01 M Maleic acid and 0.001 M MgCl2) was added. The mixture was ground with a micro-pestle and put on ice. The damaged specimens were digitally photographed on both sides and conserved in 96% alcohol, as reference material. To create a centrifuge, the blades of a small table-ventilator were removed and the tubes were stuck to the spindle with heavy duty tape. Each sample was centrifuged for 30 seconds at maximum speed. The supernatants were extracted with a 0.100 ml pipette and added to a new tube, which was centrifuged for 30 seconds and put on ice.

Occasionally the supernatant was too slimy (highly viscose and sticky) to be extracted into a 0.100 ml pipette point. In that case, the top of this pipette point was cut off with a scalpel to enable the extraction of the "slimy" supernatant into a new tube. An additional 0.050 ml homogenization buffer was added and everything was mixed by sucking it up and down into the cut pipette point. After centrifuging this mixture at maximum speed for 30 seconds, the supernatant could be extracted with a 0.100 ml pipette point. It was added to a new tube and put on ice.

The slots of a well-plate were filled with 0.010 ml supernatant each. An applicator was used to load and apply the supernatant to a cellulose acetate gel (Hillis *et al.*, 1987). Supernatants of all samples were run on a gel for 25 minutes and on an additional gel for 40 minutes. The electrophoresis was performed in a refrigerator at 4°C.

To test which allozyme systems in combination with which running buffers work best for Fungiidae, i.e. show polymorphic loci with a good resolution and activity, a preliminary study was done in the Netherlands. In total 10 allozyme systems were tested on two specimens of *Heliofungia actiniformis* (Quoy and Gaimard, 1833) and two specimens of *Fungia* (*Verrillofungia*) *repanda* Dana, 1846, viz. apartate aminotransferase (sAAT 2.6.1.1), alcohol dehydrogenase (ADH, 1.1.1.1), glucose dehydrogenase (GCDH, 1.1.1.118), glucose 6 phosphate dehy-

drogenase (G6PDH, 1.1.1.49), hexokinase (HK, 2.7.1.1), L-iditol dehydrogenase (IDDH, 1.1.1.14), isocitrate dehydrogenase (IDH, 1.1.1.42), malate dehydrogenase (MDH, 1.1.1.37), glucose-6-phosphate isomerase (GPI, 5.3.1.9), and phospho-glucomutase (PGM, 5.4.2.2). The nomenclature and IUBNC numbers are according to those of the standard of the International Union of Biochemistry (IUBNC, 1984). All allozyme systems were tested in combination with three buffers (Saccheri, 1995), i.e. TG (pH 8.5, 25mM Tris, 192 mM Glycine), TM (pH 7.8, 50 mM Tris, 20 mM Maleic acid) and P (pH 7.0, 11.6 mM Na2HPO4.2H2O, 8.4 mM NaH2PO4. H2O). The loci of the allozyme systems G6PDH, HK, MDH and GPI were polymorphic, with a good activity and a reasonable resolution using TM, TM, TG and P buffer respectively. The chemicals to test these allozyme systems were either bought in Indonesia or imported ice-packs from the Netherlands. These allozyme system-buffer combinations also gave good results during an additional test in Indonesia for specimens of *Herpolitha limax* (Esper, 1797), *Zoopilus echinatus* Dana, 1846, *Fungia* (*Danafungia*) *fralinae* Nemenzo, 1955, *F.* (*D.*) *scruposa* Kluzinger, 1879, *F.* (*Pleuractis*) *gravis* Nemenzo, 1955, *F.* (*Verrillofungia*) *repanda* Dana, 1846 and *F.* (*V.*) *scabra* Döderlein, 1901. A spider extract was used as a reference and positive control in all analyses. It showed a good activity and resolution for the allozyme system-buffer combinations described above. Unexpectedly, none of the specimens of morphs A, B or C showed any clear bands. Therefore, the allozyme system-buffer combinations were tested again for these corals. A good activity and reasonable resolution was only seen for the PGI allozyme system in combination with TG buffer. It showed one polymorphic and one homomorphic locus and was studied for the three morphs. The spider extract showed 4 bands with a high activity and good resolution and was used as a reference.

Data analyses

The resulting bands for each specimen were scored independently on two gels which had run for 20 and 40 minutes respectively. The bands that were scored twice were used for further analysis. The package of Swofford and Selander (1981), BIOSYS-1, was used to analyse the data. The exact probability test

was used to test for Hardy-Weinberg at the polymorphic locus.

Morphological investigations

All the corals used in the experiment were investigated and identified morphologically in the field and, independently, from the photographs.

Results

Morphology

Except for the morphological differences described in the introduction, two additional characters distinguishing between A and B on the one hand, and C on the other hand were noticed.

It took about 2 minutes per specimen of the morphs A and C to scrape off sufficient tissue mixed with septal skeleton pieces to fill half a 1.5 ml tube, while it only took about 15 seconds for each specimen of B, indicating that the skeletal structure of B was weaker. Furthermore, all specimens of only the morphs A and C had slimy mucous for which the protocol had to be adjusted (see "Material and methods section").

Allozyme electrophoresis

The frequencies of the 6 alleles found for the polymorphic locus of PGI are shown in table 1. The alleles A, D and F, accounting for 77% of all alleles scored for morph B, are not present in A and C. The
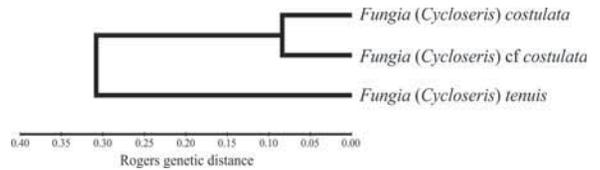

Fig. 9. UPGMA dendrogram based on Rogers' genetic distances for the three samples (cophenetic correlation = 0.99).

alleles B and C, accounting for respectively 100% and 90% of the alleles in the samples in morphs A and C, account for only 9% of the alleles in morph B.

None of the three samples A, B and C (respectively p = 0.57, 1.00 and 0.48) was significantly (α = 0.05) deviating from Hardy-Weinberg. However, when pooling the samples, the frequencies of the alleles were significantly (p = 0.02) deviating for the sample A+B+C, almost significantly (respectively p = 0.06 and p = 0.12) for A+B and B+C, and not (p = 0.35) for A+C. This indicates that the samples A, B, C and A+C can each be considered representatives of single demes, while the samples in which morph B was pooled with the morphs A and C cannot.

Nei's genetic distances (D), ranging between 0.02 and 0.32, and Rogers' genetic distances, ranging between 0.087 and 0.317, are shown in table 2. The dendrogram (fig. 9) resulting from an UPGMA on Rogers' genetic distances has a very high cophenetic correlation (0.99), indicating that it accurately reflects the pattern of genetic variation in the matrix of genetic distances (Sneath and Sokal, 1973). An UPGMA using Nei's genetic distances gave similar results.

Table 1. Frequencies of the alleles for the PGI allozyme system.

Sample Allele	*F. (C.) costulata*	*F. (C.)* **cf** *costulata*	*F. (C.) tenuis*
(N)	10	9	11
A	0.000	0.000	0.045
B	0.300	0.500	0.045
C	0.600	0.500	0.045
D	0.000	0.000	0.591
E	0.100	0.000	0.136
F	0.000	0.000	0.136

Table 2. Rogers' (below diagonal) and Nei's (above diagonal) genetic distances between the samples.

Sample	*F. (C.) costulata*	*F. (C.)* **cf** *costulata*	*F. (C.) tenuis*
F. (C.) costulata	0	0.02	0.30
F. (C.) **cf** *costulata*	0.09	0	0.32
F. (C.) tenuis	0.31	0.32	0

Discussion

The fact that the allozyme system buffer combinations that work best for *Fungia* (*Danafungia*) *fralinae*, *F.* (*D.*) *scruposa*, *F.* (*Pleuractis*) *gravis*, *F.* (*Verrillofungia*) *repanda* and *F.* (*V.*) *scabra* do not give any clear results for the *Fungia* (*Cycloseris*) specimens, could be an indication that the allozymes of the latter taxon differ considerably from those of the former taxa. Therefore it might be more appropriate to refer to *Cycloseris* as a genus. DNA-analyses of Fungiidae also support this view (Gittenberger *et al.*, 2006).

A significant deviation of Hardy Weinberg was found when the alleles of all the specimens were pooled. This indicates that they should not be considered representatives of a single panmictic population. No proof was found for a reproduction barrier between *F.* (*C.*) *costulata* offshore morphs and *F.* (*C.*) *costulata* nearshore morphs. Pooling their alleles, no deviation of Hardy Weinberg was found. They should therefore be referred to as phenotypes within *F.* (*C.*) *costulata*.

Nei's and Rogers' genetic distances and the resulting dendrogram (fig. 9) clearly show that there is very little to no gene flow between *F.* (*C.*) *tenuis* and *F.* (*C.*) *costulata* at Bone Baku reef. In total 77% of all alleles scored for *F.* (*C.*) *tenuis* were not present in the specimens of both forms of *F.* (*C.*) *costulata*, and vice versa 95% of the alleles scored for *F.* (*C.*) *costulata* were only accounting for 9% in *F.* (*C.*) *tenuis*. These results combined with the morphological differences, i.e. the roughness of the costae, the mouth coloration, skeletal strength and sliminess of the mucus, support the view that *Fungia* (*Cycloseris*) *costulata* Ortmann, 1889, and *F.* (*C.*) *tenuis* Dana, 1864, are two valid species; and that *F.* (*C.*) *costulata* has a nearshore ecomorph that may be related to low-salinity sea water.

Acknowledgements

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