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Discrimination of Anemonefish Species by PCR-RFLP Analysis of Mitochondrial Gene Fragments

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Abstract

A means of discriminating among species of clown anemonefishes, based on restriction enzyme analysis of partial mitochondrial DNA sequences, was investigated. Mitochondrial 16S rRNA and cytochrome *b* genes from 6 species (7 strains) of anemonefish (*Premnas biculeatus, Amphiprion polymnus, A. sandaracinos, A. perideraion, A. ocellaris, A. ocellaris var.* and *A. percula*) were PCR-amplified. A 623-bp portion of 16S rRNA gene was obtained from different fishes using the same pair of primers. Further investigation of this 16S rRNA fragment, by restriction endonuclease digestion with *Bfu*CI and *Rsa*I, was not able to distinguish all fishes studied, but did yield 3 different digestion patterns. The first was specific to *P. biculaetus*, the sole member of the genus *Premnas*, while the remaining two separated the *Amphiprion* species into 2 groups: 1) *A. polymnas*, *A. sandaracinos* and *A. perideraion*, and 2) *A. ocellaris*, *A. ocellaris var.* and *A. percula*. In contrast to this, restriction endonuclease digestion of a 786-bp fragment of the cytochrome *b* gene with *Hin*fI and *Rsa*I, was able to differentiate different 7 anemonefishes. This utility marker is valuable for unambiguous species/strain identification of juvenile anemonefishes.

Keywords: anemonefish, species identification, 16S rRNA, cytochrome *b*, PCR-RFLP

1. Introduction

Anemonefishes of the genus *Amphiprion* and *Premnas* (Perciformes: Pomacentridae: Amphiprioninae) are one of the most attractive marine ornamental fishes. Approximately 28 species have been recorded in the warm waters of Indian and Pacific Oceans, including Australia's Great Barrier Reef (Robertson, 1998). Typical classification and species identification of anemonefishes are based on morphological characteristics, such as tooth, shape, scalation of head and body proportions (Allen and Fautin, 1992). However, to distinguish anemonefishes in the field, colour pattern is the most important feature. Identification of newly hatched fish larvae to species level is often difficult, due to poorly defined morphological characteristics and great differing juvenile morphology in comparison to the adults (Fautin and Allen, 1997). In such cases molecular based taxonomy, particularly methods employing analysis of polymerase chains reaction (PCR) amplified DNA fragments, can provide an accurate alternative means of identification of individuals to genus, species or even strain level. This approach has been widely applied in the study of teleost fishes, because of the relative simplicity, specificity and sensitivity of the technique. Often sufficient diagnostic information can be obtained from analysis of PCR amplicons digested with restriction enzymes, generating potentially discriminatory restriction fragment length polymorphism (PCR-RFLP) markers. PCR-RFLP analysis is faster, more cost effective and more accessible than the alternative of sequencing each PCR amplicon.

Mitochondrial DNA (mtDNA) genetic markers have been widely used as a tool to distinguish within and among species (e.g. Patarnello *et al*., 1994; Chirstian *et al*., 2000; Klossa *et al*., 2002; Moyses and de Almeida, 2002; Aranishi *et al*., 2005, Hsieh *et al*., 2007; and references therein). MtDNA sequences are almost exclusively maternally inherited (Gyllensten *et al*., 1985) and the rate of evolution of the mtDNA genome is considered to be approximately ten times greater than that of the nuclear genome (Brown *et al*., 1979). In this study the use of PCR-RFLP analysis of two mitochondrial gene fragments to distinguish among six species (two genera) of anemonefish is investigated.

2. Materials and methods

2.1 Fish samples

Four different anemonefish species (5 strains) comprising *P. biaculeatus, A. sandaracinos, A. ocellaris, A. ocellaris var*., and *A. percula* were gifted from Percula Farm, Chonburi, Thailand. These

fishes were originally purchased from overseas and bred at the farm. An additional two species, *A. polymnus* and *A. perideraion*, were wild-caught of the Samaesan Islands, Chonburi, Thailand. Details of all fishes used in this study are listed in Table 1. Each species was represented by at least 3 fish. A small fin clip was obtained from each fish and preserved in 100% ethanol, which was stored at 4 °C until further analysis.

2.2 DNA extraction

Total genomic DNA was extracted from each sample using a 'PCR-ready' genomic DNA isolation method described by Meeker *et al*. (2007). In brief, tissue (about 2 mg) was placed into a microtube containing 100 µl of 50 mM NaOH and heated at 95°C for 30 min. After cooling to 4°C, one tenth volume of 1 M Tris-HCl, pH 8.0 was added. The sample was centrifuged at 12,000 rpm for 5 min. A 5-µl aliquot of supernatant was used in subsequent PCR reactions.

2.3 PCR primers and amplification

PCR of the 16S rRNA gene utilised universal primers, 16Sar-L (5'-CGCCTGTTTATCAAAAACAT) and 16Sar-H (5'-CCGGTCTGAACTCAGATCACGT), previously reported to generate a 16S rRNA gene fragment in various fish species (Palumbi *et al*., 1991). Specific cytochrome *b* gene primers, Apocyt_L2 (5'-GACCATAAACGATGCCGACT) and Apocyt_R2 (5'-GACCATAAACGATGCCGACT) were designed from a published sequence for saddleback clownfish (*A. polymnus*; GenBank accession number DQ343960) using primer3 program (Rozen and Skaletsky, 2000). These two primer pairs were used separately in PCRs with template genomic DNA from all fish species. Standard PCRs were performed in a total volume of 20 µl containing approximately 50 ng of template DNA, 10 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1x buffer plus Q-solution (Qiagen Inc., Germany), and 0.15 U of *Taq* polymerase (QiaGen Inc.). The PCR amplifications were carried out in a T-Personal Thermal Cycler (Biometra, Germany). For amplification of the 16S rRNA gene, the samples were denatured at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 56°C for 30 s, and 72°C for 40 s, with a final 10 min at 72°C on the last cycle. For amplification of the cytochrome *b* gene, the samples were denatured at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 50 s, with a final 10 min at 72°C.

Four microlitres of each PCR reaction were evaluated by electrophoresis through 1% SeaKem® LE Agarose (Cambrex Corp., USA) containing 0.5 µg ml^{-1} ethidium bromide in 0.5x Tris borate EDTA (TBE) buffer. The electrophoresis was performed at 50 V for 45 min and DNA bands were visualized and photographed under UV illumination.

2.4. RFLP pattern analysis

In order to produce potentially informative RFLP patterns an *in silico* restriction endonuclease (RE) analysis of published sequences (GenBank accession numbers AP006017, DQ343962 and AY208518) using Webcutter 2.0 software was undertaken. Restriction digestion of the PCR products of the 16S rRNA and cytochrome *b* genes were carried out individually in a 20-µl reaction mixture containing 1x enzyme buffer (New England Biolabs, Germany), 8 µl of unpurified PCR product and 5 units of each enzyme (New England Biolabs). The reaction was then incubated at 37 °C for 2 hrs. The entire reaction (20 µl) was separated through 2.0 % SeaKem® LE Agarose (Cambrex) and visualized as mentioned above. To verify the patterns obtained, all restriction analyses were performed at least twice. A double digestion with *Bfu*CI and *Rsa*I was selected for 16S rRNA amplicons, predicted to give eight fragments in *A. ocellaris* (16, 18, 59, 67, 87, 87, 105, 184 bp). A similar RE double digestion (*Hinf*I and *Rsa*I) was selected to fragment the cytochrome *b* amplicons, predicted to produce species specific patterns between *A. sandaracinos* (7, 11, 22, 42, 53, 108, 195, 286 bp) and *A. perideraion* (11, 22, 42, 53, 62, 161, 195, 233 bp).

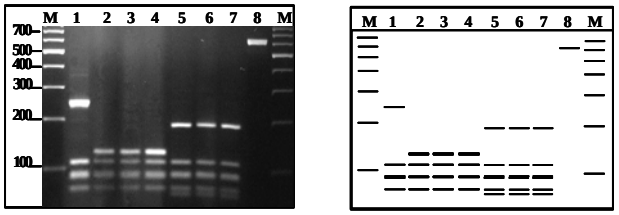
Table 1. Details of anemonefishes used in this study

Common name	*n*	Scientific name	Distribution (Allen, 1980)
Spine-cheek anemonefish	3	*Premnas biaculeatus* Bloch, 1790	Indo-Australian Archipelago
Saddleback anemonefish	5	*Amphiprion polymnus* Linnaeus, 1758	Indo-Australian Archipelago
Orange anemonefish	4	*A. sandaracinos* Allen, 1972	Northern Melanesia, Philippines, Taiwan, and Ryukyu Islands
Pink anemonefish	3	*A. perideraion* Bleeker, 1855	Western Pacific and Indo-Australian Archipelago
False percula clown anemonefish	3	*A. ocellaris* Cuvier, 1830	Eastern Indian Ocean, Indonesia, Malaysia, Philippines, and east coast of Asia to southern Japan
Black percula clown anemonefish	3	*A. ocellaris var*. Cuvier, 1853	Vicinity of Darwin, Australia
Percula clown anemonefish	3	*A. percula* Lacepéde, 1802	Melanesia and Queensland

3. Results and Discussion

Two gene regions of mitochondrial DNA from 7 anemonefishes were selected as potential species diagnostic markers. Analysis of the full length amplicons provided no discriminatory power, the size of the PCR products (c. 623 bp for 16S rRNA and c. 786 bp for cytochrome *b*) being indistinguishable among samples by the gel electrophoretic screening method employed [Fig.1(a) and (b)]. Therefore, further searching for appropriate restriction enzymes to differentiate the PCR products was carried out. Double digestion of 16S rRNA amplicons with *Bfu*CI+*Rsa*I produced three different RFLP patterns that were consistent among individuals (3-5) within each species/strain examined. Thus, all individuals from *A. ocellaris*, *A. ocellaris var.* and *A. percula* (Fig. 2; lanes 5, 6 and 7, respectively) had the same profile, *A. polymnus*, *A. sandaracinos* and *A. perideraion* (Fig. 2; lanes 2, 3 and 4, respectively) displayed a second profile, while the remaining pattern was specific to the genus *Premnas* (all *P. biaculaetus* samples; Fig. 2; lanes 1). PCR-RFLP analysis of the cytochrome *b* amplicons proved to be much more discriminatory with each species producing an apparently different diagnostic pattern (Fig. 3). Again, within each species/strain all individuals exhibited the same RFLP profile.

This preliminary analysis has identified two PCR-RFLP analyses that should prove useful for distinguishing among anemonefish at the genus and species level, and possibly even strains within species. It must be recognised, however, that the number of individuals examined per species was low. The extent of polymorphism within species is yet to be established, and if found to be present, may reduce the discriminatory power observed in this study. Any loss in discriminatory power may possibly be countered by additional RFLP assays with different restriction enzyme combinations.

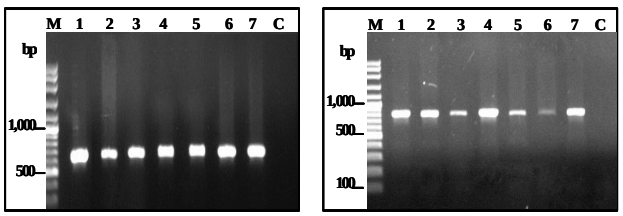


(a) (b)

Figure 2. PCR-RFLP patterns of a mitochondrial 16S rRNA gene fragment double digested with *Bfu*CI+*Rsa*I from different anemonefish species (a) and illustrated in a diagram (b). Lanes 1-7 are *Premnas biaculeatus, Amphiprion polymnus, A. sandaracinos, A. perideraion, A. ocellaris, A. ocellaris var.* and *A. percula*, respectively. Lanes 8 and M are undigested 16S rRNA amplified products and 100bp DNA marker, respectively.

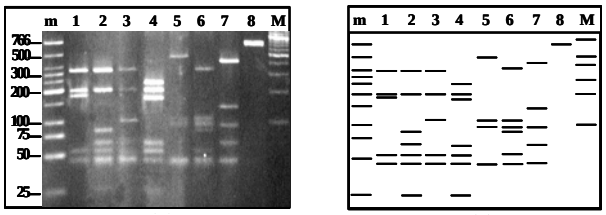
Based on 16S rRNA amplicon PCR-RFLP comparisons, the genus *Premnas* was clearly split from the genus *Amphiprion* fishes, in accordance with their morphological differences (Elliott *et al*., 1999). Within the *Amphiprion* species, the false percula clownfish (*A. ocellaris*) and the true percula clownfish (*A. percula*) shared an identical digestion profile. This suggests that they are closely-related species and, indeed, they can only be distinguished morphologically by the presence of diagnostic black vertical stripes presented in adult of *A. percula* (Fautin and Allen, 1997). The results of the present investigation agree with previous studies based on mtDNA analysis, where the true and false clownfishes were placed into the same Clade (Elliott *et al*., 1999; Santini and Polacco, 2006). The grouping of *A. polymnus*, *A. sandaracinos* and *A. perideraion* together, based on 16S rRNA PCR-RFLP screening also in agreement with the findings of Santini and Polacco (2006), which was based on the analysis of nucleotide sequences from three parts of the mitochondrial genome (D-loop segment, cytochrome *b* and 16S rRNA genes).

The RFLP patterns produced from *Hinf*I+*Rsa*I digestion of the cytochrome *b* amplicons proved to be more discriminatory. This may partly reflect the larger size of this amplicon (768 bp vs. 623 bp for 16S rRNA).



(a) (b)

Figure 1. PCR amplified products of mitochondrial 16S rRNA (a) and Cytochrome *b* (b) gene fragment from different anemonefish species. Lanes 1-7 are *Premnas biaculeatus, Amphiprion polymnus, A. sandaracinos, A. perideraion, A. ocellaris, A. ocellaris var.* and *A. percula*, respectively. Lanes C and M are negative (no-DNA) PCR-amplified control and 100bp DNA marker, respectively.



(a) (b)

Figure 3. PCR-RFLP patterns of a mitochondrial cytochrome *b* gene fragment double digested with *Hinf*I+*Rsa*I from different anemonefish species (a) and illustrated in a diagram (b). Lanes 1-7 are *Premnas biaculeatus, Amphiprion polymnus, A. sandaracinos, A. perideraion, A. ocellaris, A. ocellaris var.* and *A. percula*, respectively. Lane 8 is undigested cytochrome *b* amplified products. Lanes m and M are Low molecular weight and 100bp DNA markers, respectively.

The cytochrome *b* gene region is known to exhibit a relatively high mutation rate within the mitochondrial genome (Saccone *et al*., 2000) and this high level of polymorphism has been used to discriminate among closely related fish species (Lindstrom, 1999; Aranishi *et al*., 2005). The reliable and repeatable PCR-RFLP method as outlined above does not require costly nucleotide sequencing and all experimental analysis can be completed less than 4 hours from fish tissue sampling through to scoring RFLP profiles.

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