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SHORT COMMUNICATION

Random amplified polymorphic DNA (RAPD) fingerprinting resolves species ambiguity of domesticated clown fish (genus: *Amphiprion*, family: Pomacentridae) from India

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Clown fishes of the genus *Amphiprion* are a valuable resource from the point of view of the aquarium trade. There are about 27 species distributed widely in the Indo West Pacific region, extending from the eastern Indian Ocean to the Indonesian and Philippine archipelagos and from the coast of northwestern Australia to the Ryukyu Islands of Japan (Allen 1980; Fautin & Allen 1992). Allen (1991) used morphological similarities to designate six taxonomic complexes of closely related species. Apart from colour variation, tooth shape, scalation on the head and body proportions are used to identify the species of clown fish (Fautin & Allen 1992). The occurrence of multiple colour variants in most of the species, besides variations in colour patterns between juveniles and adults of the same species, makes unambiguous identification in clown fishes difficult.

Molecular techniques have become a major tool for systematic ichthyologists and may also be useful to fishery biologists for ratification of taxonomic problems at species and population levels (Chow, Clarke & Walsh 1993). Random amplified polymorphic DNA (RAPD) technique allows detection of DNA polymorphisms by randomly amplifying multiple regions of the genome by PCR using single arbitrary primers designed independent of target DNA sequence (Welsh & McClelland 1990; Williams, Kubelik, Livak, Rafalski & Tingey 1990; Hardys, Balick & Schierwater 1992). Random amplified polymorphic DNA markers have been used to investigate the taxonomic status of different groups of marine fishes such as striped bass (Bielawski & Pumo 1997), large mouth bass (Williams, Kazianis & Walter 1998), goatfishes (Mamuris, Stamatis, Bani & Triantaphyllidis 1999) and groupers (Govindaraju & Jayasankar 2004).

The domesticated species of clown fish at Vizhinjam Marine Aquarium on the southwest coast of India was identified as *Amphiprion chrysogaster* (Gopakumar, George & Jasmine 1999). However, the species, which was domesticated at Mandapam Marine Aquarium on the southeast coast of India, was identified as *A. sebae* (Ignatius, Rathore, Jagdis, Kandasami & Victor 2001). Since the stock of the latter was drawn from the former, the present study was carried out to ratify the species status of this important marine ornamental fish.

Caudal fin clippings were taken from eight individual clown fish each from Mandapam Marine Aquarium (65–117 mm total length) and Vizhinjam Marine Aquarium (38–108 mm total length). Liver tissues of 8 individuals of *A. chrysogaster*, collected from Mauritius waters, were obtained from Dr Joel Elliot (Biology Department, University of Puget Sound, Tacoma, WA, USA). Morphological identification of the clown fish was done based on the description of Fautin and Allen (1992). Original photographs and type specimens of domesticated clown fish species from Vizhinjam and Mandapam aquaria were sent to Dr Gerald Allen for confirmation of species identity.

Extraction of genomic DNA and RAPD amplification were done as per Govindaraju and Jayasankar (2004). Initially, a total of 15 primers from kits A and F of Operon Technologies (Alameda, CA, USA) were used to amplify the DNA. Based on repeatability and robustness of the loci generated by them, the amplification results of four primers, OPA 08 (5′ GTGACGTAGG 3′), OPA 10 (5′ GTGATCGCAG 3′), OPF 02 (5′ GAGGATCCCT 3′) and OPF 03 (5′ CCTGATCACC 3′) were the only ones analysed. The size of RAPD bands was determined by comparison with a λ DNA digested with *Eco*RI/*Hin*dIII molecular weight marker. For all the primers, presence (1) or absence (0), of a fragment was scored and RAPD patterns of individuals were compared within and between species. Bioprofil (Bio-1D, Vilber Lourmet, Cedex 1, France) was used to calculate the fragment sizes of the RAPD bands with reference to molecular size markers. The 'species-specific diagnostic' markers are defined in the present study as those RAPD bands which are exclusive to a species for a given primer.

The similarity index between all possible pairwise comparisons of individuals was calculated (Nei 1978) and the phylogenetic relationships between individuals and populations were constructed using cluster analysis. For this, the unweighted pair-group method with arithmetic (UPGMA) (Sneath & Sokal 1973) contained in the NEIGHBOR program of PHYLIP ver 3.57c (Department of Genome Science, University of Washington, Seattle, WA, USA), based on Nei's (1978) genetic distance values calculated for all primers, was used. Data resampling (1000 replicates) and matrix calculations for bootstrap analysis were performed using WinBoot, an UPGMA-based program (Yap & Nelson 1996).

In order to confirm the repeatability of the results, each tissue was amplified with each of the primers at least three times and considered the loci for further analysis only if they occurred in all the three amplifications. The four primers generated three to nine stable and clear loci in the size range of 100–3500 bp. Figure 1 shows the RAPD profiles of different individuals of domesticated species from India and Mauritius generated by two of the selected primers. The average GD (genetic distance) and SI (similarity index) values were calculated for all the four primers together. The SI value derived between Mandapam and Vizhinjam samples was 0.936, as expected for the same species, while that between them and Mauritius species was very low, 0.340.

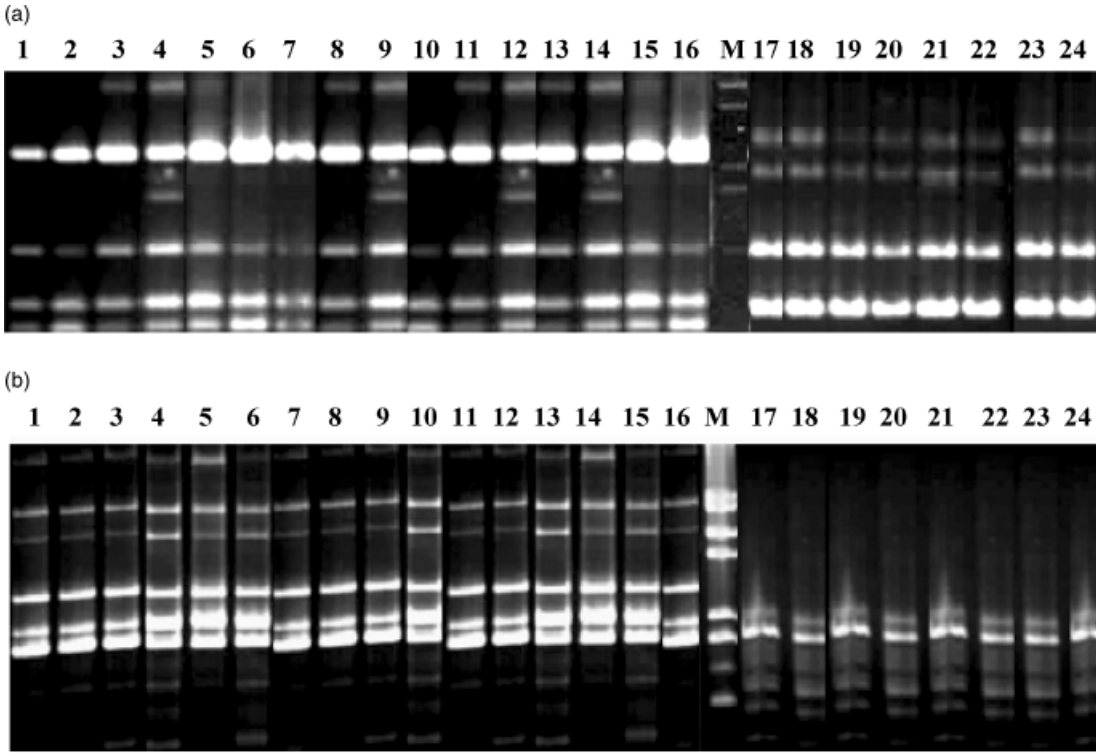

Figure 1 RAPD fingerprints of clown fish generated by Primer OPA 08 (a) and OPF 03 (b). Lanes 1–8, eight individuals of *Amphiprion sebae* from Mandapam marine aquarium; lanes 9–16, 8 individuals of *A. sebae* from Marine Aquarium of Vizhinjam; lanes 17–24, *A. chrysogaster* from Mauritius; lane M, DNA size marker, λ *Hin*dIII/*Eco*RI.

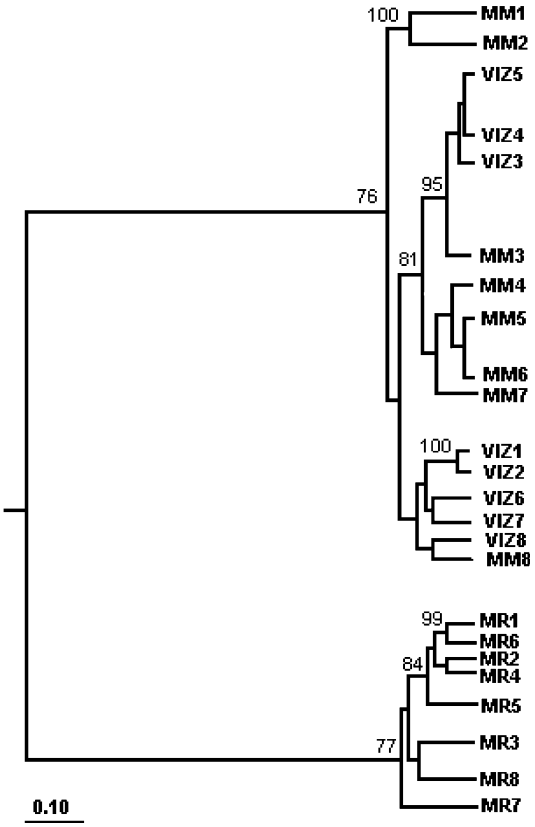

Figure 2 UPGMA dendrogram showing relationships among 24 individuals of two species of *Amphiprion*. Based on genetic distance values calculated by Nei (1978) from data for all primers. Bootstrap estimates (as percentage) are indicated above the branches. MM, Mandapam; VIZ, Vizhinjam; MR, Mauritius.

The similarity index between all possible pairwise comparisons of individuals from all primers was calculated and phylogenetic relationship between individuals of Mandapam, Vizhinjam and Mauritius samples was constructed using cluster analysis (UPGMA contained in PHYLIP 3.57c package). The results (Fig. 2) showed clustering of Mandapam and Vizhinjam samples together, clearly separated from Mauritius samples. Thus all the individuals of each species formed monophyletic species clusters. Some RAPD fragments have shown fixed frequencies and hence they can be used as species diagnostic markers in *A. sebae* and *A. chrysogaster* (Table 1). Both the species had at least one diagnostic marker from each primer, with some of the primers yielding more.

Jones and Kumaran (1980) have recorded *A. chrysogaster* from Minicoy waters. However, Gerald Allen, who is a global authority on pomacentrids, is of the opinion that *A. chrysogaster* is unlikely to be available in Indian waters but commonly occur in Mauritius waters (pers. comm.). Morphological (Fautin & Allen 1992) and RAPD fingerprinting (present study) have ratified the species status of the domesticated clown fish in the marine aquariums of Mandapam and Vizhinjam as *A. sebae*.

Table 1 Species diagnostic RAPD loci (in bp) in two species of clown fishes

Species	OPA 08	OPA 10	OPF 02	OPF 03
Amphiprion sebae	1650 and 500	2027	947	4973 and 4268
A. chrysogaster	1584	500	1375, 1000 and 900	1000

RAPD, random amplified polymorphic DNA.

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