Molecular Ecology Notes (2007) 7, 1169–1171 doi: 10.1111/j.1471-8286.2007.01818.x

PRIMER NOTE

Eight microsatellite loci in Clark's anemonefish, *Amphiprion clarkii*

SHANG-YIN VANSON LIU,* HON-TSEN YU† and CHANG-FENG DAI*
*Institute of Oceanography, National Taiwan University, Taipei, Taiwan 10617, †Graduate Institute of Zoology and Department of Life Science, National Taiwan University, Taipei, Taiwan 10617

Abstract

To determine the genetic relationship between different colour morphs (orange and black morphs) of Clark's anemonefish (*Amphiprion clarkii*) in Taiwan, we isolated eight polymorphic microsatellite loci. A large number of alleles (range, 6–30), and high levels of observed heterozygosity (range, 0.1231–0.8358) were resolved in 71 individuals from two populations, indicating that these markers should be useful in assessing the relationship between the two colour morphs of *A. clarkii*.

Keywords: *Amphiprion clarkii*, anemonefish, microsatellites

Received 21 January 2007; revision accepted 4 April 2007

The colouration pattern of tropical reef fish has often been used for taxonomic purposes. Nonetheless, the roles of sexual selection and colour patterns on genetic differentiation and speciation are not completely understood among reef fishes. Only a few studies have focused on the phylogenetic relationship between closely related species with different colour morphs (McMillan *et al*. 1999; Bernardi *et al*. 2002; Messemer *et al*. 2005). However, the results cannot still clarify the role of colouration in reef fish speciation. Among the Amphiprioninae, melanism has been reported in several species between different geographical locations or host sea anemones (Fautin & Allen 1992), especially Clark's anemonefish (*Amphiprion clarkii*) which is widely distributed in the Indo-Pacific and has a wide range of hosts (Allen 1972). Bell *et al*. (1982) used allozyme polymorphism to study the latitudinal colour variations of *A. clarkii* throughout Japan and the Ryukyu Archipelago. The results indicated that genetic clines were absent, and they concluded that this morphological variation might not be genetic. However, due to limited resolution, allozyme polymorphism might not be capable of resolving fine-scale differences, and highly polymorphic markers should be developed to clarify the relationship between different colour morphs of *A. clarkii* populations. Eight polymorphic microsatellite loci were developed from the *A. clarkii* genome to investigate the relationship between orange and black colour morphs of *A. clarkii* in Taiwan.

Correspondence: Chang-Feng Dai, Fax: +886-2-23916693; E-mail: corallab@ntu.edu.tw

Fish muscle (< 2 g) was treated with DNA extraction buffer (0.4 M NaCl and 200 mm EDTA; pH 8.0). Proteinase K (0.1 mg/mL) was added to the samples and incubated at 55 °C overnight. The samples were then centrifuged, and the supernatant was extracted with equal volumes of phenol:chloroform (1:1) twice. The final supernatant was precipitated with ethanol.

Genomic DNA (200 μg) was digested with *Sau*3AI (New England Biolabs) and fractioned in a 2.5% ultrapure agarose gel (Gibco). DNA in a size range of 300–900 bp was isolated, purified with GFX PCR DNA and a gel band purification kit (Amersham Pharmacia Biotech), and ligated into the plasmids, PUC18/*Bam*HI/BAP (Amersham), according to the manufacturer's protocols. Ligated plasmids were transformed into competent cells (ECOS-101, Yeastern Biotech). One thousand and six hundred recombinant clones containing inserts were transferred to Hybond-N + nylon membranes (Amersham), which were hybridized to a set of oligonucleotide probes, including $(AC)_{10}$ $(AT)_{10}$ $(AAG)_{10}$, and $(AG)_{10}$. Probes were labelled with a DIG Oligonucleotide Tailing Kit (Roche). Hybridization was performed at 55 °C for 24 h in standard hybridization buffer, consisting of 5 × SSC, 0.1% N-lauroylsarcosine, 0.2% SDS, and 1% blocking reagent (Boehringer Mannheim). The membranes were washed twice, each for 5 min at 45 °C with a solution of 2 × SSC and 0.1% SDS, and then twice, each for 15 min at 65 °C with a solution of 0.1 × SSC and 0.1% SDS. Chemiluminescent detection was performed with a DIG Luminescent Detection Kit for nucleic acids (Roche). Exposure times ranged from 20 to 30 min. In total,

Table 1 Eight polymorphic microsatellite loci isolated from *Amphiprion clarkii*, with primer sequences and characteristics of each locus (bp, base pair; T_a, annealing temperature). Repeated structures and lengths were derived from the sequence of the original cloned allele. Observed (H_O) and expected (H_E) heterozygosity of the Hardy–Weinberg equilibrium among populations from two localities in Taiwan ($n = 71$) were tested, *$P < 0.05$; **$P < 0.01$

Locus	Repeat	Primer sequences (5′–3′)	Size (bp)	T_a (°C)	No. of alleles	H_O	H_E	GenBank Accession no.
AC137	$(AC)_{19}$	F: GGTTGTTTAGGCCATGTGGT R: TTGAGACACACTGGCTCCT	273	57	29	0.8358	0.9496	EF141332
AC277	$(GT)_7GA(GT)_{11}$	F: TTTGCTCTTGTGGGGAGTTC R: TGTTCTCTAGTGGGGGTGGA	226	53	24	0.8254	0.8978	EF141333
AC522	$(AC)_{44}$	F: GCTTTCAATGGACCTGTGCT R: TGGCATAACTTCAGTTTGTTGC	203	55	29	0.8269	0.9501	EF141334
AC626	$(TC)_6(AC)_{20}$	F: CACACATGCACACACCTTGA R: TAATTGAGGCAGGTGGCTTC	230	59	21	0.7611	0.8635	EF141335
AC915	$(AC)_9$	F: TTGCTTTGGTGGAACATTTGC R: TCTGCCATTTCCTTTGTTC	227	51	7	0.1231**	0.4545	EF141336
AC1240	$(AC)_{17}$	F: TAACACAGCCCTGCACATTC R: ATTGGTGCGAGGACAACTCT	282	57	30	0.2157**	0.9264	EF141337
AC1359	$(AC)_9$	F: TGAAATACGATGCGGAAACA R: GAAGGCGAATACGTGGGATA	242	62	6	0.5080*	0.6660	EF141338
AC1578	$(AC)_9$	F: CAGCTCTGTGTGTGTTTAATGC R: CACCCAGCCACCATATTAAC	251	54	7	0.2881**	0.4552	EF141339

64 positive clones were sequenced using a DYEnamic ET Dye Terminator Kit (MegaBACE) (Amersham) following the manufacturer's protocols and analysed on linear polyacrylamide (LPA) gels with a MegaBACE 500 automated sequencer (Molecular Dynamics).

The online program, PRIMER 3.0 (http://www.genome.wi.mit.edu), was used to design primers from flanking regions of the microsatellite loci. Nineteen pairs of primers were designed, only eight of which could efficiently be amplified. Loci were assayed on populations of *A. clarkii* from two localities in Taiwan ($n = 71$). Polymerase chain reactions (PCR) of the eight loci were performed on an MJ Research gradient thermocycler with a gradient procedure (50–65 °C) to determine the best annealing temperature for each locus. The best annealing temperatures ranged from 51 to 62 °C among the eight loci (Table 1). PCR conditions were optimized as follows: 95 °C for 5 min, then 30 cycles of 45 s at 94 °C, 40 s at the annealing temperature (51–62 °C), and 45 s at 72 °C, with a final extension cycle for 1 min at 72 °C. Standard reaction conditions after optimization were 25 ng of each primer, 1.5–3 mM $MgCl_2$, 10 × PCR buffer, 2.5 nM dNTPs, 1 U of *Taq* DNA polymerase, and 2–5 ng DNA in a 25-μL reaction volume. Primers were labelled with fluorescent dye (TAMRA, FAM, and HEX), and the PCR products were visualized and sized by automated detection with gel electrophoresis using a MegaBACE 500 automated sequencer and analysed with GENETIC PROFILER 2.0 (Amersham). The number of alleles per locus ranged from six to 30 (Table 1). We used GENEPOP 3.1 (Raymond & Rousset 1995) to test for linkage disequilibrium (LD) and to estimate the expected and observed heterozygosities. No combination of the eight loci showed a significant linkage. The results of the Hardy–Weinberg test showed significant departure from expectations in four of the eight polymorphic loci (Table 1). The departure might have been due to the presence of null alleles or Wahlund's effect (Wahlund 1928). However, null alleles were unlikely since nonamplified samples were rare. Wahlund's effect might have been the cause because we pooled samples from two localities. A cross-species amplification test was also conducted. Four species of pomacentrids including *Amphiprion perideraion*, *Amphiprion frenatus*, *Pomacentrus moluccensis*, and *Stegastes faciolatus* were examined by PCR amplification at the best annealing temperature for the eight loci. However, none of the loci could be amplified in the other species. This indicates that the eight polymorphic loci are species-specific.

Acknowledgement

This study was supported by a grant (NSC95-2611-M002-011) from the National Science Council, Taiwan to C.-F. D.

References

Allen GR (1972) *The Anemonefishes: Their Classification and Biology*. TFH Publications, Neptune City, New Jersey.

Bell LJ, Moyer JT, Numachi (1982) Morphological and genetic variation in Japanese populations of the anemonefish *Amphiprion clarkii*. *Marine Biology*, **72**, 99–108.

Bernardi G, Holbrook SJ, Schmitt RJ, Crane NL, DeMartini E (2002) Species boundaries, populations and colour morphs in the coral reef three-spot damselfish (*Dascyllus trimaculatus*) species complex. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **269**, 599–605.

Fautin DG, Allen GR (1992) *Field Guide to Anemonefishes and their Host Sea Anemone*. Western Australian Museum, Perth, Australia.

McMillan WO, Weigt LA, Palumbi SR (1999) Color pattern evolution, assortative mating, and genetic differentiation in brightly colored butterflyfishes (Chaetodontidae). *Evolution*, **53**, 247–260.

Messemer V, Herwerden LV, Munday PL, Jones GP (2005) Phylogeography of color polymorphism in the coral reef fish *Pseudochromis fuscus*, from Papua New Guinea and the Great Barrier Reef. *Coral Reefs*, **24**, 392–402.

Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Wahlund S (1928) Zusammensetzung von Population und Korrelationserscheinung vom Standpunkt der Vererbungslehre aus betrachtet. *Hereditas*, **11**, 65–106.