Application of the RAPD technique in tilapia fish: species and subspecies identification

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Random Amplified Polymorphic DNA (RAPD) analysis was applied to three species of the tilapia genus *Oreochromis* and four subspecies of *O. niloticus*. Thirteen random 10-mer primers were used to assay polymorphisms within and between populations. Different RAPD fragment patterns were observed for different species, although not always for different subspecies. Evidence is presented that RAPD markers might be useful for systematic investigation at the level of species and subspecies.

Keywords: DNA, *Oreochromis*, polymorphism, RAPD, systematics, tilapia.

Introduction

Tilapia are cultured extensively throughout the world, especially in Africa and the Far East. Most of the important tilapiine species used in aquaculture are members of the *Oreochromis* genus, as many members of this taxon grow well under diverse culture conditions (Fryer & Iles, 1972; Pullin & Capili, 1989). Despite its commercial importance, resources have only recently been devoted to the development of improved strains of tilapia. Moreover few efforts have been made to assess the relative value of different methods for genetic characterization of tilapia germplasm.

The most recent classification of tilapiine species is based on reproduction, development, feeding, structural characteristics and biogeography (Trewavas, 1983). However, such characters are of limited value for identification purposes because they show considerable interpopulation variation and differences between species are small (Fryer & Iles, 1972; Abban, 1988). Protein electrophoresis has been extensively used to discriminate species of tilapia (Kornfield *at al.,* 1979; McAndrew & Majumdar, 1983, 1984; Abban, 1988; Sodsuk & McAndrew, 1991) and their hybrids (Macaranas *et al.,* 1986) but this technique could not discriminate subspecies of *O. niloticus* (Seyoum, 1989, cited in Seyoum & Kornfield, 1992; Seyoum, 1990). Mitochondrial DNA markers have been used success-fully to identify the subspecies of *O. niloticus* (Capili, 1990; Seyoum & Kornfield, 1992) but little effort has yet been devoted to the analysis of nuclear DNA for this purpose.

The development of random amplified polymorphic DNA (RAPD) markers, generated by the polymerase chain reaction (PCR), allows the examination of genomic variation without prior knowledge of DNA sequences (Williams *et al.,* 1990, 1993; Welsh & McClelland, 1990; Hadrys, 1992). The number and the size of amplified fragments depend on length and sequence of short, single and arbitrary primers. Priming sites are randomly distributed throughout a genome and polymorphisms in such sites result in differing amplification products, detected by the presence and absence of fragments. Such polymorph-isms are inherited in a Mendelian fashion and can be used as genetic markers. The method has been success-fully used to detect variation between strains of bacteria and rice (Welsh & McClelland, 1990), mice (Welsh *et al.,* 1991), *Gliricidia* (Chalmers *et al.,* 1992), closely related species of black Aspergilli (Megneg-neau *et al.,* 1993), and cocoa (Russell *et al.,* 1993) and between species of parasitic protozoa (Tibayrenc *et al.,* 1993).

This paper provides evidence that RAPD markers can be used to discriminate between three widely culti-vated species of tilapia and between several subspecies of *Oreochromis niloticus*, probably the species with greatest commercial importance. An assessment is also made of the utility of RAPD analysis for systematic analysis of tilapia.

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Materials and methods

Species and subspecies studied

The species and subspecies used in this study are held in aquaria at the University College of Swansea, Wales. Their sources are given in Table 1. *O. niloticus* (baobab) is a commercial strain of *O. niloticus* with possible hybrid ancestry. It is not recognized as a subspecies but is designated as one for convenience in this study.

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DNA extraction

DNA was prepared from fin tissue following the method described by Hillis & Moritz (1990) with some modifications. Approximately 50 mg of the caudal fin tissue was cut into small pieces and suspended in 500 μL STE (0.1 M NaCl, 0.05 M Tris and 0.01 M EDTA, pH 8). After adding 30 μL SDS (10 per cent) and 30 μL proteinase K (10 mg mL^{-1}), the mixture was incubated at 50°C for 30 min. DNA was purified by successive extraction with phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), respectively. DNA was precipitated with ice-cold absolute ethanol and washed with 70 per cent ethanol. The pellet was dried and resuspended in 150 μL TE (10 mM Tris-HCl, 1 mM Na$_2$EDTA.H$_2$O, pH 7.2).

Amplification conditions and electrophoresis

A set of 13 decamer primers from Operon Technologies was used in this study (Table 2). The amplification conditions were based on Williams *et al.* (1990) with some modifications. Amplification reactions were performed in 500 mM KCl, 100 mM Tris (pH 9.0 at 25°C), 1 per cent Triton X-100, 2.5 mM MgCl, 100 μM each of dATP, dTTP, dGTP and dCTP (Pharmacia), 5 pmoles of 10-base primer, 0.5 units of *Taq* DNA polymerase (Promega Biotec.), and 20 ng of genomic DNA in a final volume of 25 μL. The mixture was overlaid with the same volume of mineral oil. DNA amplification was performed in a thermal cycler (Hybaid, U.K.). For the first cycle, denaturation, annealing and extension were 94°C for 2 min, 35°C for 1 min and 72°C for 2 min, respectively. Denaturation time was decreased to 30 s for the following 44 cycles. One negative control (absence of template DNA) was performed for each set of amplifications.

Approximately 10 μL of amplification products were separated on 5 per cent vertical nondenaturing polyacrylamide gels in TBE buffer (0.89 M Tris, 0.89 M boric acid and 0.11 M EDTA, pH 8.3). Gels were fixed with 10 per cent ethanol and 0.5 per cent acetic acid solution twice for 3 min, stained with 0.1 per cent silver

Table 2 Sequence and operon codes of the random primers used to study variation in *Oreochromis species*

Primer codes	Sequence (5' to 3')
OPA 03	AGTCAGCCAC
OPA 04	AATCGGGCTG
OPA 05	AGGGGTCTTG
OPA 07	GAAACGGGTG
OPA 08	GTGACGTAGG
OPA 10	GTGATCGCAG
OPA 12	TCGGCGATAG
OPA 13	CAGCACCCAC
OPA 17	GACCGCTTGT
OPA 19	CAAACGTCGG
OPB 08	GTCCACACGG
OPC 02	GTGAGGCGTC
OPC 11	AAAGCTGCGG

Table 1 Species and subspecies used in this study and their source

Species and subspecies	Abbreviation	Source
Oreochromis aureus	Aur	Lake Manzala, Egypt
Oreochromis mossambicus	Mos	Aquarist stock
Oreochromis niloticus		
O. n. vulcani (vulcani)	Vul	Lake Vulcani, Kenya
O. n. baringoensis (baringo)	Bar	Lake Baringo, Kenya
O. n. niloticus (manzala)	Nil	Lake Manzala, Egypt
O. n. (baobab)	Bao	Baobab Fish Farm, Kenya

Common names of *O. niloticus* subspecies are given in brackets.
Nomenclature according to Trewavas (1983).
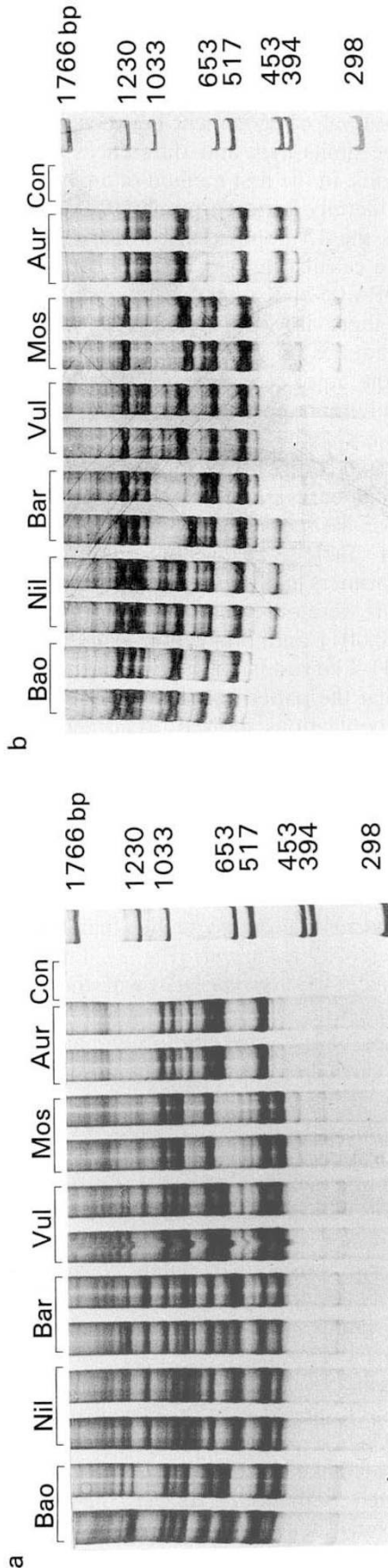
nitrate solution for 10 min, rinsed twice with distilled water, and then developed in an alkaline solution (1.5 per cent NaOH, 0.1 per cent $NaBH_4$ and 0.15 per cent CH_2O). A total of four individuals per species and sub-species (two males and two females) were analysed. Silver staining of polyacrylamide gels was found to be far superior to ethidium bromide staining of agarose gels in terms of the number of fragments that could be resolved clearly.

Analysis

The RAPD patterns of individuals were compared within and between populations. Fragments were scored as 1 if present or 0 if absent. The index of similarity between individuals was calculated using the formula:

$$S_{xy} = 2n_{xy}/(n_x + n_y),$$

where n_{xy} is the number of fragments shared by individuals x and y and n_x and n_y are the number of fragments scored for each individual (Lynch, 1990). Within population similarity (S) is calculated as the average of S_{xy} across all possible comparisons between individuals within a population.

Between population similarity, corrected for within population similarity, is:

$$S_{ij} = 1 + S'_{ij} - 0.5(S_i + S_j),$$

where S_i and S_j are the values of S for populations i and j, respectively and S'_{ij} is the average similarity between randomly paired individuals from populations i and j (Lynch, 1990). It is possible for the value of S_{ij} to exceed 1.

S'_{ij} was also converted to a measure of genetic distance (D_{ij}) using the equation:

$$D'_{ij} = -\ln[S'_{ij}/\sqrt(S_i S_j)], \text{(Lynch, 1991)}.$$

D'_{ij} values were used to construct dendrograms using the unweighted pair-group method of analysis (UPGMA) (Sneath & Sokal, 1973).

The statistics can be calculated for each primer separately or for the combined data for all primers.

Results

All the primers examined produced different RAPD fragment patterns (Fig. 1). The number of fragments generated per primer varied between six and 17. All primers gave species-specific RAPD patterns. Sub-

Fig. 1 RAPD patterns from each of six populations using (a) primer OPA 04 and (b) OPA 07. Samples (reading from the right hand lane): size markers; negative control (no template), *O. aureus, O.mossambicus, O. n. vulcani, O. n. baringoensis, O. n. niloticus, O. n.* (baobab).

Table 3 Estimated similarity (S_{ij}) for each primer between the species of *Oreochromis* and the subspecies of *O. niloticus*

Primers	Aur/Mos	Aur/Vul	Mos/Vul	Aur/Bar	Mos/Bar	Vul/Bar	Aur/Nil	Mos/Nil	Vul/Nil	Bar/Nil	Aur/Bao	Mos/Bao	Vul/Bao	Bar/Bao	Nil/Bao
OPA 03	0.666	0.665	0.891	0.732	0.832	0.952	0.800	0.842	0.862	0.942	0.664	0.873	1.006	0.958	0.906
OPA 04	0.727	0.833	0.909	0.800	0.714	0.800	0.857	0.615	0.615	0.941	0.825	0.941	1.000	0.804	0.711
OPA 05	0.444	0.546	0.716	0.505	0.697	0.812	0.500	0.666	0.686	0.886	0.537	0.627	0.957	0.815	0.900
OPA 07	0.900	0.900	0.857	0.848	0.848	0.905	0.860	0.812	0.812	0.886	0.952	0.857	0.909	0.905	0.866
OPA 08	0.531	0.222	0.578	0.256	0.575	0.881	0.459	0.588	0.569	0.816	0.486	0.972	0.752	0.762	0.684
OPA 10	0.703	0.815	0.733	0.745	0.800	0.753	0.570	0.615	0.645	0.833	0.780	0.836	0.878	0.893	0.834
OPA 12	0.821	0.772	0.785	0.798	0.820	1.002	0.729	0.772	0.862	0.962	0.718	0.757	1.005	0.971	0.923
OPA 13	0.727	0.615	0.666	0.615	0.666	1.000	0.615	0.666	0.666	1.000	0.615	0.615	1.000	1.000	1.000
OPA 14	0.461	0.333	0.666	0.461	0.750	0.933	0.382	0.704	0.704	0.967	0.437	0.745	0.967	0.908	0.969
OPA 19	0.615	0.783	0.672	0.808	0.616	0.977	0.851	0.641	0.667	0.990	0.733	0.833	0.855	0.825	1.071
OPB 08	0.913	0.691	0.590	0.853	0.784	0.792	0.813	0.785	0.792	1.000	0.828	0.784	0.792	1.000	1.000
OPC 02	0.888	0.903	0.947	0.838	0.872	0.930	0.843	0.881	0.896	0.934	0.881	0.916	0.879	0.882	0.953
OPC 11	0.727	0.739	0.739	0.740	0.740	0.853	0.800	0.800	0.812	0.913	0.783	0.696	0.881	0.867	0.868
Mean	0.702	0.678	0.750	0.692	0.747	0.891	0.698	0.722	0.737	0.928	0.711	0.804	0.914	0.891	0.899
SD	0.157	0.207	0.120	0.182	0.091	0.083	0.171	0.098	0.106	0.059	0.156	0.113	0.084	0.076	0.110

species-specific patterns were obtained for some but not all primers. Values of similarity (S_{ij}) between populations for each primer are given in Table 3. Two analyses were carried out on the data in Table 3 to assess the level of agreement between primers in the pattern of similarities and differences in population comparisons. In the first method of analysis, the correlation in identity between primers OPA 03 and OPA 04 across the 15 population comparisons was calculated. The calculation was repeated for primers OPA 03 and OPA 05 and so on for all possible primer pairs of which there are 78 in total. Nineteen values were significant at the 1 per cent level and a further 12 significant at the 5 per cent level. All the significant values were positive and the average correlation was 0.482. In a factor analysis on the matrix of identity values, the first principal component explained 45 per cent of the variance in identity. In the second method of analysis a two-way analysis of variance was carried out on the values of Table 3 using the two-way interaction between primers and population pairs as error. Significant effects were observed both for primers ($F_{12,168} = 9.9$, $P < 0.001$) and population pairs ($F_{14,168} = 11.4$, $P < 0.001$). The results of the two methods of analysis suggest that the pattern of similarities and differences between populations showed broad agreement across primers but that the overall level of similarity varied between primers.

The highest value of within-population similarity (S) was obtained for *O. mossambicus*, the lowest for *O. n.* (baobab) (Fig. 2). The similarities between the subspecies of *O. niloticus* (except between *O. n. vulcani* and *O. n. niloticus*) are clearly higher than those between the species of *Oreochromis* (Fig. 3). The dendrogram (Fig. 4) links *O. n. mossambicus* with the subspecies of *O. niloticus*, with *O. aureus* as outgroup. To assess the significance of this grouping a compatibility analysis has been carried out by constructing separate dendrograms for each of the 13 primers. Because there were four *O. niloticus* subspecies, there were $4 \times 13 = 52$ possible dendrograms involving *O. aureus*, *O. mossambicus* and one of the *O. niloticus* subspecies. Of these, the majority (a total of 25) had *O. aureus*, rather than another species, as outgroup. If the expectation that the three species are equally likely to be outgroup is assumed as the null hypothesis, the expected number of dendrograms with *O. aureus* as outgroup is $52/3 = 17.33$ and $\chi_1^2 = 5.088$ ($P < 0.05$) and the null hypothesis can be rejected. The favoured outgroup is thus *O. aureus*. Of the three subspecies (disregarding *O. n.* (baobab)), *O. n. baringoensis* and *O. n. niloticus* are more closely linked with *O. n. vulcani* as outgroup (Fig. 4). Applying compatibility analysis, the number of individual primer dendrograms with *O. n. baringoensis*,

Fig. 2 Average similarity (S) across primers within species of *Oreochromis* and subspecies of *O. niloticus* with 95 per cent confidence intervals.
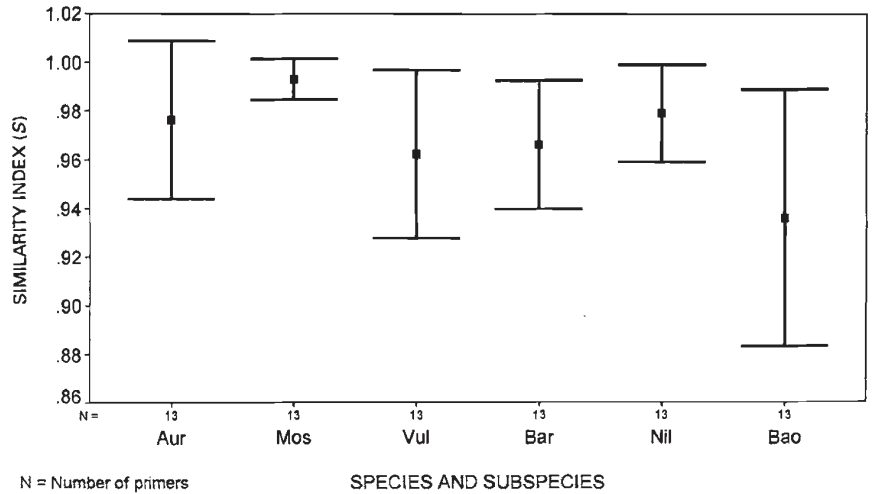
N = Number of primers

Fig. 3 Similarity (S_{ij}) across primers between species of *Oreochromis* and subspecies of *O. niloticus* with 95 per cent confidence intervals.
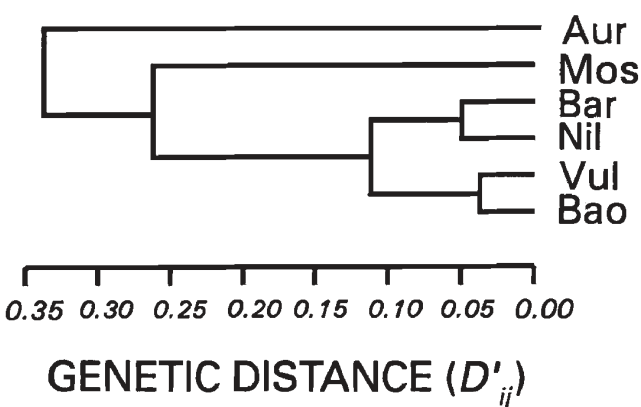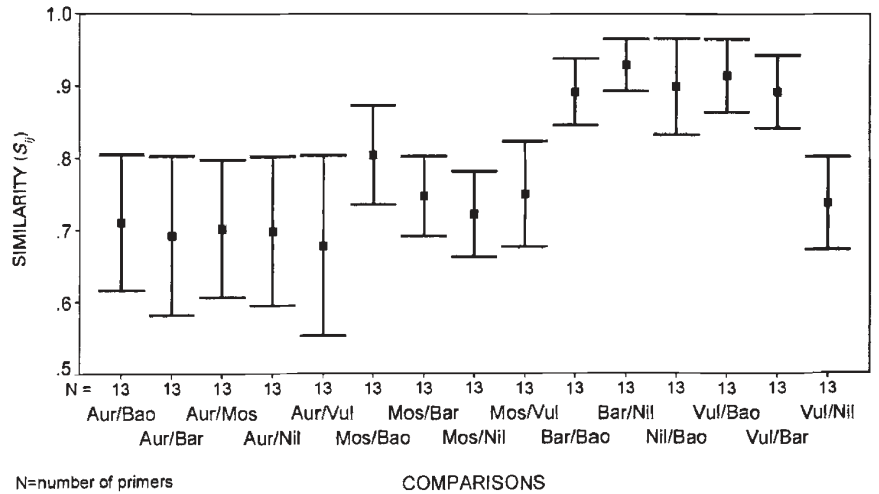
N=number of primers

GENETIC DISTANCE (D'_{ij})

Fig. 4 UPGMA dendrogram of the species of *Oreochromis* and the subspecies of *O. niloticus* based on values of genetic distance (D'_{ij}) calculated from data for all primers.

O. n. vulcani and *O. n. niloticus* as outgroup are zero, eight and five, respectively. Under the null hypothesis that the three dendrograms should be equally frequent, $\chi_2^2 = 7.544$ $(P < 0.05)$. Thus the hypothesis that *O. n. baringoensis* is the outgroup can be rejected. *O. n. vulcani* is favoured as the outgroup since it occurs in the largest number of individual primer dendrograms.

Discussion

In common with other molecular techniques, RAPD analysis has been successful in identifying markers which distinguish tilapia species, although individual primers differ significantly in the amount of inter-population variation they detect. RAPD analysis has

also identified DNA markers which can distinguish subspecies of *Oreochromis niloticus* and in this respect has been more successful than allozyme analysis (Seyoum, 1989, cited in Seyoum & Kornfield, 1992; Seyoum, 1990). Mitochondrial DNA analysis has also revealed differences between subspecies of *O. niloticus* (Seyoum & Kornfield, 1992) but might have some disadvantages in studies of population differentiation compared with nuclear markers. This is because of evidence that mtDNA gene flow can occur across hybrid zones, in the absence of nuclear gene flow, in some animals (Ferris *et al.*, 1983; Tegelstrom, 1987). The tilapia species studied here hybridize readily in captivity, therefore the opportunity for mtDNA introgression is great.

Intrapopulation RAPD variation was detected with all primers. This suggests that RAPD analysis might be more sensitive than mtDNA analysis which has failed to reveal variation within tilapia populations (Capili, 1990; Seyoum & Kornfield, 1992). In the present study, levels of intrapopulation variation are expected to be low because most of the subspecies used were founded, and have been propagated, from relatively small numbers of individuals. The population of *O. mossambicus* is descended from a single sib-mating and has the lowest level of variation.

In the widely accepted classification of tilapia of Trewavas (1983), *O. aureus* and *O. niloticus* are placed in a different subgenus from *O. mossambicus*. The allozyme studies of McAndrew & Majumdar (1984) and Sodsuk & McAndrew (1991), favoured either this classification or the closer linking of *O. mossambicus* with *O. aureus*. The RAPD results are at variance with these studies in suggesting a closer relationship between *O. mossambicus* and *O. niloticus*. In the mtDNA study of Seyoum & Kornfield (1992), *O. n. niloticus* was more similar to *O. n. vulcani* than to *O. n. baringoensis*. The compatibility analysis gives least support of all to this hypothesis. The evidence of highly significant differences between population pair similarity values provides support for the reliability of the technique as a tool for detecting genetic differences between populations.

RAPD analysis has been used for constructing trees in other organisms (Chalmers *et al.*, 1992; Kaemmer *et al.*, 1992; Megnegneau *et al.*, 1993; Tibayrenc *et al.*, 1993). We have observed that small alterations in PCR parameters or quality of target DNA can radically alter RAPD patterns (see also, Devos & Gale, 1992; Williams *et al.*, 1993). Thus there may be reason to view with caution systematic conclusions based on RAPD analysis alone. On the other hand, the possibility of carrying out compatibility analysis with unlimited numbers of primers, each detecting variation

at several regions in the genome, provides an advantage over other techniques. Even if some primers amplify identical regions of the genome or if the technique itself is noisy, it should be possible to build up quickly a consensus from patterns of interpopulation variation.

Acknowledgements

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