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Development of polymorphic microsatellite DNA loci for characterizing *Oreochromis shiranus* subspecies in Malawi

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Summary

Six sets of microsatellite primers were developed from *Oreochromis shiranus* nuclear DNA. These cross-primed in species of the genera *Sarotherodon* and *Tilapia*, and were polymorphic in most of the species. The total number of alleles ranged from 18 to 30 per locus; the mean heterozygosity per population ranged from 0.51 ± 0.12 to 0.82 ± 0.03, which is higher than reported in allozyme studies. Five sets of primers were used to characterize five putative populations of *O. shiranus*, an indigenous mouth brooding tilapia which has been widely distributed in fish farms and reservoirs in Malawi. Two postulated subspecies of *O. shiranus* (subspecies *shiranus* and subspecies *chilwae*) have been difficult to distinguish morphologically. Lake Chilwa, Lake Chiuta and Bunda Reservoir populations cluster together as *O. sh. chilwae*, while the Lake Malombe and Bishop Reservoir populations form a second cluster as *O. sh. shiranus*. The assignment of the Chiuta and Chilwa populations of *O. sh. chilwae* to a single subspecies is consistent with the fact that the two lakes were a single open lake until about 8000–9000 BP. There is no connection between them and the Lake Malawi–Malombe drainage system where the *O. sh. shiranus* subspecies is found.

Introduction

The distribution of the several indigenous species of tilapias found in Malawi has been outlined by Msiska (1988). The genus *Tilapia* is represented in Malawi by *T. rendalli* (Boulenger); the subgenus *Oreochromis Oreochromis* comprises *O. O. shiranus* [subdivided into *O. O. sh. shiranus* (Boulenger) and *O. O. sh. chilwae* (Trewavas)], *O. O. mossambicus* (Peters) and *O. O. placidus* (Trewavas). The *O. Nyasalapia* subgenus is composed of *O. Ny. karongae* (Trewavas), *O. Ny. lidole* (Trewavas), *O. Ny. squamipinnis* (Gunther) and *O. Ny. saka* (Trewavas). All of these *Nyasalapia* species are endemic to lakes Malawi and Malombe (Fig. 1). Contrary to the species distinctions listed above, multivariate morphometric analysis suggests that there are only three species of *O. Nyasalapia* (Turner and Robinson 1990). Cultivation of *O. shiranus* is widespread in Malawi, where the government has banned the importation of exotic species. The two subspecies are believed to have evolved about 9000 years BP (Furse et al. 1979) from ancestral *O. shiranus*, which existed in the Rovuma River into which the ancient Chilwa–Chiuta lakes drained via the Lugenda River (Fig. 1) into the Indian Ocean. Together with *O. mossambicus* and *O. placidus* they belong to the *mossambicus* group of tilapias (Fryer and Iles 1972). Trewavas (1983) reports that *O. sh. shiranus* is found in Lake Malawi and its tributary rivers, streams, lagoons and the upper Shire River including Lake Malombe, while *O. sh. chilwae* is confined to Lake Chilwa (Fig. 1). To date it has not been possible to ascribe the Lake Chiuta population with certainty to either of the subspecies because of its intermediate morphological characteristics. Questions have therefore been raised as to whether the differences between the two subspecies are genetically fixed. For instance, Trewavas (1983) doubts as to whether the Lake Chilwa population is genetically different from the Lake Chiuta population because there was an oppor-

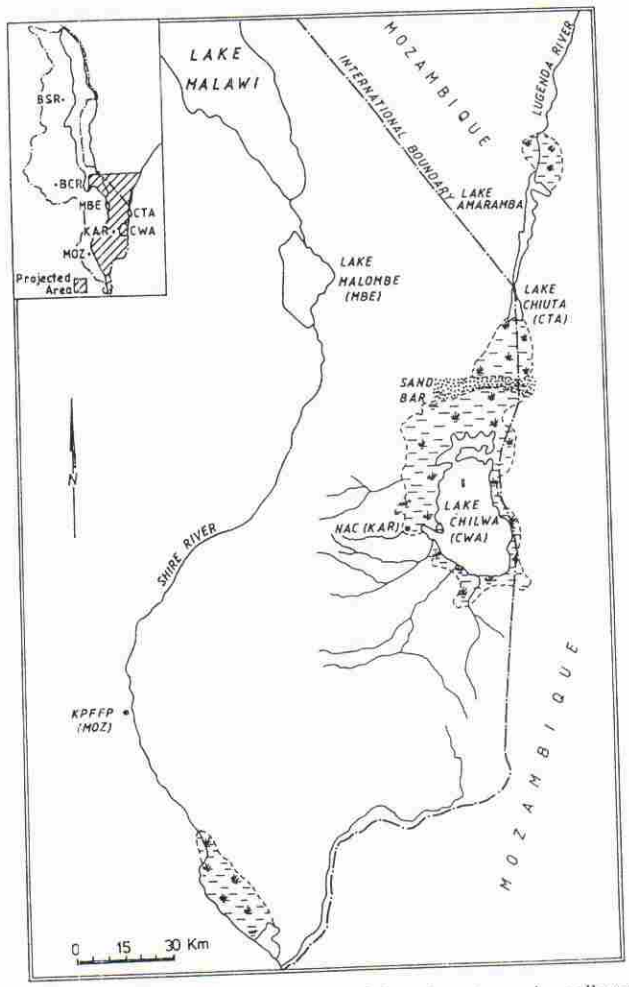

Fig. 1. Lakes and sampling locations of *Oreochromis* species collected in Malawi. The population abbreviations (in brackets) are defined in Table 1

tunity for the two populations to have diverged from a common ancestor.

Unlike the *O. Nyasalapia* subgenus where morphological, biochemical and molecular studies recently differentiated the species (Turner and Robinson 1990; Sodsuk et al. 1995), there have been no studies of *O. shiranus* to address the phylogenetic questions at the subspecies level or the questions that Trewavas (1983) raised in her monograph. The Malawian government and aquaculture industry are currently designing genetic management procedures for the various species and subspecies populations that are being domesticated. The consequences of the taxonomic confusion are that strains having desirable production characteristics cannot be ascribed with certainty to any subspecies. Accidental hybridization due to the mixing of species and subspecies is occurring and may in the future destroy the genetic differentiation in both wild and domestic populations (Ambali 1996).

The most common biochemical technique that has been used to identify and characterize tilapias is allozyme electrophoresis (see Gregg et al. (1998) and references therein). Some of these applications have successfully discriminated among species despite the low resolution and sensitivity, especially at the subspecies level (Cruz et al. 1982; Seyoum 1990). Microsatellites or simple sequence repeats consist of repeated 1–6 bp nucleotide motifs occurring with an average spacing of 7–10 kbp in the eukaryotic genome (Tautz 1989; Wright 1993). They have become popular markers because they are abundant in the eukaryotic genome, they show a high level of allelic variability, they are codominant, and only minute quantities of tissue are required for their analysis (Wright and Bentzen 1994). All of these attributes have made microsatellites become markers of choice in population genetics research. We have developed six microsatellite primers for tilapias based on *O. shiranus* DNA; in the present study they were tested for cross-priming in other species and the amplified loci were used to determine the genetic relationships of putative *O. shiranus* subspecies in Malawi.

Materials and methods

A summary of the sample sources for the *Oreochromis* species and the number of individuals analysed is provided in Table 1 and Fig. 1. Blood samples of *O. shiranus* were collected from the Chilwa, Chiuta and Malombe lakes and the Bunda and Bishop reservoirs. In addition to *O. shiranus*, other tilapia species were analysed to determine the cross-priming range of the primers developed in this study. *Oreochromis karongae* was collected from Lake Malawi and identified while in ponds at the National Aquaculture Centre (G. F. Turner, personal communication). *Oreochromis mossambicus* was collected from broodstock obtained from the lower course of the Shire River. *Oreochromis niloticus* samples were the Chitralada strain from Thailand. Samples of *T. rendalli* were collected from Malawi, while those of *O. placidus, O. aureus, O. hornorum, Sarotherodon galilaeus* and *T. zilli* were obtained from blood samples of a few representative individuals from various sources collected by the Marine Gene Probe Laboratory. Approximately 0.3–0.5 mL of blood was drawn along the lateral line of the fish using a syringe rinsed with 0.5 M ethylene diamine tetraacetic acid (EDTA) and preserved in 0.5 mL of 100% ethanol. The DNA was extracted using the phenol extraction procedure (Brooker et al. 1994). DNA analysis was carried out using the procedure outlined in Ruzzante et al. (1996).

Table 1
Sources of samples analysed in this study

Species	Code	n	Source	Country
O. sh. chilwae	CWA	53	Lake Chilwa	Malawi
O. shiranus sp.	CTA	50	Lake Chiuta	Malawi
O. sh. shiranus	MBE	50	Lake Malombe	Malawi
O. shiranus sp.	BCR	50	Bunda College Reservoir	Malawi
O. shiranus sp.	BSR	50	Bishop's Residence Reservoir	Malawi
O. mossambicus	MOZ	40	Shire River	Malawi
O. karongae	KAR	40	NAC*	Malawi
O. niloticus	NIL	30	?	Thailand

*NAC, National Aquaculture Center

The BIOSYS-1 computer program (Swofford and Selander 1989) was used to estimate the mean number of alleles and heterozygosity per species samples. Because the statistical distribution of alleles and heterozygosity was not known, we used mean $\pm$ 2 standard errors (SE) as the approximation of 95% confidence intervals in order to determine whether the mean number of alleles and heterozygosity were statistically different between populations.

Analysis of population differentiation and genetic relatedness was carried out on five populations of *O. shiranus* sp. The DIPLOIDL program in GENEPOP (version 1.2; Raymond and Rousset 1995) was used to calculate pairwise population F_{ST} based on Weir and Cockerham (1984). The genetic distance between populations based on the proportion of shared alleles was estimated using the MICROSAT.C program (Minch et al. 1996). Patterns of genetic relatedness between subspecies populations were examined using cluster and ordination analyses in the NTSYS-PC program (version 1.80; Rohlf 1992). A phylogenetic tree was constructed using neighbour joining in the SAHN program. The goodness of fit of the cluster analysis was determined by computing the cophenetic correlation between the cophenetic value matrix and the original distance matrix using the MXCOMP program (Rohlf 1992). Nonhierarchical patterns among the samples were analysed using principal components analysis following the arcsine transformation of the allele frequencies to determine the clusters in plots of the first two components (Rohlf 1992; Phelps et al. 1994; Sokal and Rohlf 1994). Mantel's test was carried out to assess the goodness of fit of the principal components (Rohlf 1992).

Results

Microsatellite sequences

Sequences for six polymorphic dinucleotide microsatellites obtained from putative clones are presented in Table 2. According to the classification of Weber (1990), five of the repeat sequences were perfect, while one was compound. The GT/CA repeat sequence length ranged from eight to 33 repeats, while the linked GA repeat sequence was seven repeats long. Primer sequences designed from the regions flanking the tandem repeats are also presented in Table 2.

Cross-priming among species

The six *O. shiranus* primers were cross-primed with DNA from other tilapia species of three genera, namely, *Oreochromis, Sarotherodon* and *Tilapia* (Table 3). All loci were polymorphic in the *Oreochromis* species available in Malawi, namely *O. shiranus, O. mossambicus, O. karongae* and *O. placidus*. Polymorphism was also observed in other *Oreochromis* species not available in Malawi (*O. aureus, O. hornorum* and *O. niloticus*),

Table 2
Microsatellite sequences, primers designed from flanking regions and optimum annealing temperatures (T_{ann})

Locus	Cloned microsatellite	Forward primer sequence	Reverse primer sequence	T_{ann}
Os-7	$(TG)_{12}$	5′-TGTCTGCTGCCTCGGCCTG-3′	5′-ACTGTGCCGCATCGCCAG-3′	58
Os-7R	$(GT)_{33}$	5′-AGAGGAAATGAGCAGCCTC-3′	5′-GATGCGGCAACAGTTATGTC-3′	54
Os-25	$(CA)_{16}$	5′-TTGTGAAATTGCATTGCACTC-3′	5′-AACTCCCTTTGATCCTCTGC-3′	53
Os-64	$(TG)_{12}(GA)_7$	5′-CAGTGTCTTCAGTTCCTTGC-3′	5′-CAGAAGCATCTTATTGATGAC-3′	54
Os-74	$(CA)_{32}$	5′-GAACCGATTTAACTAGAACC-3′	5′-GAGTGCTTTGAACTAGTGC-3′	49
Os-75	$(TG)_{31}$	5′-AGCCTAAAATAATGGAATCAC-3′	5′-CCACAGAGTCATGGTTCAC-3′	49

Table 3
Locus variability in the various species of tilapia

Species	Os-7	Os-7R	Os-25	Os-64	Os-74	Os-75
O. aureus	+	+	+	+	+	–
O. hornorum	+	+	+	+	+	+
O. karongae	+	+	+	+	+	+
O. mossambicus	+	+	+	+	+	+
O. niloticus	+	+	+	+	+	+
O. placidus	+	+	+	+	+	+
O. shiranus	+	+	+	+	+	+
S. galilaeus	–	–	+	–	NP	+
T. rendalli	+	+	+	+	NP	NP
T. zilli	+	+	+	+	+	–

+, polymorphic; –, monomorphic; NP, no product.

with the exception of *O. aureus* where locus Os-75 was monomorphic. *Sarotherodon galilaeus* showed polymorphism at loci Os-25 and Os-75 and monomorphism at the other four loci. Both substrate brooders, *T. rendalli* and *T. zilli,* were polymorphic at four loci. While *T. zilli* was polymorphic at locus Os-74 and monomorphic at locus Os-75, there was no visible product at either locus in *T. rendalli.*

Variability

The six sets of primers varied considerably in the quality of amplified product; primer Os-74 did not produce sharp bands and was not used in this analysis. The level of variability is presented in Table 4. All loci were highly polymorphic in all populations with the mean number of alleles ranging from 5.6 ± 1.1 to 13.2 ± 3.2 and the mean heterozygosity ranging from 0.51 ± 0.12 to 0.82 ± 0.04. The mean heterozygosity was not significantly different between populations at the 95% confidence interval.

Genetic relationship and population differentiation

The proportions of shared allele distance measures are presented in Table 5; the resultant dendogram constructed using the neighbour-joining method is presented in Fig. 2(A). There are two major clusters: CWA, BCR and CTA populations cluster together representing *O. sh. chilwae*; and MBE and BSR representing *O. sh. shiranus*. The cophenetic correlation was 0.89, considered a good fit (Rohlf 1992). The fact that the two closest wild populations from the lake are CWA and CTA confirms the appropriateness of a single subspecific ranking for the populations from lakes Chiuta and Chilwa (Trewavas 1983).

A plot of principal components is presented in Fig. 2(B). As in the neighbour-joining dendogram, the two samples nearest each other were the populations of *O. sh. chilwae*. The separation of the samples on the first principal component (37.1% of variance) is very similar to the neighbour-joining dendogram of the proportion of shared allele distance and confirms the conventional taxonomy. The correlation between the matrix of distance among all pairs of points and the matrix of genetic distance among species was 0.88, considered a good fit (Rohlf 1992).

The level of interpopulation differentiation as measured by F_{ST} is presented in Table 5 (above diagonal). The pairwise values show a similar pattern to that of genetic distance where F_{ST} values were low in populations belonging to the same subspecies, as indicated by the dendogram.

Discussion

Cross-priming

The cross-priming of primers has been reported in several other taxa; for example, cattle (*Bos taurus*) primers amplify microsatellite loci in goats (*Capra hircus*; Pepin et al. 1995), brown trout (*Salmo trutta*) primers amplify in rainbow trout (*Oncorhynchus mykiss*; Estoup et al. 1993), Atlantic cod (*Gadus*

Table 4
Number of alleles at a locus (total), mean ± standard error number of alleles (A ± SE) and observed heterozygosity (Het ± SE)

Population	Os-7	Os-25	Os-7R	Os-64	Os-75	a ± SE	Het ± Se
CWA	5	6	14	4	15	8.8 ± 2.2	0.51 ± 0.12
CTA	6	16	21	5	18	13.2 ± 3.2	0.68 ± 0.09
MBE	6	11	22	6	17	12.4 ± 3.1	0.67 ± 0.06
BCR	8	10	14	6	14	10.2 ± 2.1	0.68 ± 0.01
BSR	8	10	13	4	11	9.6 ± 1.6	0.71 ± 0.03
MOZ	8	3	6	3	8	5.6 ± 1.1	0.54 ± 0.10
KAR	10	8	17	6	10	10.2 ± 1.9	0.62 ± 0.07
NIL	7	6	7	5	8	6.8 ± 0.5	0.82 ± 0.04
Total	18	19	30	16	30		

Table 5
Mean ± standard error (SE) proportion of shared alleles (below diagonal) and pairwise F_{ST} values of *Oreochromis shiranus* populations (above diagonal)

	CWA	CTA	MBE	BCR	BSR
CWA		0.081	0.282	0.077	0.230
CTA	0.559 ± 0.098		0.156	0.041	0.113
MBE	1.639 ± 0.253	1.166 ± 0.199		0.159	0.061
BCR	0.488 ± 0.112	0.494 ± 0.108	1.218 ± 0.250		0.110
BSR	1.240 ± 0.243	1.004 ± 0.147	0.637 ± 0.108	1.002 ± 0.115	

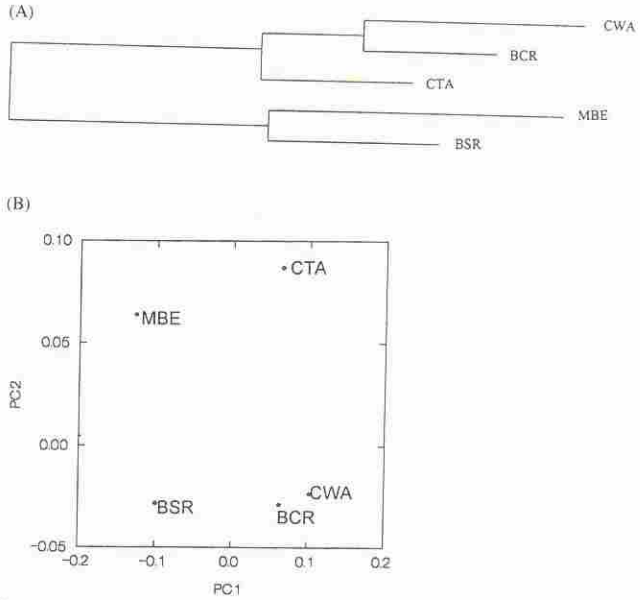

Fig. 2. (A) Neighbour-joining dendogram of the shared allele distance. (B) Plot of tilapia species against values for the first two principal components

morhua) primers amplify microsatellite loci in haddock (*Malanogrammus aeglefinus*) and pollock (*Pollachius virens*; Brooker et al. 1994), and human (*Homo sapiens*) microsatellite primers amplify in chimpanzees (*Pan troglodytes*; Garza et al. 1995). This study has demonstrated that *Oreochromis* primers amplify DNA of other tilapia genera: *Sarotherodon* and *Tilapia*. The intensity of the bands on the autorads varied among loci, with a very low yield of the polymerase chain reaction (PCR) product amplified at locus Os-74. Work carried out in our laboratory (W. Kamonrat, unpublished data) has shown that the quality of product from amplification of microsatellites can be enhanced by the addition of Tween 20 to the cocktail. With this supplement it has been possible to expand the number of loci in use to include Os-74 which is at present yielding products of sufficient quality for analysis. The complete set of primers has been employed in the cross-hybridization study and studies of pedigree analysis (S. Gadagkar, unpublished data).

Variability

The most variable loci were those that had long dinucleotide repeat arrays. For instance, the microsatellites flanked by primer sets Os-75 and Os-7R were 31 and 33 repeats long in their original isolates, respectively, and showed the highest variability. A direct relationship between the variability of the microsatellites and the number of repeats has been reported in humans (Weber 1990). This relationship tends to be more obvious in sequences of 12–20 repeats and weakens in sequences of more than 21 repeats (Weber 1990). Loci Os-25, Os-7 and Os-64 were relatively less variable than loci Os-7R and Os-75. The correlation between array length and variability suggests that the mutation rate may be correlated with array size, although the structure of the repeat may also be important (Weber 1990). The nonperfect array at locus Os-64 might have attributed to the low variability. In the sequence of the original isolate, there was a change in dinucleotide repeats motifs from (TG) to (GA). The fact that interruptions in repeat sequences reduce variability at a locus has been observed in humans (Weber 1990).

The monomorphism observed in the other species listed in Table 3 is not claimed to be definitive because of the limited number of samples that were analysed. Inferences based on interlocus comparison would not be appropriate because it was not unusual for an individual to be homozygous at one locus and heterozygous at another.

The level of heterozygosity observed in the microsatellite loci analysed (range 0.51 ± 0.12 to 0.82 ± 0.03) is much higher than that observed in allozymes, e.g. Sodsuk et al. (1995) observed heterozygosity values of 0.08 for *O. sh. shiranus* and 0.108 for *O. karongae*. This probably reflects the higher mutation rates at microsatellite loci (Terauchi and Konuma 1994; Watkins et al. 1995).

The relationship between genetic distance and the geological history of the lakes

The results show that there were genetic differences between the Lake Malombe population and the Chilwa–Chiuta lake populations of *O. shiranus*. Msiska (1988) speculated that the population in Lake Chiuta is *O. sh. shiranus*. However, the present study shows that it is closer to *O. sh. chilwae* than to *O. sh. shiranus*. This supports an earlier hypothesis by Trewavas (1983, p. 356) that there is no genetic difference at the subspecies level between Chilwa and Chiuta lake populations. Hence, the two populations belong to the subspecies *O. sh. chilwae*. The DNA data are explained in part by geological evidence that lakes Chilwa and Chiuta were a single open lake with an area of about 5500 km^2, draining into the Indian Ocean via the Lugenda River in Mozambique. The two lakes are now separated by a sand bar formed during the early Holocene phase (8000–9000 BP) or earlier, by an easterly movement of beach sand across the northern end of Lake Chilwa (Fig. 1; Lancaster 1979).

Conclusion

The microsatellite markers developed in this study showed polymorphism across several genera of tilapias. The variability profile produced at the five loci varied both within individuals and within species. The general trend observed was that loci which were developed from sequences flanking long tandem arrays showed higher variability than those loci which were made from sequences flanking shorter arrays. The lack of reliable procedures for identifying cultured species in Malawi has deterred proper broodstock handling procedures and the devel-

opment of improved strains. The microsatellite DNA markers used in this study have been able to distinguish the accepted subspecies of *O. shiranus* previously postulated on morphological grounds (Trewavas 1983). The low genetic differentiation of *O. shiranus* in lakes Chilwa and Chiuta is consistent with the recent geological union of these lakes.

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