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Genetic structure of *Oreochromis* populations of selected reservoirs of Sri Lanka: a molecular approach

De Silva M.P.K.S.K.[1], Hettiarachi S.[2] and Mather P.B.[3]

[1] *Department of Zoology, University of Ruhuna, Matara, Sri Lanka*
[2] *Department of Botany, University of Ruhuna, Matara, Sri Lanka*
[3] *School of Natural Resources Science, Queensland University of Technology, Brisbane, Australia*
Correspondence: kumududs@zoo.ruh.ac.lk

Abstract
Oreochromis mossambicus and *O. niloticus* are the species that contribute mainly to the fishery production in reservoirs of Sri Lanka. The two *Oreochromis* species have been proven to interbreed resulting hybrids and introgressed individuals. The effect of hybridization on genetic diversity of *Oreochromis* populations in Sri Lankan reservoirs is not known. Knowledge of genetic structuring of *Oreochromis* populations is an important aspect in adopting management strategies to enhance the reservoir fishery. Four microsatellite loci were used as molecular markers to reveal the genetic structure of *Oreochromis* populations of six reservoirs.

All populations showed low allelic diversity and heterozygosity levels in microsatellite loci studied possibly due to small founder populations and repeated population bottlenecks. Most populations did not conform to Hardy Weinberg Expectations, which could be considered as a result of positive assortive mating. Results of the present study illustrate the necessity of improvement of genetic diversity of *Oreochromis* populations and possible strategies are suggested.

Keywords: Fishery management, Hardy Weinberg, hybridization, microsatellite DNA, mitochondrial DNA

Introduction

Since their introductions *Oreochromis mossambicus* and *Oreochromis niloticus* have been well established in Sri Lankan reservoirs. They provide the most significant contribution to the inland fishery. Hybridisation/ introgression is a common phenomenon among tilapia species and have been reported in many aquaculture systems and reservoirs (Trewavas, 1983; Moreau, 1986; Gregg *et al.*, 1987). Hybridisation leads to mixing of genetic characters of the two species and this gene introgression may change the genetic variability present in pure species and also

could produce progenies, which have different genetic traits to the parents. Biochemical studies and molecular studies have shown that hybridisation between *O. mossambicus* and *O. niloticus* have taken place in Sri Lankan reservoirs too (De Silva and Ranasinghe, 1989; De Silva *et al.*, 1999). During the past three decades after the introductions of *Oreochromis* species, the event of hybridisation and the backcrossing should have taken place immensely. The effect of hybridisation on genetic diversity of the *Oreochromis* population in Sri Lankan reservoirs is not known.

Studies have revealed that different levels of hybrids (hybridity levels) are present in Sri Lankan reservoirs (De Silva *et. al.*, unpublished data). This shows that mating between *O. mossambicus* and *O. niloticus*, hybrids and the parents, and hybrids and hybrids have taken place in reservoirs. As randomly mating individuals in a geographic isolation could be considered as a separate genetic stock, *Oreochromis* individuals in a reservoir therefore could be treated as a separate stock.

Biochemical markers had been the standard tool for genetic studies of fish populations for many years (Verspoor and Hammer, 1991). In recent years, mitochondrial and nuclear DNA techniques have been proven to be more powerful in analysing population structure in fish species, especially where protein polymorphism show little or no differences (Estoup *et al.*, 1998). Microsatellite markers are a widely applicable DNA technology. They have been shown highly polymorphic in teleost fish (Coughlan *et al.*, 1998). Microsatellite loci can be scored relatively easily using a combination of Polymerase Chain Reaction (PCR) amplification followed by electrophoresis to separate alleles that differ in length as a result of differences in the number of tandem repeats.

Genetic diversity is an important determinant in a population. Fish populations with low genetic variability often show poor culture performances *viz.*, in growth, disease resistance, fecundity, viability of eggs and high mortality, and thereby determines the biology, population dynamic parameters of the fish stocks and ultimately the fishery productivity in a reservoir. Therefore knowledge of genetics of population structure is crucial to the long-term fisheries conservation. Present study was carried out to explore and compare the genetic variability of *Oreochromis* populations in six reservoirs of Sri Lanka.

Materials and Methods

Samples of *Oreochromis* species were collected from six reservoirs situated in different parts of the dry zone in Sri Lanka (Figure 1) from October, 1999 to March, 2000. Random samples of fish were obtained from fishermen at landing sites. Fish were caught by gill nets as part of the commercial fisheries in individual reservoirs. Sample sizes for each reservoir used for molecular studies are as follows.

Reservoir	No
Chandrikawewa	45
Tabbowa	56
Lunugamwehera	66
Nuwarawewa	46
Minneriya	48
Ridiyagama	55

A small piece of white muscle tissue was removed from each individual and the tissue samples were preserved in 70% alcohol. DNA extraction was performed according to Doyle and Doyle (1987). Microsatellite primers developed for *O. niloticus* by Lee and Kocher (1996) and trialed for *O. mossambicus* (Agustin, 1999) were used. The loci selected were diagnostic for *O. mossambicus* and *O. niloticus* and belonged to different linkage groups. The four diagnostic loci used in this study possessed unique alleles that showed very different electrophoretic mobilities for the two species (Table 1).

Table 1 - Specifications of microsatellite primers used (Lee and Kocher, 1996)

Locus	Primer sequence A-Forward Primer B- Reverse Primer	Linkage group	Size (bp)	Repeat sequence	Annealing temperature (^{0}C)
UNH 190	A-CGCGATCGAGCATTCTAA	lg21	167	$(CT)_6(CA)_{20}$	50^0 C
	B-TGTCTGCACGCGCTTTTGT				
UNH 146	A-CCACTCTGCCTGCCCTCTAT	lg4	122	$(CA)_{10}$	55^0 C
	B-AGCTGCGTCAAACTCTCAAAAG				
UNH 106	A-CCTTCAGCATCCGTATAT	lg14	134	$(CT)13(CA)_{20}$	55^0 C
	B-GTCTCTTTCTCTCTGTCACAAG				
UNH 216	A-GGGAAACTAAAGCTGAAATA	lg23	124	(CA)12	50^0 C
	B-TGCAAGGAATATCAGCA				

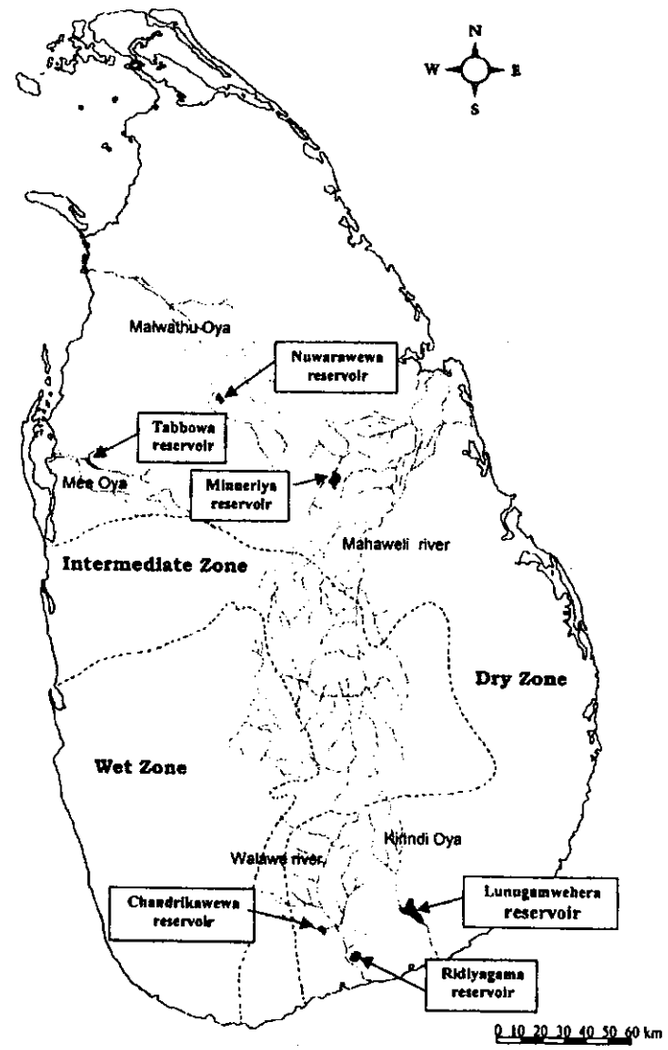


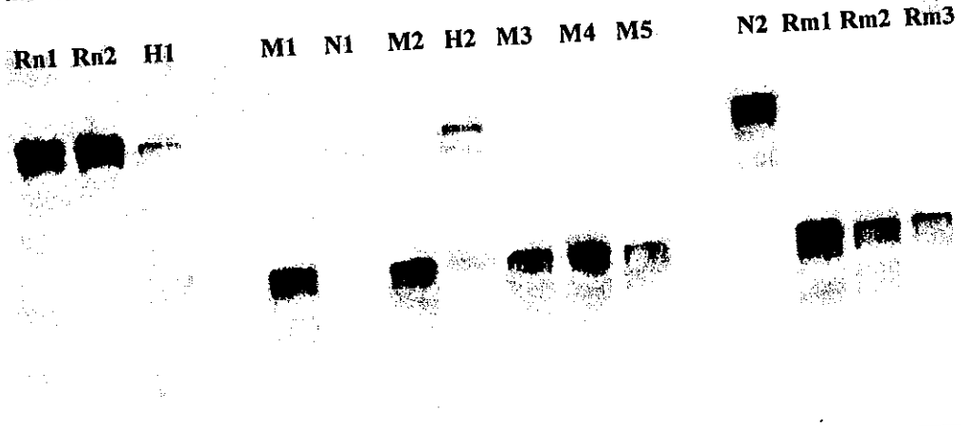
Figure 1 - Map of Sri Lanka showing six reservoirs selected for the study, their feeding rivers and major climatic zones

As no known pure lines of *Oreochromis* populations exists or maintained in Sri Lanka, pure strains of *O. niloticus* from Israel and Fiji and three samples of pure *O. mossambicus* from Malaysia, Singapore and Australia served as reference samples as they possessed identical banding patterns to patterns observed in Sri Lankan *O. mossambicus and O. niloticus*, respectively. Reference DNA samples from pure non-Sri Lankan lines of *O. mossambicus* and *O. niloticus* were used in each microsatellite PCR and gel run.

Microsatellite amplifications were performed in 0.5 ml sterile eppendorf tubes containing 10 µl reaction volume. Reaction mixtures contained template DNA (~100ng), 1.5 X T^{th} Reaction buffer (Biotech), dNTPs each at 2 mM final concentration (dCTP labelled ^{32}P, added just before the PCR) (Boehringer-Mannheim), $MgCl_2$ at 2 mM final concentration (Biotech), 1 µM of each primer and 0.02 units of T^{th} polymerase (Biotech). Amplification was undertaken in a programmable mini-thermocycler (Bresatec) according to the following PCR programme.

1) 94^0 C for 1 minute, 2) 50^0 C - 55^0 C for 1 minute depending on annealing temperature of primer, 3) 72^0 C for 2 minutes for 34 cycles and 4) final extension at 72^0 C for 10 minutes and 5) cooling to storage temperature (4^0 C).

PCR Products were electrophoresed in 5% polyacrylamide gel using 1 X TBE as running buffer, gel was dried, exposed to autoradiography film overnight and then the film was developed (Figure 2).



Rn1 and Rn2	- *O. niloticus* from Israel and Fiji (Chitralda), respectively
Rm1, Rm2 and Rm3	- *O. mossambicus* from Malaysia, Singapore and Australia
N1 and N2	- *O. niloticus* samples
M1 to M5	- *O. mossambicus* samples
H1 and H2	- Hybrid samples

Figure 2- Autoradiograph showing Microsattelite phenotypes of *O. mossambicus*, *O. niloticus* and hybrids/introgressed individuals obtained for locus UNH 190.

Indices of genetic variation including mean number of alleles per locus, proportion of polymorphic loci, mean heterozygosity (H_0 and H_e) and Hardy Weinberg estimates were estimated using POPGENE version 1.21 (Yeh *et al.*, 1997).

Differences in mean heterozygosity estimates among populations were assessed by Kruskal-Wallis non-parametric tests.

Results

Most populations did not conform to Hardy Weinberg equilibrium at most microsatellite loci. Only four tests (marked as *) of 21 (19.5%) conformed to Hardy Weinberg expectations (Table 2). All populations showed very low levels of genetic diversity at all microsatellite loci studied. Pure *O. mossambicus* individuals and pure *O. niloticus* individuals had a maximum of three alleles per locus at the most variable loci. Individuals belonging to the same species had the same common alleles in all populations. Three loci were monomorphic (UNH 190, UNH 146, and UNH 106) in pure *O. mossambicus* individuals in all reservoirs except for a single individual in Tabbowa reservoir. Loci 216 and 190 were monomorphic in all pure *O. niloticus* individuals. When *O. mossambicus*, *O. niloticus* and hybrids/ backcrosses were considered as a single interbreeding population, none of the loci were monomorphic for all populations except Chandrikawewa reservoir population (Table 2). Percentages of polymorphic loci in six populations are presented in Table 3. A small number of rare alleles were observed in Chandrikawewa and Tabbowa reservoirs but not observed in other populations. Average number of alleles per locus was low and no apparent differences were observed among populations. Mean number of alleles per locus ranged between 3.5 ± 0.58 and 3.25 ± 0 .96 except in the Chandrikawewa reservoir population 2 ±2 (Table 3).

Table 2 - Results of Hardy-Weinberg Tests given as p-values (significance at 5% level indicated by* after Bonferroni adjustments)

Reservoir / Locus	Chandrikawewa	Tabbowa	Lunugamwehera	Nuwarawea	Minneriya	Ridiyagama
190	mml	0.00000[c]	0.00000[d]	0.00000[c]	*0.29303[c]	0.00000[d]
146	mml	0.00004[b]	0.00000[b]	0.00001[b]	*0.03717[b]	0.00000[b]
106	mml	0.00000[b]	0.00000[b]	*0.00350[b]	0.00000[b]	0.00000[b]
216	.00204[a]	0.00000[c]	0.00000[b]	0.00039[c]	*0.45248[c]	0.00030[c]

mml- monomorphic loci, [a]df = 10; [b]df = 6; [c]df = 3; [d]df=1

All reservoirs showed a deficiency in observed heterozygote numbers compared with that expected under Hardy Weinberg except for the Chandrikawewa reservoir population (Table 2). Significant differences were not observed in heterozygosity estimates among reservoirs ($\chi^2 = 7.935$, df = 4, p = 0.094).

Table 3 - Genetic variability at four microsatellite loci in all reservoir populations (Standard Deviation in parentheses)

Reservoir	% of polymorphic loci	Mean no of observed alleles	Mean observed heterozygosity	Mean expected heterozygosity
Chandrikawewa	25	2 (2.0)	0.22 (0.43)	0.15 (0.30)
Lunugamwehera	100	3.5 (1.0)	0.31 (0.10)	0.62 (0.09)
Minneriya	100	3.5 (0.577)	0.45 (0.11)	0.59 (0.13)
Nuwarawewa	100	3.5 (0.577)	0.17 (0.06)	0.32 (0.11)
Tabbowa	100	3.5 (0.577)	0.15 (0.24)	0.23 (0.17)
Ridiyagama	100	3.25 (0.957)	0.27 (0.13)	0.60 (0.11)

Discussion

Genotypic frequencies at microsatellite loci in most populations did not satisfy Hardy Weinberg equilibrium. Only one population (Minneriya) consistently conformed to Hardy Weinberg equilibrium but only at three of the four loci examined. Deviations from Hardy Weinberg equilibrium resulted in all instances from homozygote excess, which corresponded to higher than expected number of pure species genotypes. The best explanation of the result is that individuals of the *Oreochromis* populations do not mate randomly but preferentially with their own species or that hybrids have lower fertility or viability than do with pure species crosses. Both pure species have characteristic reproductive behaviours in nest building, mating and parental care. Male breeding colouration also has shown an important cue in mate choice. Seehausen and Alphen, 1998). These factors may also been contributed to the preferential mating with the own species. Positive assortive mating may explain the deficit of heterozygotes (Poteaux *et al.*, 1998) observed in most populations leading to Hardy Weinberg disequilibrium.

Hybridisation studies often report low or no non-existent viability of hybrids leading to little contribution to the next generation (Brunson and Robinette, 1987; Coyne and Orr, 1989a). While it is obvious that *O. mossambicus* and *O. niloticus* will hybridise and backcross regularly in nature where the opportunity exists, no studies have

determined if this occurs randomly and hybrids and/ or backcrosses have equivalent viability as do non hybrid individuals in the same population. If hybrids and/ or backcrosses do not possess equivalent viability, fecundity or fertility to pure species individuals then this could have a significant impact on population structure and affect Hardy Weinberg equilibrium estimates. This process would also affect the distribution of alleles among genotypes, which would influence deviations from Hardy Weinberg.

Null alleles at genetic loci may also cause deviations from Hardy Weinberg equilibrium (Callen *et al.*, 1993; Pemberton *et al.*, 1995). Primers used in this study were developed for *O. niloticus* but were also used to screen *O. mossambicus* and hybrid individuals. If null alleles were present at microsatellite loci due to mutations in the priming sites in *O. mossambicus* genomes then this could also have influenced the rates of deviation from Hardy Weinberg equilibrium. There is however, no simple method available to determine whether null alleles were present in some individuals screened here or to quantify their relative frequencies in different sampled populations

As a whole all sampled populations had very little genetic variability with low number of alleles per locus, and low mean heterozygosity levels (Table 2). In all populations studied, pure *O. mossambicus* and *O. niloticus* were homozygous for three (UNH 190, UNH 146, UNH 106) and two (UNH 216, UNH 190) loci, respectively. Therefore, the contribution of pure species to the population heterozygosity estimates was very low. In contrast, true hybrids are heterozygotes for all loci and backcrosses were comparatively more heterozygous than 'pure' individuals. Therefore relative heterozygosity levels observed in the sampled populations were determined primarily by input from hybrids. Populations with higher hybrids numbers (Minneriya) or populations like Lunugamwehera and Ridiyagama with approximately similar proportions of the three groups (*O. mossambicus*, *O. niloticus* and hybrids), showed much higher mean heterozygosity levels (Table 3) than populations which had none or very few hybrids (Chandrikawewa, Nuwarawewa and Tabbowa). Mean heterozygosity ranged from 0.15 ± 0.24 in Tabbowa samples to 0.45 ±0.11 in Minneriya samples.

Different 'hybridity' levels found in the six populations studied indicated that the hybrids found in these reservoirs are both viable and fertile (De Silva *et al.* unpublished data). However, there is no simple way of equating the relative viability or fertility of the hybrids found. Therefore, it's possible that some alleles, especially

rare alleles, have been lost from populations due to non-viable hybrids. Presence of rare alleles in Chandrikawewa and Tabbowa populations that consist of a majority of pure *O. mossambicus* individuals may therefore, be due to negligible levels of hybridisation.

Mean number of alleles per locus was low and showed no marked variation among populations (Table 3). Chandrikawewa had the least number of alleles per locus. It has also been shown in many studies that populations which have originated from small founder populations (Franklin, 1980) and which have been exposed to severe bottle necks (Taylor *et al.*, 1994; Tarr *et al.*, 1998) during their ancestry, experience loss of or lack of allelic variability. The number of individuals of *O. niloticus* and *O. mossambicus* originally introduced to Sri Lanka as founder populations is not known but may have been very small as is the case with introductions to other countries. According to Welcomme (1988) and Agustin (1999), introduction of tilapia to the Pacific region and Australasia originated from a founder population of five individuals introduced from Indonesia. In Sri Lanka, introduced stocks may also have been exposed to repetitive population bottlenecks after introduction especially in years with exceptionally dry seasons. During dry periods many of the reservoirs in Sri Lanka dry up and fish either die or concentrate in scattered pools in the centre (De Silva, 1988). Although the effect of drying up of reservoirs on the flora and fauna is little understood, it is possible that a significant percentage of fish in reservoirs is either harvested by fishermen or dies via natural causes. Therefore, each generation starts from a small gene pool with consequent reduced genetic variability. In summary, this study revealed that all reservoir populations showed low genetic variability due to positive assortive mating, population bottlenecks and lack or loss of viability and fertility in hybrids and backcrosses. These factors operating simultaneously or in isolation could have contributed to the relatively low genetic variability in all six populations studied.

Low genetic diversity may impact on the long term productivity of the reservoir fisheries if it results in poor adaptive potential and long term increased inbreeding may produce inbreeding depression effects. It is difficult to gauge whether the relative productivity of the *Oreochromis* stocks exploited in the reservoir fishery could benefit from the introductions of new germ plasm. Agustin (1999) has shown relatively high level of genetic diversity in native populations of *O. mossambicus* obtained from different localities in Africa. It has been shown that dominance of *O. mossambicus* or *O. niloticus* varies among six reservoirs named in the present study (De Silva *et al.* unpublished data). Therefore, selection of most suitable species

(*O. mossambicus* or *O. niloticus*) for introduction to Sri Lanka is a problem because no comparative study has been done to evaluate the most suitable species for Sri Lankan conditions. Introduction of germ plasm is a possible option to improve the genetic variability of *Oreochromis* populations. It needs a well- planned project with a thorough review of feasibility, pilot studies, monitoring and evaluation programs with scientific background to investigate the best direction to take.

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References

Agustin, L.Q., 1999. Effects of population Bottlenecks on levels of genetic diversity and patterns of differentiation in feral populations of *Oreochromis mossambicus*. Ph.D. thesis, School of Life Sciences Center of Molecular Biotechnology, Queensland University of Technology, Brisbane, 4001, Australia.

Brunson, M.W. and Robinette, H.R., 1987. Reproductive isolation between a hybrid sunfish and its parental species. Prog. Fish Cult. 49: 296-298.

Callen, D.F., Thompson, A.D., Shen, Y., Phillips, H.A., Richards, R.I., Mulley, J.C. Sutherland, G.R., 1993. Incidence and origin of null alleles in the $(AC)_n$ microsatellite markers. Am. J. Hum. Genet. 52: 922-927.

Coughlan, J.P., Imsland, A.K., Galvin, P.T., Fitzgerald, R.D., Naevdal, G., Cross, T.F., 1998. Microsatellite DNA variation in wild populations and farmed strains of turbot from Ireland and Norway: a preliminary study. J. Fish Biol. 52: 916-922.

Coyne, J.A. and Orr, H.A., 1989. Two rules of speciation. In: Speciation and its consequences, D. Otte and J.A. Endler (eds.), Sinauer, Sunderland, MA.

De Silva, C.D. and Ranasinghe, J., 1989. Biochemical evidence of hybrid gene introgression in some reservoir populations of tilapia in southern Sri Lanka. Aquacult. Fish. Manage. 20: 269-277.

De Silva, M.P.K.S.K., Baker, N., Mather, P.B., 1999. Microsatellites as Genetic Markers for Identification of Exotic Cichlids and their Hybrids in Sri Lanka- A Preliminary Study. Sri Lanka J. Aquat. Sci. 4: 83-90.

De Silva, S.S., 1988. Reservoirs of Sri Lanka and their fisheries. FAO fisheries technical paper 298, Rome, Italy.

Doyle, J.J. and Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tipsier. Phytochem. Bull. 19: 11-15.

Estoup, A., Rousset, F., Michalakis, Y., Cornuet, M., Adriamanga, M., Guyomard, R., 1998. Comparative analysis of microsatellite and allozyme markers: a case study investigating microgeographic differentiation in brown trout (*Salmo trutta*). Mol. Ecol. 7: 339-353.

Franklin, O.H., 1980. Evolutionary change in small populations. In: Soule M.E. and Wilcox B.A. (Eds.). Conservation Biology: An Evolutionary- Ecological Perspective. Sinauer Associates, Sunderland MA. pp135-150.

Gregg, R.E., Howard, J.H., Shonhiwa, F., 1997. Introgressive hybridization of tilapias in Zimbabwe. J. Fish Biol. 52: 1-10.

Lee, W.J. and Kocher, T.D., 1996. Microsatellite DNA markers for genetic mapping in *Oreochromis niloticus*. J. Fish Biol. 49:169-171.

Moreau, J., 1986. Inland fisheries in Madagascar. In: Reports and papers presented at Indo- Pacific fishery commission expert consultation on inland fisheries of the larger Indo-Pacific Islands, Bangkok, Thailand, 4-9 August 1986. T. Petr (Ed.) FAO Fisheries Report 371(Supplement), FAO, Rome. pp 72-103.

Pemberton, J.M., Slate, J., Bancroft, D.R., Barret, J.A., 1995. Non-amplifying alleles at microsatellite loci: a caution for parentage and population studies. Mol. Ecol. 4: 249-252.

Poteaux, C., Beaudou, D., Berrebi, P., 1998. Temporal variations of genetic introgression in stocked brown trout populations. J. Fish Biol. 53: 701-713.

Seehausen, O. and Alphen, J.J.M.V., 1998. The effect of male colouration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). Behav. Ecol. Sociobiol. 42: 1-18.

Tarr, C.L., Conant, S., Fleischer, R.C., 1998. Founder events and variation at microsatellite loci in an insular passerine bird, the Laysan finch (*Telespiza cantans*). Mol. Ecol..7: 719-731.

Taylor, A.C., Sherwin, W.B., Wayne, R.K., 1994. Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiorhinus krefftii*. Mol. Ecol. 3: 277-290.

Trewavas, E., 1983. Tilapiine Fishes of the Genera *Sarotherodon*, *Oreochromis* and *Danakilia*. British Museum of Natural History, London.

Verspoor, E. and Hammar, J., 1991. Introgressive hybridization in fishes: the biochemical evidence. J. Fish Biol. 39 (Supplement A): 309-334.

Welcomme, R.L., 1988. International introductions of inland aquatic species. FAO Fisheries Technical Paper 294: pp194-203

Yeh, F.C., Yang, R.C., Boyle, T.B.J., Ye, Z.H., Mao, J.X., 1997. POPGENE Ver. 1.21, the user friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.