Biochemical genetic markers to identify hybrids between the endemic *Oreochromis mossambicus* and the alien species, *O. niloticus* (Pisces: Cichlidae)

RD Moralee[1], FH van der Bank[1]* and BCW van der Waal[2]
[1] *Department of Zoology, Rand Afrikaans University, PO Box 524, Auckland Park 2006, South Africa*
[2]*Department of Biology, University of Venda, PO Bag X 5050, Thohoyandou 0950, South Africa*

Abstract

The invasion of exotic *Oreochromis niloticus* into the Limpopo River system (in the vicinity of the Vembe Nature Reserve, Northern Province, South Africa) was studied. *Oreochromis niloticus* was found in pools in the nearly dry river bed, as well as in dams alongside the river. Pure *O. mossambicus* was also found at these localities, as well as individuals that appeared hybrid-like. Starch gel-electrophoresis of muscle tissues and the resolution of protein loci using specific histochemical techniques identified the pure species as well as verifying the presence of hybrids. Various morphological characteristics were studied in order to identify hybrids. Red tilapias from the aquariums at the Rand Afrikaans University were also analysed to determine if they are hybrids or mutants. This is the first account of hybrids between the above-mentioned species in South Africa and there is no evidence that the red tilapias are hybrids.

Introduction

Skelton (1993) described the Nile tilapia (*Oreochromis niloticus*) in Southern Africa as a fodder fish, introduced from Israel before 1955 for aquaculture. He reported that this species was distributed in rivers of the Cape flats area, southwest Cape, KwaZulu-Natal and Kariba basin in Zimbabwe; the natural range includes the Nile basin, Rift Valley lakes and certain West African rivers. On 20 November 1996, three adult specimens were collected in Manxeba Pan (22°22'40"S, 31°12'40"E) situated in the Pafuri region of the Kruger National Park close to the Mozambique border and 750 m south of the Limpopo River (Van der Waal and Bills, 1997). *Oreochromis niloticus* was added to the list of exotic species in the Limpopo River and its tributaries. The alien species occurred in the lower Limpopo River, which gave dam walls a new meaning. It could resist the assault by alien tilapias and/or hybrids if foreign tilapias are not introduced in the dams. This is important since hybridisations and/or introgressions occur as a consequence of man's actions to change geographic or ecological barriers (Agnèse, 1998).

Oreochromis niloticus directly competes with our native Mozambique tilapia *(O. mossambicus*) for food and breeding place, and hybridisation has been reported elsewhere (Agnèse, 1998). Thus the indigenous Mozambique tilapia may lose its genetic purity and be replaced by hybrid wild populations throughout most of its natural range in time (Van der Waal, 1997). However, molecular markers provide a powerful means of determining the occurrence and extent of hybridisation, and these markers (unlike morphological characters) typically possess simple modes of expression and inheritance (Nason et al., 1992). Electrophoretic markers to identify the above-mentioned pure tilapia species are reported in McAndrew and Majumdar (1983) and Pouyaud and Agnèse (1995). These authors did not include hybrids in their studies. However, hybrids exhibit character coherence (i.e. parental characteristics remain associated in hybrid progenies) (Rieseberg and Ellestrand 1993; Rieseberg, 1995), and the use of allozyme studies to identify hybrids are well documented (e.g. Van Vuuren et al., 1989; Van der Bank and Van Wyk, 1996). Hybridisation between the above-mentioned tilapia species can also produce what is known as red tilapia. Red tilapias from the aquariums at the Rand Afrikaans University (RAU) were also analysed to determine their genetic composition (i.e. to determine if they are mutants of *O. mossambicus* or hybrids). The latter specimens originated from experiments at RAU, were distributed to many parts of South Africa and interbreeds with *O. mossambicus* (Ferreira, 1998). The aims of this study were to verify the presence of *O. niloticus* upstream in the Limpopo River, in the vicinity of the Vembe Nature Reserve, to determine whether hybridisation with *O. mossambicus* had occurred and to verify the genetic integrity of the red tilapia at RAU.

Material and methods

Fourteen *O. niloticus* specimens were collected in May 1998, on the farm Den Staat, using hook and line. No *O. mossambicus* were caught. The aforementioned dam was sampled again in June 1998 using gill and seine nets. Pools within the nearly dry Limpopo River bed were also sampled in similar fashion (Fig.1). A total of 102 individuals were collected: five *O. mossambicus* individuals were collected at Loskop Dam (25°26'S, 29°21'E) and included as control samples, 23 *O. mossambicus*, 31 *O. niloticus*, 32 juveniles (of which the species-specific characteristics were not yet expressed), and 11 hybrid-appearing individuals were collected at Den Staat for electrophoretic analyses. Muscle tissue was dissected and preserved in liquid nitrogen for allozyme analysis; DNA samples were prepared from muscle tissue and stored in 70% ethanol and voucher material was stored in 10% formalin. Representatives of these fish and DNA samples will be deposited at the JLB Smith Institute of Ichthyology. Muscle tissue was also dissected from three red tilapia specimens from the RAU aquariums

* To whom all correspondence should be addressed.
☎(011) 489-2450; fax (011) 489-2286; e-mail: fhvdb@na.rau.ac.za
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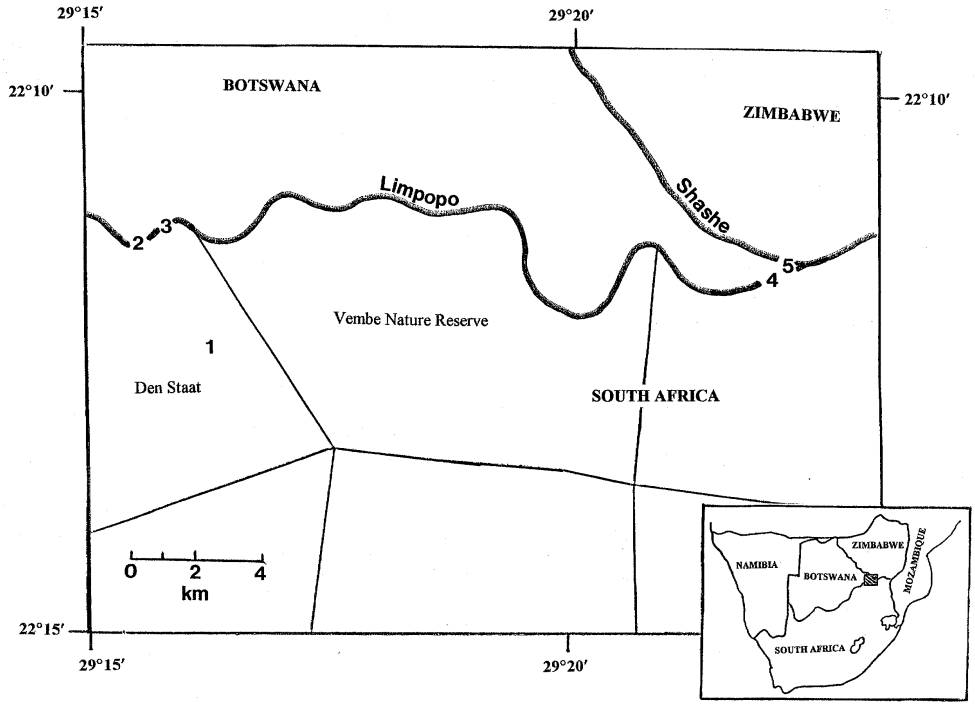

Figure 1
Map of the Vembe area, Northern Province (South Africa), showing the sampling localities.
1 = locality 1 (22°12'28"S; 29°16'44"E),
2 = locality 2 (22°11'50"S; 29°15'23"E),
3 = locality 3 (22°11'25"S; 29°15'53"E),
4 = locality 4 (22°11'38"S; 29°22'04"E),
5 = locality 5 (22°11'32"S; 29°22'03"E)

and used immediately for allozyme analysis.

Allozyme analysis of muscle tissue was done using the methods described by McAndrew and Majumdar (1983). These authors have published biochemical genetic data that can be used as markers to identify pure *O. mossambicus* and *O. niloticus*. Continuous tris-citrate (CTC) and tris-EDTA-borate (TEB) buffers, gels and biochemical genetic markers as described in McAndrew and Majumdar (1983) were used. The enzymes stained for included aspartate aminotransferase (AAT; E.C. no. 2.6.1.1), adenylate kinase (AK; E.C. no. 2.7.4.3), creatine kinase (CK; E.C. no. 2.7.3.2), esterase (EST; E.C. no. 3.1.1.-), glucose-6-phosphate isomerase (GPI; E.C. no. 5.3.1.9), l-lactate dehydrogenase (LDH; E.C. no. 1.1.1.27), and superoxide dismutase (SOD; E.C. no. 1.15.1.1).

Morphological data were recorded in an attempt to determine if hybrid identification would be possible. This included counting the dorsal and anal fin spines and rays, lateral scales, and gill rakers on the lower part of the first gill arch. The length and width of the last four dorsal fin spines were measured, and x-rays of the fin spines were taken to determine if they were different for the taxa studied. Standard length (in centimetres) and mass (in grams) were measured to one decimal place. Age was determined by counting the annuli on scales. Morphometric data were analysed using a discriminant component analysis using the statistics programme SPSS3.

Results and discussion

Staining for CK and GPI using CTC gels gave the best results to identify the pure tilapia species and hybrids (Table 1; Fig. 2). Fixed allele differences at these loci were also reported by McAndrew and Majumdar (1983) and Pouyaud and Agnèse (1995) for the pure species. The observed enzyme activity at GPI in muscle tissue corresponds to two loci whose products hybridise to form a heterodimer of intermediate mobility (McAndrew and Majumdar 1983; Fig. 2). It was not possible to identify juveniles from anatomical characteristics in the present study, but allozyme analyses identified four as being hybrids. The results also showed that

TABLE 1
ALLELE FREQUENCIES FOR ALLELE CLASSES AT POLYMORPHIC LOCI

Locus	Allele mobility	*O. mossambicus*	Juveniles and hybrids	*O. niloticus*
CK	95 100 95/100	1.000	0.250 0.375 0.375	1.000
GPI-A	95 100 95/100	1.000	0.313 0.500 0.187	1.000
GPI-B	-10 10 -10/10	1.000	0.071 0.786 0.143	0.250 0.750

three of the larger specimens provisionally identified as possible hybrids from their morphology, were in fact *O. mossambicus*, and a further two were *O. niloticus*. Only five adults were hybrids. The hybrids had both alleles of the parent species at the CK and GPI protein coding loci (Table 1; Fig. 2), and hybrid characteristics varied from no markings on the body with bars on the tail, to two spots on the body with no bars on the tail. The measurements and method of identifying possible hybrids are similar to those reported in Van der Waal and Bills (1997).

Only five adult hybrids were identified by electrophoresis from which morphometric data could be determined. This sample size was too small to classify identification ranges for external features. We obtained the following ranges: 19 to 24 rakers on the first gill arch, 31 to 34 lateral scales, 15 to 17 dorsal spines, 10 to 13 dorsal rays, three anal spines, and nine to ten anal rays. The morphometric data of the two pure species were also compared to those of the

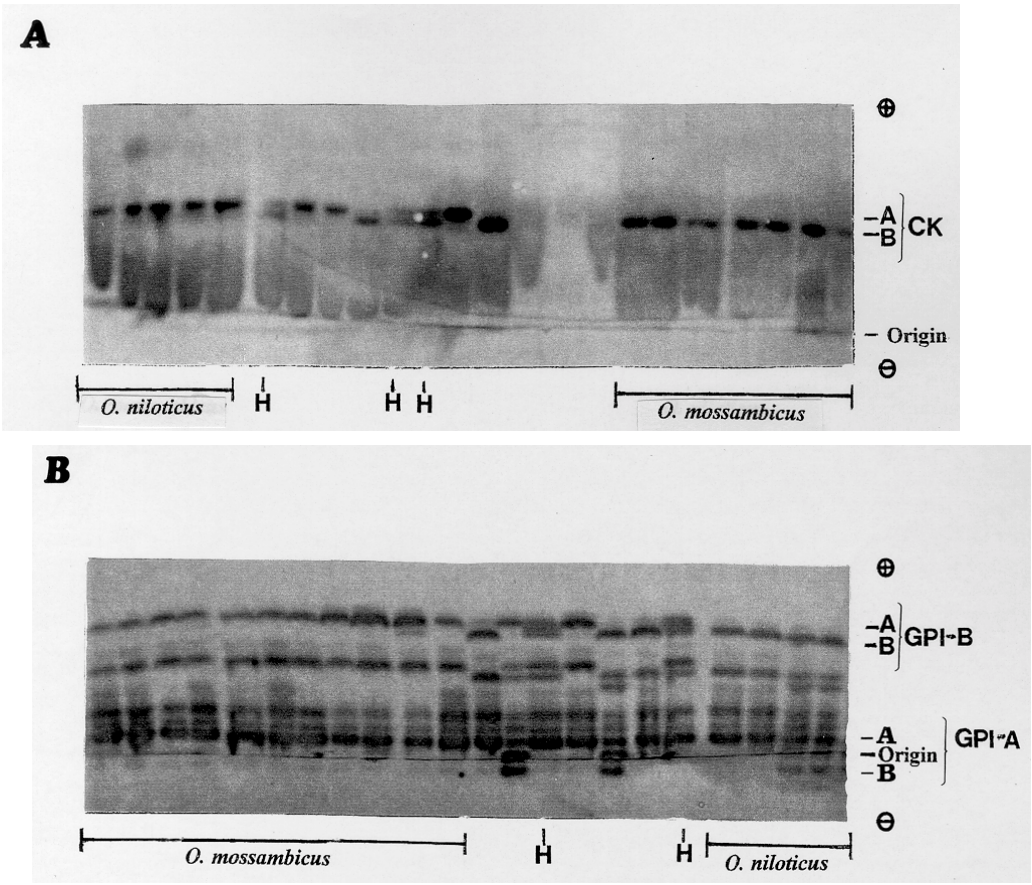

Figure 2
CTC-starch gels with O. niloticus, O. mossambicus *and hybrid (H) muscle samples stained for A) CK and B) GPI respectively*

hybrids. Four hybrids, 15 *O. mossambicus*, and 33 *O. niloticus* individuals were used for the comparisons. The hybrid values are intermediate to those of the two pure species in regard to the number of dorsal rays, lateral line scales and the number of rakers on the lower part of the first gill arch. The range of the above hybrid characteristics overlaps with those of the two pure species and can therefore not be used for identification purposes.

Each sample was identified by starch gel-electrophoresis and the numbers of each pure species and hybrids and the localities at which they were found are summarised. Locality 1 (Fig. 1) was a fish-pond used for commercial fishing on the farm Den Staat. Of the 123 specimens sampled at this locality for anatomical studies, 91% were *O. niloticus* between the ages of two and four years, 7% were *O. mossambicus* (two to three years old) and 2% were two-year-old hybrids. Locality 2 was a pool in the Limpopo River bed next to a water pump supplying the farm. Of the 95 specimens sampled at this locality, 98% were *O. niloticus* (two to three years old), 1% were two-year-old *O. mossambicus* and 1% were three-year-old hybrids. Locality 3 was a pool in the river bed alongside a weir at the edge of the farm. Of the 25 specimens sampled at this locality, 20% were *O. niloticus*, 72% were *O. mossambicus* and 8% were hybrids. All these specimens were juveniles. Locality 4 was a large pool in the river bed at the Shashe confluence. Of the eight specimens sampled at this locality, 13% were *O. niloticus* aged four years old, 62% were *O. mossambicus* between the ages of one and two years and 25% were two-year-old hybrids. Locality 5 was a much smaller pool at the Shashe confluence. Six juveniles were sampled at this locality, of which 33% were *O. niloticus*, 67% were *O. mossambicus* and there were no hybrids. In total, 257 specimens were sampled, of which 83% were the alien *O. niloticus*, 14% were *O. mossambicus* and 3% were hybrids.

It is evident that *O. niloticus* is the most abundant species found in the fish-ponds on the farm Den Staat (Locality 1) and in the pool (Locality 2) of the nearly dry Limpopo River bed just below the farm. These two localities are connected since water is pumped from Locality 2 for use on the farm. At the aforementioned localities, 205 adult *O. niloticus* specimens were found. The juveniles found at Locality 3 is evidence that these fish are breeding in the pools of the nearly dry river bed. The source of *O. niloticus* upstream in the Limpopo River is uncertain, but by comparing the ages of fish caught at Localities 1 and 2 it seems likely that they are breeding in the fish-ponds at Den Staat, and the juveniles could have escaped through the water pipes. During the 1996 floods, there was also a direct connection between the fish-ponds and the Limpopo River (Hodgson, 1998). At the Shashe confluence, approximately 10 to 15 km downstream from Den Staat, only one large *O. niloticus* individual was found. Due to its age (four years old) it is unlikely that the source of this exotic specimen is the fish-ponds at Den Staat, but rather similar ponds bordering the Shashe River in Zimbabwe. Nile tilapia are found in dams in the Umzingwane River, running into the Limpopo 70 km downstream of the Sashe/Limpopo confluence (VDW, pers. obs.), and the invasion from that source may have already occurred. We also expect hybrid vigor (i.e. hybrids are usually larger than any of the pure species). However, the hybrids we identified were two years old or younger; their standard lengths were therefore less than those of the *O. niloticus* individuals.

Special attention was given to the dorsal spines. It appeared from a superficial examination that hybrids had alternating thick and thin spines, but comparative analyses of the spinal ratios proved inconclusive. X-ray analysis of the dorsal fin spines revealed no difference. They were hollow for the two pure species as well as the hybrids. Comparative ratios of the length and width of the last four dorsal spines, as well as the ratio of the standard

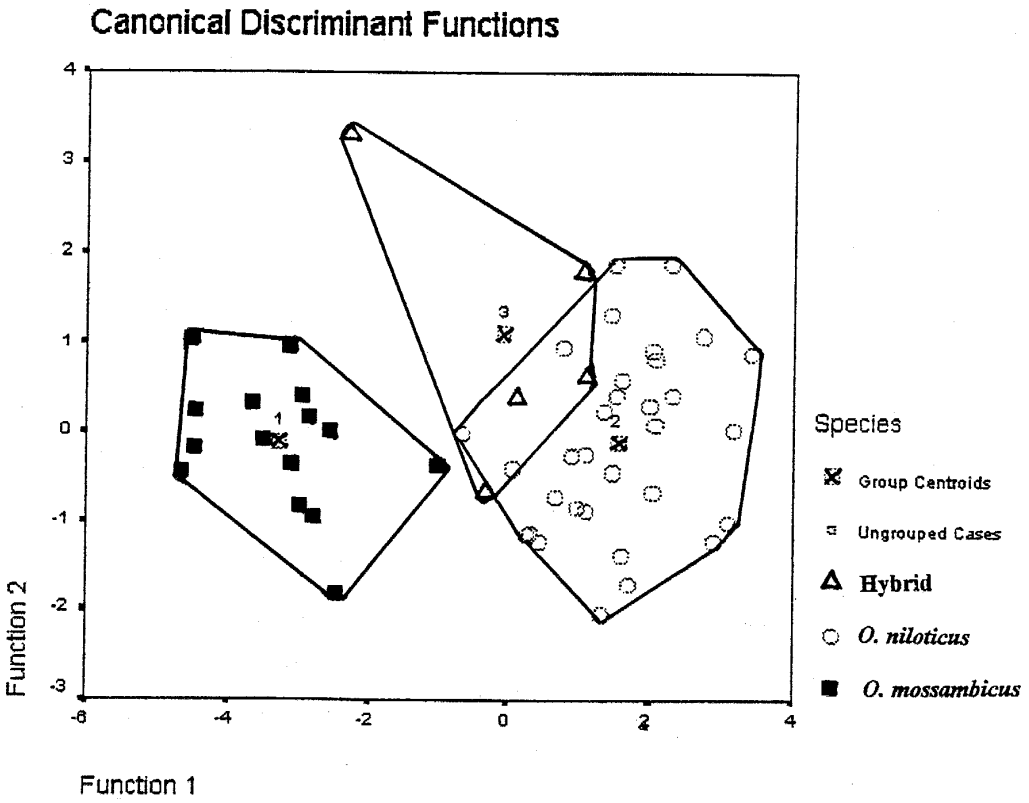

Figure 3
Discriminant component analysis of meristic characters for 1) O. mossambicus, *2)* O. niloticus *and 3) hybrids*

length to the length of the last four dorsal spines showed that the hybrid values were intermediate to those of the two pure species in most, but not all of the cases.

The discriminant component analysis was done using the following variables: the number of lateral scales, the number of gill rakers on the lower portion of the first gill arch, the number of dorsal fin spines and rays, and the number of anal fin spines and rays. Figure 3 illustrates that there is a distinction between the two pure *Oreochromis* species with respect to the canonical variable function (Function 1). The secondary function, however, has no effect on species separation. The hybrid values are intermediate to those of the pure species, but overlap with the ranges of the pure species. The hybrid values are more closely linked with those of *O. niloticus* than *O. mossambicus*. Discriminant component analysis can therefore not be used to identify hybrids from their meristic values.

The exotic species *O. niloticus* was only collected once in the Limpopo River before 1998 (Van der Waal and Bills, 1997). The latter authors collected these fishes in November 1996, and six months later we caught approximately 100 *O. niloticus* individuals in a single pool within the nearly dry Limpopo River bed. When the rains come and the river begins to flow again, these aliens will escape and be free to hybridise at will. Quite often, the F_1 generations are viable but the gender ratio of the descendants is unbalanced (Agnèse et al., 1998) or they are sterile, but they can also be fertile. These authors reported that there are numerous cases of hybridisation under natural conditions. However, these hybridisations have not always been easy to observe or prove. Since the development of genetic techniques, the characterisation of tilapia species has been advanced and it is easier today to prove the existence of natural tilapia hybrids. These natural tilapia hybrids can be classified in three categories: those following species' introduction, those following man-made perturbations of the environment, and those which are truly natural (Agnèse et al., 1998). The latter can be excluded for the fishes in the present study, but both of the first two categories are possibilities because *O. niloticus* was introduced and/or could have escaped from dams upstream.

Although it is now possible to identify F_1 hybrids, it would be more difficult to identify successive generations if the hybrids are fertile and interbreed with the parent species. We do not know if the hybrids are sterile. However, the hybrid index (Campton and Utter, 1985) can then be used to identify such individuals and to provide evidence for hybridisation. The index measures the relative probability that the combined genotype for a particular fish at several loci could have arisen by random mating within each of the species, and it can be used to classify individual fish based on the allele frequencies at the diagnostic loci.

Red tilapia (*Oreochromis* spp.) is a hybrid produced by the inter-breeding between *O. niloticus* and *O. mossambicus* (Fitzgerald, 1979), or it could be a mutant of *O. mossambicus* (Ferreira, 1998). The biology and behaviour of red tilapia is extremely similar to that of the common mouthbreeding tilapia. Red tilapia is also omnivorous, reproductive and euryhaline, and is highly resistant to diseases. Having a glorious reddish colouration and lacking black colouration on the peritoneum, red tilapia look very similar to sea bream (*Chrysophrys major*), and are highly preferred by the consumers (Liao and Chang, 1983). It is for these reasons that red tilapias are cultured, and similarly why biologists dread that they escape into natural waters. There is, however, a large misconception whereby any tilapia that appears red, orange, gold, or pink in colour is termed a "red tilapia", and that they are always hybrids. Red tilapia can be formed when two individuals from the same species, which have mutations for colour, are crossed. The progeny is then also crossed, and so the red colour is bred into a pure species. This was the case with the RAU red tilapia (Ferreira, 1998). Allozyme analysis in the present study confirmed that they were *O. mossambicus*, and their morphological data corresponded to that of *O. mossambicus* as defined by Skelton (1993). Natural red tilapias usually do not survive as they are soon eradicated through natural selection by predatory birds and larger fish.

The end result of hybridisation between any two tilapia species is unpredictable. In most cases, the hybrids in fact have a lower adaptive value than either of the two pure species and these hybridisations usually lead to the disappearance of one of the two pure species and eventually the hybrids as well (Agnèse et al., 1998). For example, in Lake Itasy, in Madagascar, *O. macrochir* was introduced in 1958 and *O. niloticus* in 1961. In 1965 and 1966 intermediate specimens between these two species were harvested and named 3/4 tilapia (Daget and Moreau, 1981). These hybrid individuals had a noticeable pharyngeal bone resembling that of *O. niloticus* but a morphology closer to that of *O. macrochir*. Between 1963 and 1969, the hybrid population in the captures went from 5% to 74%. *Oreochromis macrochir* was considered a vanished species in 1971. Finally, the *O. niloticus* became predominant. On the contrary, in Lake Ihema, Rwanda, *O. macrochir* was introduced near the end of the 1960s, after the introduction of *O. niloticus* in the 1940s. Hybrids were observed in the 1970s. From 1983 to 1987, the proportion of *O. niloticus* decreased from 30 to 20%, that of the hybrids increased from 10 to 20%, and the *O. macrochir* population remained stable at 60% (Micha et al., 1996).

In another example, Welcomme (1967), in the mid-1960s described the existence of hybrids between *O. niloticus* and *O. variablis*. These hybrids were all males. Other authors also suspected hybridisation with *O. esculentus*. Since then these two species have disappeared from Lake Victoria and *O. niloticus* is suspected of being the cause of these disappearances. The double experiment of the introduction of *O. niloticus* and *O. mossambicus* in Lakes Itasy in Madagascar and Ihema in Rwanda also shows that one cannot predict which species will win the competition. The consequences of the elimination of a species after hybridisation with another species are often also not known. In particular, the vanished species may have left some of its genes in the established species (Agnèse et al., 1998).

Conclusions

Freshwater fishes are the most threatened of all vertebrate groups exploited by humans. About 160 species are endangered and about one species per year becomes extinct. The threats include water abstraction, pollution, overfishing and the impacts of exotic species (Pullin, 1997). Our study indicated that hybrids between the exotic and endemic tilapias have occurred. Further sampling is needed to determine the extent to which *O. niloticus* has spread in the Limpopo River, and pools in the nearly dry Limpopo River bed containing *O. niloticus* individuals need to be treated with rotenone ($C_{23}H_{22}O_6$) to destroy them. Individuals in ponds alongside the Limpopo River (e.g. at Den Staat) must also be destroyed. Distribution throughout the whole river needs to be monitored and the public should be made aware of the danger of releasing exotic, invasive fish into river systems and dams. In addition, water abstraction from the Limpopo River (and all rivers for that matter) should also be prohibited because it is a vector for alien fish dispersion. As the Limpopo is an international river, all monitoring and eradication should be done in consultation with neighbouring countries. Immediate action is necessary since the river itself will spread the aliens when it flows again in the wet season. It is possible that the introduction of non-indigenous species can bring about a form of extinction by hybridisation and introgression (Rhymer and Simberhoff, 1996). On the other hand, the contaminated ponds may dry to destroy the aliens (as reported in Van der Waal and Bills, 1997) including the hybrids.

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References

AGNÈSE J-F (1998) Genetic impacts of some fish species introductions in African freshwaters. In: *Genetics and Aquaculture in Africa.* Editios de 1‘ Orstom Institut Francais de recherche scientifique pour le dêveloppement en cooperation. Collectia colloques et sêminaires, Paris, 1998.

AGNÈSE J-F, ADÈPO-GOURÈNE B and POUYAUD L (1998) Natural hybridisation in tilapias. In: *Genetics and Aquaculture in Africa*. Editios de 1‘ Orstom Institut Francais de recherche scientifique pour le dêveloppement en cooperation. Collectia colloques et sêminaires, Paris, 1998.

CAMPTON DE and UTTER MF (1985) Natural hybridization between steelhead trout (*Salmo gairdneri*) and coastal cutthroat trout (*Salmo clarci clarci*) in two Puget Sound streams. *Can. J. Fish. Aquatic. Sci.* **42** 110-119.

DAGET J and MOREAU J (1981) Hybridation introgressive entre deux especes de Saretherodon (Pisces, Cichlidae) dans un lac de Madagascar. *Bull. Mus. Nat. Hist.* **4** 689-703.

FERREIRA JT (1998) Personal communication. Rand Afrikaans Univ., PO Box 524, Auckland Park, 2006.

FITZGERALD WJ (1979) Red-orange tilapia – A hybrid that could become a world favorite. *Fish Farming Inst.* **6** 26-27.

HODGSON G (1998) Personal communication. Owner: Den Staat farm. Private individual.

LIAO I-C and CHANG S-L (1983) Studies on the feasibility of red tilapia culture in saline water. In: Fischelson L and Yaron Z (eds.) *Int. Symp. on Tilapia in Aquaculture*. Tel Aviv Univ. Publishers, Tel Aviv, Israel. 524-533.

McANDREW BJ and MAJUMDAR KC (1983) Tilapia stock identification using electrophoresic markers. *Aquacult.* **30** 249-261.

MICHA JC, CUVELIER R, TILQUIN C, MURAILLE B, BOURGEOIS M and FALTER U (1996) *Comparative growth of hybrids (F_1, F_2 and F_3) of* Oreochromis niloticus *(L.) and* O. macrochir *(Blgr).* 354-360.

NASON JD, ELLESTRAND NC and ARNOLD ML (1992) Patterns of hybridization and introgression in populations of oaks, manzanitas, and irises. *Am. J. Bot.* **79** 101-111.

POUYAUD L and AGNÈSE J-F (1995) Phylogenetic relationships between 21 species of three genera *Tilapia*, *Sarotherodon* and *Oreochromis* using allozyme data. *J. Fish Biol.* **47** 26-38.

PULLIN RSV (1997) International concerns on fish diversity and genetic resources management. In: Pullin RSV, Casl CMV, Abban EK and Falk TM (eds.) *Characterisation of Ghanaian Tilapia Genetic Resources for Use in Fisheries and Aquaculture*. *ICLARM Conf. Proc.* **52** 58.

RHYMER JM and SIMBERHOFF D (1996) Extinction by hybridization and introgression. *Annu. Rev. Ecol. Syst.* **27** 83-109.

RIESEBERG LH (1995) The role of hybridization in evolution: Old wine in new skins. *Am. J. Bot.* **82** 944-953.

RIESEBERG LH and ELLESTRAND NC (1993) What can morphological and molecular markers tell us about plant hybridization? *Critical Rev. Pl. Sci.* **12** 213-241.

SKELTON PH (1993) *A Complete Guide to the Freshwater Fishes of Southern Africa*. Southern Book Publishers. 325-330.

VAN DER BANK FH and VAN WYK B-E (1996) Biochemical genetic markers to identify hybrids between *Aloe arborescens* and *A. ferox* (Aloaceae). *S. Afr. J. Bot.* **62** 328-331.

VAN VUUREN NG, MULDER PFS, FERREIRA JT and VAN DER BANK FH (1989) The identification of hybrids of *Barbus aeneus X B. kimberleyensis* and *Labeo capensis X L. umbratus* in Hardap Dam, SWA/Namibia. *Madoqua* **16** 27-34.

VAN DER WAAL BCW (1997) Vreemde kurpers bedreig die bloukurper in die Limpopo. *Stywe Lyne/ Tight Lines* (December) 50-51.

VAN DER WAAL BCW and BILLS R (1997) *Oreochromis niloticus* in the Limpopo System. *Ichthos* **52** 14-16.
WELCOMME RL (1967) Observation on the biology of the introduced species of tilapia in Lake Victoria. *Rev. Zool. Bot. Afr.* **124** 249-279.
WOODRUFF DS (1989) Genetic anomalies associated with *Cerion* hybrid zones: The origin and maintenance of new electromorphic variants called hybrizymes. *Biol. J. Linn. Soc.* **36** 281-294.