ALLOZYME VARIATION WITHIN AND DIFFERENTIATION BETWEEN POPULATIONS AND GENERA OF REPRESENTATIVES OF THE TIGERFISH FAMILY

by

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The best of all things is to learn.
Money can be lost or stolen,
health and strength can fail,
but what you have committed to your mind
is yours forever.

Louis L'Amour

For what I've learned, appreciation to my two promoters Prof. Herman van der Bank and Prof. Gert Steyn.

For Karel, thank you for your loving support and interest.

For colleagues, family and friends, thank you for the continual support and interest.

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ABSTRACT

Since the systematics in the family Characidae from southern Africa were only reliant on some morphological traits, the determination of the genetic variation, genetic distances and phylogenetic relationships using different molecular techniques led to the formulation of this study. The first aim of the study was to describe the amount and pattern of genetic variation within and between populations. The analysis of the isozymes provided the first account of the amount, pattern and distribution of genetic variation within this family. A general introduction on the history, distribution, descriptions, taxonomy and some ecological notes from the literature on southern African Characidae species is included in Chapter 1. The second chapter dealt with the *Hydrocynus* genus that represents, morphologically, the largest member of this family. Other members of the Characidae family are relatively small species that do not exceed 300g. Twenty-five enzyme coding loci in two populations of *H. vittatus*, from Namibia and South Africa, were analyzed by horizontal starch gel-electrophoresis. Electrophoretic analysis of liver, white muscle, heart and testis samples revealed genetic variation at 20% (Upper Zambezi River, Namibia) and 36% (Olifants River, South Africa) of the protein coding loci studied. Average heterozygosity values ranged from 1.9% (Upper Zambezi River) to 4.6% (Olifants River), with a genetic distance value of 0.005 between these populations. The low amount of genetic variability in the former population compared to that of other fish species from the same geographical area, and to that of *H. vittatus* from the Olifants River, can be attributed to restricted gene flow due to isolation. Although morphological differences exist between the two populations, the extent thereof is not sufficient to regard them as subspecies.

The second aim of the study was to provide information on the comparison of the genetic variability between two populations of *Brycinus lateralis*. This aspect is decribed in chapter three of the study and included genetic variation, gene flow

and possible inbreeding determinations within these populations. Two populations from the Upper Zambezi and Cuando Rivers were studied by horizontal starch and polyacrylamide gel electrophoresis. Gene products of 21 protein coding loci, using whole fish samples, revealed genetic variation at 12 and 14 loci with average heterozygosity values of 0.177 (±0.038) and 0.217 (±0.046) respectively for the populations from the Upper Zambezi and Cuando Rivers. The relative high amount of genetic variation could be essential to allow fish to adapt to environmental changes associated with isolation. Other freshwater fish species from the same geographical area show lower genetic variation. The unbiased genetic distance between the two populations was 0.143, and indicates a high degree of genetic drift between isolated populations. Non-random mating probably occurred within sub-populations (F_{ST}=0.22) and little evidence of gene flow between the two populations was detected (F_{IS}=0.78).

The third part (Chapter 4) of the study considered the systematic status and the phylogenetic relationships of the southern African Characidae species, in particular those of the genus *Brycinus*. At present, no comparative genetic data are available with respect to their classification and phylogeny. Other African species were also included for outgroup comparisons in this part of the study. Thirteen species representing three families and five genera were studied using allozyme and DNA sequence data. Allozyme data indicated a basal position of the genus *Hydrocynus* within the Characidae, but failed to support the monophyly of the genus *Brycinus*. However, cytochrome-b analysis confirmed a monophyletic origin of this genus and all other genera involved, and thus supports the current classification of southern African species of the family Characidae. Moreover, mtDNA sequence data also supported the validity of all three characiform families present in southern Africa.

A morphological analysis was also performed on some of the species of the family Characidae to possibly aid in supporting the genetic relationships as was found in the previous chapter. Only limited characters were obtained for this comparative study. The cladogram confirms the allozyme and mtDNA data sets with clear distinction between the families Hepsetidae and Characidae. The monophyly of the

two *Brycinus* species was also supported.

Lastly the biochemical and genetic tools as applied in the previous chapters contributed to the establishment of a species identification service, the only of its kind in Africa as well as a focal laboratory for genetic characterization of animals. A service is rendered for the determination of the origin of blood, meat and meat products to the different meat industries, for the export and import of meat and meat products and to the consumer. Another valuable contribution to the African continent is the identification of the origin of species in blood and meat for forensic science. The techniques developed through this study now contributes to the solving of stock theft and poaching cases in collaboration with the South African Police Service. Training courses in species identification are also offered to other African countries. The second outcome of this study is the achievement of being the focal point for genetic characterisation of farm animal genetic resources for the Southern African Development Community (SADC) region comprising 13 countries. Techniques for the determination of the genetic status of populations and breeds important for the conservation and utilization of southern African indigenous animals are now established. The activities of these services are described and the importance to the relevant stakeholders are discussed.

The data of this study contributed to several manuscripts that have been submitted to recognised international scientific journals:

1) **KOTZE, A., VAN DER BANK, F.H. & STEYN, G.J.,** 1998. Allozyme variation in two populations of *Hydrocynus vittatus* (Pisces, Characidae). *S. Afr. J. Anim. Sci.* 28, 153-160; *Proc. 15th Congr. S. Afr. Genet. Soc.*, Stellenbosch 1-3 July 1996 (oral presentation).

2) **KOTZE, A., VAN DER BANK, F.H. & STEYN, G.J.,** 2001. Allozyme variation in two populations of *Brycinus lateralis* (Pisces, Characidae). *S. Afr. J. Anim. Sci.* Submitted and accepted.

3) **KOTZE, A., FALK, T. & VAN DER BANK, F.H.,** 2002. Systematic status and phylogenetic relationships of southern African Characidae species: results of allozyme and mtDNA studies. *Comp. Biochem. Physiol.* Submitted.

Some important contributions on the industrial application of the biochemical and genetic tools for species identification and genetic chracterisation are listed below:

1) **KOTZE, A. & JERLING, S.,** 1994. Identification of raw meats using electrophoretic patterns. *Proc. 33rd Congr. S. Afr. Anim. Prod. Soc.*, Warmbaths, 28-31 March, 103.

2) **KOTZE, A. & MULLER, G.H.,** 1994. Genetic relationships in southern African cattle breeds. *Proc. 5th World Congr. Genet. Applied Livestock Prod.*, Univ. Guelph, Ontario, Canada, 7-12 August 1994, 21: 413-416.

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8) **KOTZE, A.,** 1998. The genetic charcterization of animals using basic biochemical techniques. *Proc. 6th World Congr. Genet. Applied Livestock Prod.,* Armidale, Australia, 16-23 January 1998, 28, 123.

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11) **KOTZE, A.,** 1999. Meat species identification. *Int. Meat & Manufac. Congr.,* Kyalami, 6-8 June 1999 (poster).

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13) **SARGENT, L., VAN DER BANK, F.H. & KOTZE, A.,** 2000. Genetic variation in blood proteins within and between 19 sheep breeds from southern Africa. *S. Afr. J. Anim. Sci.* 29(3), 245.

14) **KOTZE, A., HARUN, M., OTTO, F. & VAN DER BANK, F.H.,** 2000. Genetic relationships of indigenous cattle breeds from Mozambique. *S. Afr. J. Anim. Sci.* 30(2), 92-97.

OPSOMMING

Die sistematiek van die familie Characidae van suidelike Afrika was slegs op morfologiese kenmerke gebaseer. Hierdie studie is dus geformuleer om die genetiese variasie, genetiese afstande en filogenetiese verwantskappe, met die gebruik van verskeie molekulêre tegnieke, te bepaal. Die eerste doelwit van die studie was om die hoeveelheid en patroon van genetiese variasie binne en tussen bevolkings te bepaal. Die iso-ensieme wat beskryf word, is die eerste weergawe van die aantal, patroon en verspreiding van die genetiese variasie binne die familie. 'n Algemene inleiding wat handel oor die geskiedenis, beskrywings, taksonomie en enkele ekologiese aantekeninge uit die literatuur oor siudelike Afrika Characidae spesies is saamgevat in Hoofstuk 1. Die tweede hoofstuk handel oor die *Hydrocynus* genus wat morfologies, die grootste lid van hierdie familie is. Ander lede van die Characidae familie is relatiewe klein spesies wat nie groter as 300g word nie. Vyf-en-twintig ensiem-koderende loki in twee *H. vittatus* bevolkings van Namibië en Suid-Afrika onderskeidelik, is geanaliseer met behulp van stysel-gel-elektroforese. Die elektroforetiese analise van lewer, spier, hart en testis monsters het genetiese variasie by 20% (Bo Zambezirivier) en 36% (Olifantsrivier, Suid-Afrika) van die proteïen loki getoon. Gemiddelde heterosigositeitswaardes was tussen 1.9% (Bo Zambezirivier) en 4.6% (Olifantsrivier), met 'n genetiese afstandswaarde van 0.005 tussen die bevolkings. Die lae genetiese variasie in eersgenoemde bevolking in vergelyking met ander visspesies van dieselfde geografiese area, en in vergelyking met *H. vittatus* van die Olifantsrivier, kan toegeskryf word aan beperkte geenvloei as gevolg van isolasie. Alhoewel morfologiese veskille tussen die twee bevolkings voorkom, is die mate daarvan nie voldoende om hulle as subspesies te beskou nie.

Die tweede doelwit (hoofstuk 3) van die studie was om die genetiese veranderlikheid tussen twee bevolkings te vergelyk om sodoende inligting te bekom van die genetiese variasie, geen vloei en moontlike inteling in bevolkings. Twee *B. lateralis* popluasies afkomstig van die Bo-Zambezi en Kwando riviere is ondersoek met

behulp van horisontale stysel en poli-akrielamied jel-elektroforese. Geen-produkte van 21 loki vanaf heel vismonsters, het genetiese variasie by 12 en 14 loki getoon met gemiddelde heterosigositeitswaardes van 0.177(±0.038) en 0.217 (±0.046) onderskeidelik vir die populasies van die Bo-Zambezi en Kwando riviere. Die relatiewe hoë genetiese variasie kan voordelig wees vir aanpassing by omgewingsveranderinge wat gepaard gaan met isolasie. Ander varswatervisspesies van dieselfde geografiese omgewing toon laer genetiese variasie. Die genetiese afstand tussen die twee bevolkings was 0.143 wat dui op 'n graad van genetiese verskuiwing tussen geisoleerde bevolkings. Nie-willekeurige paring het waarskynlik in sub-bevolkings plaasgevind (F_{ST}=0.22) en min geenvloei tussen die twee bevolkings (F_{IS}=0.78) is waargeneem.

Die derde doelwit van die studie (hoofstuk 4) was om die sistematiese status en die filogenetiese verwantskappe van die Characidae spesies van suidelike Afrika te bepaal, veral die spesies van die genus *Brycinus*. Huidiglik is geen vergelykende genetiese data beskikbaar oor die klassifikasie en filogenie nie. Ander Afrika spesies is ook ingesluit om mee te vergelyk as buite-groepe in hierdie gedeelte van die studie. Dertien spesies verteenwoordigend van drie families en ses genera is ondersoek. Iso-ensiem data het 'n basale posisie vir die genus Hydrocynus binne die Characidae aangedui, maar kon nie die monofilie van die genus *Brycinus* staaf nie. In teenstelling daarmee het die sitochroom-b analise 'n monofiletiese oorsprong van hierdie genus en al die ander betrokke genera gestaaf. Die huidige klassifikasie van die suidelike Afrika spesies van die familie Characidae word dus gestaaf. Gepaardgaande, staaf die mtDNA volgorde data ook die geldigheid van al drie die characiform families teenwoordig in suidelike Afrika.

'n Morfologiese analise is ook uitgevoer op sommige van die spesies van die Characidae familie om moontlik die genetiese verwantskappe te ondersteun soos in die vorige hoofstuk bepaal is. Slegs beperkte kenmerke is verkry vir hierdie vergelykende studie. Die kladogram ondersteun die iso-ensiem en mtDNS datastelle met duidelike verskille tussen die Hepsetidae en Characidae families. Die monofilie van die twee *Brycinus* spesies word verder ook gestaaf.

Laastens het die biochemiese en genetiese hulpmiddelde soos toegepas in die vorige hoofstukke bygedra tot die daarstelling van 'n spesie identifikasie diens, die enigste van sy soort in Afrika asook 'n fokus laboratorium vir genetiese karakterisering van diere. 'n Diens word gelewer aan die verskillende vleis industrieë, vir die uitvoer en invoer van vleis en vleis produkte en aan die verbruiker vir die bepaling van die spesie oorsprong van bloed, vleis en vleisprodukte. 'n Ander waardevolle bydrae tot die Afrika kontinent is die identifikasie van die spesie oorsprong in bloed en vleis vir die forensiese wetenskap. Die tegnieke wat ontwikkel is tydens hierdie studie dra nou by tot die oplos van vee- en wilddiefstal in samewerking met die Suid Afrikaanse Polisiediens. Opleidingskursusse vir spesie identifikasie word ook aangebied aan ander Afrika lande. Die tweede uitvloeisel van die studie is die prestasie om as die fokus laboratorium vir genetiese karakterisering vir plaasdier genetiese bronne binne die Suidelike Afrika Ontwikkelingsgemeenskap streek wat uit 13 lande bestaan, erken te word. Tegnieke vir die bepaling van die genetiese status van bevolkings en rasse, belangrik vir die bewaring en gebruik as deel van die suidelike Afrika inheemse diere, is nou in plek. Die aktiwiteite van hierdie dienste word beskryf en die belang daarvan vir die verskeie rolspelers word bespreek.

Die data van hierdie studie het verder bygedra tot verskeie publikasies wat in erkende internasionale wetenskaplike joernale verskyn het:

1) **KOTZE, A., VAN DER BANK, F.H. & STEYN, G.J.,** 1998. Allozyme variation in two populations of *Hydrocynus vittatus* (Pisces, Characidae). *S. Afr. J. Anim. Sci.* 28, 153-160; *Proc. 15th Congr. S. Afr. Genet. Soc.*, Stellenbosch 1-3 July 1996 (seminaar).

2) **KOTZE, A., VAN DER BANK, F.H. & STEYN, G.J.,** 2001. Allozyme variation in two populations of *Brycinus lateralis* (Pisces, Characidae). *S. Afr. J. Anim. Sci.* Ingedien en aanvaar.

4) **KOTZE, A., FALK, T. & VAN DER BANK, F.H.,** 2002. Systematic status and phylogenetic relationships of southern African Characidae species: results of allozyme and mtDNA studies. *Comp. Biochem. Physiol.* Ingedien.

Van die meer belangrike bydraes vir die industriële toepassing van die biochemiese en genetiese hulpmiddele vir spesie identifikasie en genetiese karakterisering word hieronder gelys:

1) **KOTZE, A & JERLING, S.,** 1994. Identification of raw meats using electrophoretic patterns. *Proc. 33rd Congr. S. Afr. Anim. Prod. Soc.,* Warmbaths, 28-31 March, 103.

2) **KOTZE, A & MULLER, G.H.,** 1994. Genetic relationships in southern African cattle breeds. *Proc. 5th World Congr. Genet. Applied Livestock Prod.,* Univ. Guelph, Ontario, Canada, 7-12 August 1994, 21: 413-416.

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9) **STRYDOM, P.E. & KOTZE, A.,** 1999. Characterization of indigenous African cattle breeds in relation to carcass characteristics. *S. Afr. J. Anim. Sci.* 70(2), 241.

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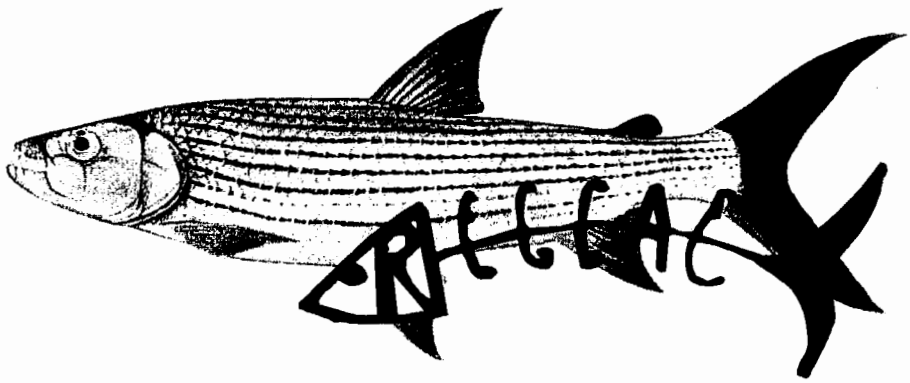
CHAPTER 1

Introduction



CHAPTER 1

Introduction

1.1 HISTORY OF FRESH WATER FISH TAXONOMY IN SOUTH AFRICA

The first descriptions of animals in the publication of the tenth edition of Linnaeus's *Systema Naturae* in 1758 did not contain descriptions of any indigenous southern African fish species. Burchell in 1822 first described the indigenous yellowfish and barbel. After that period, Smith conducted expeditions into the interior in 1834-36. Peters later led an expedition to the lower Zambezi and described many new species. In 1861 the French Consul at the Cape, F. Castelnau, described freshwater fishes from the Cape as far as the Okavango Delta. He also described the well-known tigerfish. Several European scientists, among them Boulenger from the British Museum, made important contributions during the 19th and early 20th centuries. The first comprehensive catalogue was written by dr. Gilchrist and his assistant mr. WW Thompson (1913-17) from the South African Museum. Revisions were done by Barnard and later Jubb described new species and clarified and identified others. The book: "Freshwater Fishes of Southern Africa" was published in 1967 by Jubb and during the same time other authors followed, such as Jackson: "The Fishes of Northern Rhodesia", 1961; Bell-Cross's "The Fishes of Rhodesia", 1961 and the reprint with Minshull "The Fishes of Zimbabwe", 1988. Prof. Skelton has emerged as Jubb's successor and is now regarded as the leading expert on the freshwater fishes of southern Africa. The book: "A complete Guide to the Freshwater Fishes of Southern Africa", 1993, has contributed significantly to information on indigenous fishes from this area. All the above information was taken from the publication by Skelton (1993).

Over hunderds of years information was gathered which contributed to surveys, studies on the biology of species and for collections of specimens in museums. The

South African Institute for Aquatic Biodiversity in Grahamstown, contains most of these collections. Systematic ichthyology in southern Africa during recent years is focussed on revisions and studies of phylogenetic relationships. New approaches and techniques are being applied and studies of early life history, behaviour, cytogenetic, biochemical and DNA technologies are advancing knowledge on our indigenous fish species. It is hoped that this study on the family Characidae of southern Africa will contribute to such valuable information.

This chapter includes: 1) the descriptions, taxonomy, ecology and distribution of the Characidae species occurring in the region, 2) a short review on the analysis of genetic data for systematic purposes and the determination of phylogenetic relationships as will be applied in this study and 3) the aims of the study.

1.2 DISTRIBUTION, DESCRIPTIONS, TAXONOMY AND SOME ECOLOGICAL NOTES ON SOUTHERN AFRICAN CHARACIDAE SPECIES

The characins is a large order of freshwater fishes from Africa and South and Central America (Figure 1). At one stage they were considered to be more closely related to the cypriniform fishes but it is now understood that they are more closely related to the catfishes (Brewster, 1986). Some like the tigerfishes and the small, attractive neon tetras are well-known while others like the piranha from South America are notorious.

Gery (1977) in the key to the New World characiforms descibed and redescribed most of the species from South America and Africa. These descriptions are mainly based on anatomical studies to determine the relationships between the different genera and species. The family name Characidae is derived from the classical Greek meaning "pointed stake" and probably refers to the sharp-pointed teeth of many species. It is easy to distinguish characins from the similar shaped cyprinids as the family has sharp teeth on the jaws and a small adipose fin (Skelton, 1993).

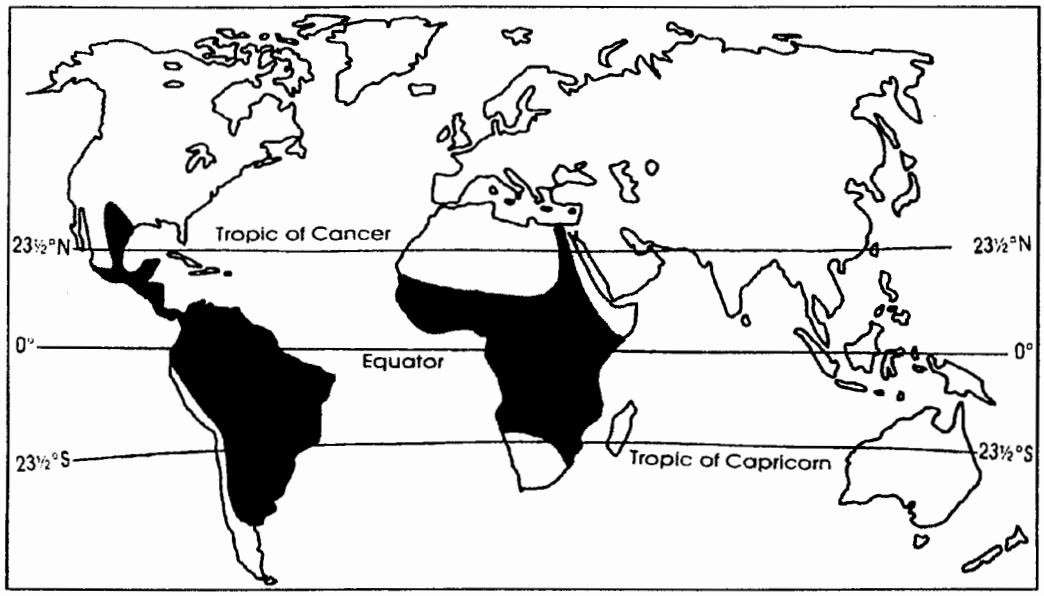


FIGURE 1. Map of the world indicating distribution of the family Characidae (Skelton, 1993).

Although the characids are more primitive, the histomorphology and ultrastructure of their hepatopancreas are similar to that of the cyprinoid and siluroid representatives of the cypriniform fishes (Geyer *et al.*, 1996). Few of the Characidae species are of current economic importance, but all play an important part in the ecology of the river systems where they occur, either as shiny shoaling species, bait, or as forage feeders. Some species are attractive in aquariums while some have the potential to be used in aquaculture.

The African characins are divided into four families with a contrasting number of 18 to 20 genera and over 100 (Skelton, 1993) to 200 species (Gery, 1977). The distribution of the different genera and species is indicated in Figure 2. In southern Africa family members are confined to tropical waters. Currently three families, viz Distichodontidae, Characidae and Hepsetidae, with only 12 species are recognised,. Within the Characidae family five genera namely *Brycinus, Rhabdalestes, Micralestes, Hemigrammopetersius* and *Hydrocynus* and six species occur in southern Africa.

FIGURE 2. Distribution of the family Characidae in Africa (Skelton, 1993)

The genus *Brycinus* Valenciennes, 1849 represents small to moderate sized shoaling fishes, similar to miniature tigerfishes. It is swift swimmers and is regarded by many an angler as a nuisance by stripping bait from hooks. It is frequently used as bait for tigerfish. There are an estimated 30 species in Africa with only two species occurring in southern Africa, namely *B. imberi* and *B. lateralis*.

Brycinus imberi (Peters, 1852) is distributed throughout the east coast rivers from the Phongola northwards to the Rufigi in Tanzania. This species is absent from the Upper Zambezi System. It is widespread in the Zaire system and coastal water bodies of West Africa (Figure 4). It occurs in a wide variety of habitats including larger rivers and floodplain pans and lagoons. *Brycinus imberi* feeds mainly on aquatic and terrestrial invertebrates, various seeds and plant material. Breeding occurrs during summer when it migrates to spawning sites after the rains (Paugy, 1980). The males mature at a smaller size (110mm forklength FL) than the females (120mm FL). The females can

reach a total FL of 180mm (Skelton, 1993). The lifespan of *B. imberi* is estimated at five years. The body is a silvery colour with yellow fins. The adipose fin as well as the top half of the eye are orange. The black spot behind the head and large dash on the caudal base are notable characteristics, but are not always distinct in life (Figure 3). The South African and Zimbabwean angling records are 0.3 kg. In Malawi the size is much smaller than the southern African species where the species reach an adult weight of only 0.13kg.

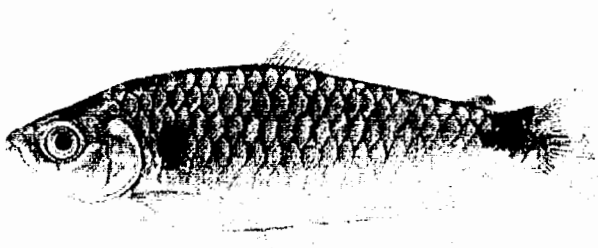


FIGURE 3. *Brycinus imberi*

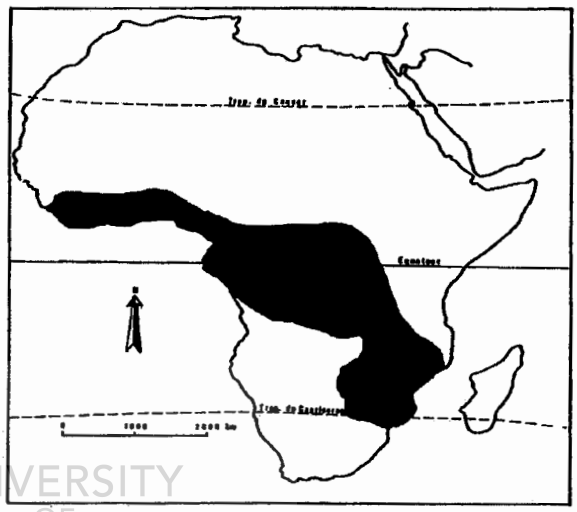


FIGURE 4. Distribution of *B. imberi* in Africa (Paugy, 1980)

Brycinus lateralis (Boulenger, 1900*),* is also known as the striped robber. It is distributed throughout the Zambezi System, Okavango, Cunene and Buzi Rivers and the St. Lucia Catchment in Kwazulu-Natal (Figure 6). Some *Brycinus* species are also found in the Zaire System (Luapula*). Brycinus lateralis* occurs in shoals in clear, slow-flowing or quiet, well-vegetated waters (Skelton, 1993). The species migrates upstream during the rainy season to breed. The dashtail *(Barbus poechii)* and the threespot barb *(Barbus trimaculatus)* show close similarity with *B. lateralis*. This phenomenon suggests mimicry between the three species. The diet consists mainly of small aquatic and terrestrial organisms. Characteristic external features are a fusiform body, leading rays of the anal fin that extend in males forming a rounded edge and the straight edge observed in immature males and females. The head is more or

less equal to the body depth and the mouth is terminal. Both jaws have two rows of sharp tricuspid teeth. A silvery, prominent black caudal dash surrounded by yellow and a yellow adipose fin include more characteristics of *B. lateralis* (Figure 5). The standard body length can be up to 140mm (Skelton, 1993). Both *B. imberi* and *B. lateralis* are often used as bait for catching tigerfish and largemouth breams *(Serranochromis spp)*. Quite often the species is caught in subsistence fisheries.

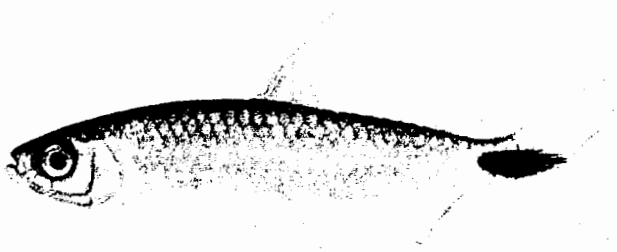


FIGURE 5. *Brycinus lateralis*

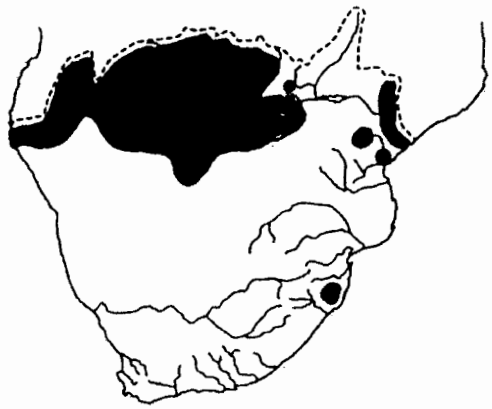


FIGURE 6. Distribution of *B. lateralis* in southern Africa (Skelton, 1993)

Balon (1971) described both *Brycinus* species as belonging to the genus *Alestes*. Before 1971, *B. lateralis* occurred mainly in the Upper Zambezi River System and the Kafue River, but has never been found in the Middle Zambezi River System. In the Middle and Lower Zambezi River Systems it is replaced by *B. imberi*. Both species have never been found together. After the creation of Lake Kariba in 1963 a large increase in the number of *B. imberi* and specimens of *B. lateralis* were found. Findings from 1965 to 1968 proved unambiguously the almost exclusive occurrence of *B. lateralis*. From catches during this period, thirty-five thousand *Brycinus* (*Alestes*) were collected of which only five were *B. imberi*. Both the species differ significantly in size, with *B. lateralis* being bigger than *B. imberi*. The sudden dominance of *B. lateralis* questioned the species validity and the possible creation of a neothenic

stock were raised. It was however proved by selected plastic and meristic characters that both species differ so significantly in many aspects, that it becomes impossible for a second species to be an ecotype or morpha of the same species. A possible explanation was found in the different spawning biologies of the two species. *Brycinus imberi* is better adapted to riverine conditions and to water without vegetation. This species found such conditions until 1963 when the operating water level was reached in Lake Kariba, but when the water level was artificially lowered, *B. imberi* lost its spawning grounds. *Brycinus lateralis* on the other hand, is known to spawn on submerged flora irrespective of the variation of the water level. This provided free space and exceptionally good spawning conditions for this species. It resulted that even individual specimens driven over the edge of the Victoria Falls could create a prolific population in a short time (Balon, 1971).

The genus *Micralestes* Boulenger 1899 is small silvery characins with distinctive sharp multicuspid teeth and a pair of teeth behind the outer row on the lower jaw. Africa hosts approximately 14 species with only one species occurring in the southern African region (Skelton, 1993).

Also known as the silver robber, *M. acutidens* (Peters, 1852) is widespread in the Cunene, Okavango, Zambezi and east coast rivers south to the Pongola and throughout the Zaire River System (Skelton, 1993) (Figure 8). The species was also noted in the Mlawula Nature Reserve in Swaziland (Clay, 1976; Hyslop, 1991). It occurs in shoals in clear, flowing or standing open water. *Micralestes acutidens* is omnivorous and often feeds on winged insects and zooplankton from surface waters. It reaches maturity within a year and the lifespan is estimated at three years. It is partial spawners with moderate fecundity with the female laying less than 700 eggs. Shoals of *M. acutidens* usually migrate upstream after the first summer rains where it breeds throughout the summer months. Some prominent characteristics are the compressed and fusiform body, the extended trailing edge of the anal fin in the males and the straight, slightly concave trailing edge of the anal fin in females. The

upper jaw has two rows of very distinctive sharp multicuspidate teeth. *Micralestes acutidens* can be described as silvery coloured fish with broad iridescent stripes along the body, pale yellow or orange fins and a dorsal fin with a distinctive black tip. The anal and pelvic fins have a white leading edge (Figure 7). *Micralestes acutidens* can reach a standard length of 80mm and makes an attractive aquarium fish for larger aquariums. It can also be utilised as a forage fish and as bait for tigerfish and pike (Skelton, 1993).

FIGURE 7. *Micralestes acutidens*

FIGURE 8. Distribution of *M. acutidens* in southern Africa (Skelton, 1993)

The genus *Rhabdalestes* Hoedeman, 1951 is small slender characins with sharp multicuspid teeth which are visible in two rows, one in the upper jaw and a single row in the lower jaw. The leading rays of the male anal fin are thick and curved with a dark bar along the base. There are seven species in this genus with one occurring in southern Africa.

Rhabdalestes maunensis (Fowler, 1935) occurs in the Kunene, Okavango, Upper Zambezi and Kafue River Systems (Figure 10). A similar, possible identical species, *R. rhodesiensis* occurs in the Zambian Zaire System (Lakes Bangweulu and Mweru and the Luapula River). *Rhabdalestes maunensis* occurs in shoals in shallow, vegetated marginal and floodplain habitats and feeds on small aquatic insects and other

invertebrates. It is a partial spawner and breeds when the water levels are high. The body of *R. maunensis* is typically compressed but more slender than other robbers and with pointed fins. In males, the leading ray of the anal fin expands and re-curves whereas females have a normal fin with a straight or slightly concave trailing edge. The body is translucent while the head is silvery with a bluish green iridescent band extending along the body. The black band along the base of the anal fin is characteristic of the species. The yellow adipose fin and the yellow caudal fin with the black edge are other distinguishable characters (Figure 9). The standard length of *R. maunensis* is approximately 60mm. This species is attractive in larger aquariums (Skelton, 1993).

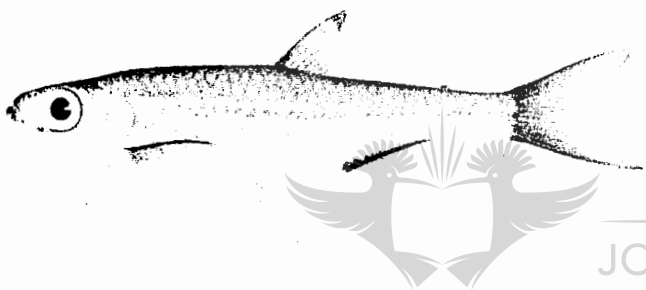


FIGURE 9. *Rhabdalestes maunensis*

FIGURE 10. Distribution of *R. maunensis* in southern Africa (Skelton, 1993)

The genus *Hemigrammopetersius* Pellegrin, 1926 consists of small African characins similar to *Rhabdalestes* but with an incomplete lateral line. The lower jaws projects beyond the upper jaws. There are four species known in Africa with one species, *H. barnardi* (Herre, 1936) being restricted to the Lower Zambezi, Pungwe and Buzi River Systems, the Upper Shire River and Lakes Malawi, Chilwa and Chiuta (Figure 12). It occurs in shoals in marginal vegetation of rivers and lakes and feeds on small insects and invertebrates. The body is compressed, fusiform and in males the leading ray of the anal fin expands and recurves. In females the fin is normal and the trailing edge straight. The teeth are tricuspid and arranged in single rows on both jaws. The body

of *H. barnardi* is translucent olive with a silvery head and abdomen and an iridescent green stripe along the body. The dorsal and caudal fins are sooty, with a yellow base (Figure 11). A black band above the anterior base of the anal fin passes across the midrays of the anal fin (Skelton, 1993). The standard length of the species is 40mm and it can be used as a potential aquarium species.

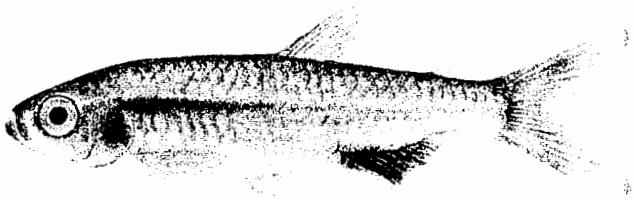


FIGURE 11. *Hemigrammopetersius barnardi*

FIGURE 12. Distribution of *H. barnardi* in southern Africa (Skelton, 1993)

The most well-known genus of the Characidae family is *Hydrocynus* Cuvier 1816. The name *Hydrocynus* means 'water dog' and is an apt description for these active, brightly striped, streamlined fishes, with typically large, sharp, widely spaced, interlocking teeth. The limbs of the lower jaw are hinged together, allowing the gape to expand laterally when striking prey. Tigerfishes are large, specialised predators regarded by many as the finest freshwater game fishes in the world. For example, the goliath tigerfish (*Hydrocynus goliath*) from the Zaire River System, is reputed to reach a standard length of 1.5m and can weigh up to 50 kg (Skelton, 1993).

Five species are currently recognised, of which one, *H. vittatus* Castelnau, 1861 occurs in southern Africa. This tigerfish species occurs in the Okavango, Zambezi and the warm water areas of the Lowveld and the coastal systems south to the Pongola River (Jubb, 1967; Van Loggerenberg, 1983; Winemiller and Kelso-Winemiller, 1994)).

Gaigher (1966) reported on the ecology of the tigerfish and decribed the occurrence of the fish specifically in the Incomati River System. It was common in the Incomati River up to Komatipoort and in the Sabie River up to the eastern border of the Kruger National Park (Figure 14). Only a few were found in the Crocodile River at Crocodile Bridge and in the Sabie River at Lower Sabie and Skukuza. Previous records indicate that before 1966 this species was more abundant in the higher reaches of the Komati and Crocodile Rivers. During June 1964 a hailstorm in the catchment areas of the Komati and Crocodile Rivers killed hundreds of *H. vittatus*. This appears to have eliminated the tigerfish which occurred above certain weirs in these streams as no reports of *H. vittatus* catches have since been received from these areas. *Hydrocynus vittatus* was very scarce higher up in the system and none was found above a weir at Coopersdal. It was absent in the Lomati and Sand Rivers (Gaigher, 1966). Although still widespread and common in certain areas like the Zambezi River System, numbers have declined in some rivers due to pollution, water extraction and obstructions like weirs and dams that prevent their passage (Skelton, 1993). In South Africa, the distribution range of *H. vittatus* has diminished over the past 50 years mainly as a result of migration barriers and reduced river flow. It was previously believed that *H. vittatus* migrates to the lowlands of Mozambique where it presumably spawns during December and January along the shallow, grass covered fringes of lakes and small streams (Pienaar, 1978). Van Loggerenberg (1983) stated that the construction of large irrigation dams such as the Fanie Botha Dam in the Letaba River created the established *H. vittatus* populations able to survive the winter outside the Kruger National Park. The large volume of water in such a dam provides a more temperature stable environment during the winter as opposed to the original river where only pools would have occurred. Steyn *et al.* (1996) however proved this wrong. According to Engelbrecht and Hoffman (1994) no tigerfish populations were found in the Letaba River outside the Kruger National Park. A few records were noted at the junction of the Letaba River and one at the Prieska Weir (1987).

Hydrocynus vittatus prefers warm, well-oxygenated water, tending to frequent the surface layers where it often falls prey to the swooping African fish eagle (Skelton, 1993). The piscivorous tigerfish of the Pongolo flood plain, which comprises 11% of

the fish population (Bruton and Kok, 1980), had the highest level of DDT that is used for indoor malaria control in the northen part of KwaZulu Natal. This species can thus contribute to the monitoring of polluted water systems and the environment (Bouwman *et al.*, 1990).

In Lake Kariba *H. vittatus* initially occurred near the shore and in river estauries until the introduction of the Lake Tanganyika sardine or kapenta *(Limnothrissa miodon)* allowed them to feed on the open waters of the lake. In notes of Badenhuizen (1966) it was clear that after the formation of Lake Kariba tigerfish was dominant in the Zambezi River. During the first year the growth rate accelerated drastically but decreased in the following years. This slower growth rate probably indicated a gradual returning stability of the tigerfish population in relation to its environment (Badenhuizen, 1966). The population dynamics of *H. vittatus* in Lake Kariba indicated that separate age groups do occur at annual intervals. Gaigher (1970) indicated that the females were distincly larger than the males and that the females grew faster. The males also appear to have a shorter lifespan.

Hydrocynus vittatus is notably absent from the Cunene, Kafue, Lake Malawi and the Upper Save-Runde River Systems, but is found northwards in Zaire, Lake Tanganyika, Rufigi and the large Nilo-Sudanian rivers in North and West Africa (Paugy and Guegan, 1989). It seems that *Hydrocynus vittatus* has evidently been successful in these areas as it was able to adapt to breeding in suitable affluent rivers around the lakeshores (Coulter, 1966).

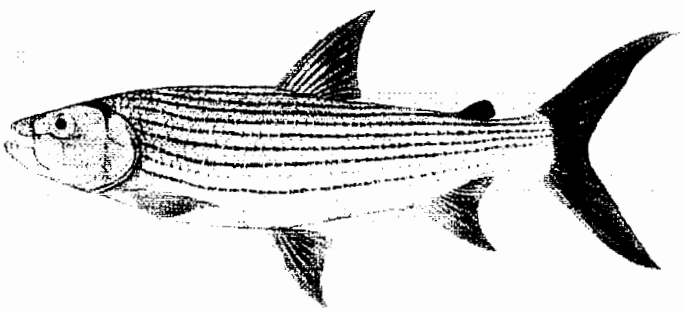


FIGURE 13. *Hydrocynus vittatus*

FIGURE 14. Distribution of *H. vittatus* in southern Africa (Skelton, 1993)

The body of the *H. vittatus* is fusiform with pointed fins which are caudally deeply forked. The head is large with bony cheeks and strong jaws, each with a series of eight large, protruding, sharply pointed teeth. The eyes have vertical adipose sleeves. Juveniles are silvery with distinctive parallel stripes that are visible above a size of 50 mm SL. The adult colouring is striking with a silvery body and head, a bluish sheen on the back and a series of parallel longitudinal black stripes. The adipose fin is black while the caudal fin varies from yellow to blood red at full intensity, with black trailing edges. The other fins are also yellow to red especially towards their bases (Figure 13). The tip and trailing edge of the dorsal fin is black. Males and females are similar in form with females reaching a length above 700 mm FL and males 500 mm FL. The South African angling record is 5.88 kg, the Zimbabwian record stands at 15.507 kg while the Malawian record is 7.03 kg (Skelton, 1993). The tigerfish is a major angling gamefish but also plays an important role as commercial species in Lake Kariba.

Hydrocynus vittatus breeds during the summer and adults migrate up or down stream to suitable spawning sites along flooded banks and lake shores. The fecundity is extremely high with as many as 780 000 ova in large females of 650-700 mm FL. Males mature at 2-3 years of age and at a average length of 300-400 mm FL. Most breeding females exceed 400 mm FL and are generally older fish than the male group (Winemiller and Kelso-Winemiller, 1994). Juveniles (30 mm FL) are pelagic, staying near surface waters during the day and descending at night. Larger juveniles change their habit and occupy marginal areas with vegetational cover and later, at a size more than 60-80 mm FL, revert to open water habitats. *Hydrocynus vittatus* attain lengths of 160-200 mm FL in their first year and up to 300 mm FL by the end of the second year. Most often the larger fishes are females with a lifespan of eight to nine years. Tigerfish are predators throughout their lifetime. Newly hatched fry less than 10 mm FL start feeding on small invertebrates, zooplankton and insects. Adults become exclusive fish feeders. Tooth development keeps pace with the changing diet of the young tigerfish. Fry of 10-25 mm FL have conical teeth that are replaced at a size of 25-35 mm FL by tricuspid teeth and again by conical teeth at the time when the diet is becoming increasingly piscivorous. Whole sets of teeth are replaced

at intervals throughout the lifespan of the tigerfish (Winemiller and Kelso-Winemiller, 1994).

Restocking of traditional *H. vittatus* waters was thus far only successful in the Kruger National Park (Steyn and Van Vuren, 1991). This species is very sensitive to handling and transportation over long distances. *Hydrocynus vittatus* could not be bred in captivity for many years and it was only as recent as 1996 that researchers from the Rand Afrikaans University were able to induce reproduction and the development of embryos and larvae. It was found that the successful artificial spawning of *H. vittatus* was being hampered by factors such as unsynchronised maturation of both sexes, discrepancy between the numbers of mature males and females, shortened breeding seasons and restricted access to breeding stock in general (Steyn and Van Vuren, 1991). In an induced reproduction study, gonadotropin releasing hormones (GnRh) were used in combination with dopamine receptor antagonists. Human chorionic gonadotropin (HCG) was administered in conjunction with catfish pituitary gonadotropin. Both sexes were successfully stripped and the eggs were inseminated artificially. *Hydrocynus vittatus* eggs are small (0.65mm diameter), demersal and slightly adhesive. Hatching occurs 22.5 hours after insemination and free embryos are pelargic and display continuous vertical movement for a period of three days. From these results as well as field observations, it can be concluded that *H. vittatus* spawns on sandy substrates in the vicinity of aquatic vegetation (Steyn *et al.*, 1996). According to Balon (1984; 1990), most fishes belong to the reproductive guild of non-guarding, egg scattering pelagic spawners that is characterised by small nutrient-poor ova produced in high numbers, delayed embryonic differentiation and a long larval period terminated by metamorphosis. *Hydrocynus vittatus* conform to these characteristics but additionally has negatively boyant eggs that are slightly adhesive for bentic or epibiotic incubation (Steyn *et al.*, 1996).

Hydrocynus vittatus feeds on whatever prey is most abundant at a particular time, but slender-bodied shoaling fishes like robbers (*Brycinus* and *Micralestes)*, minows

(*Barbus*) and the sardine (*Limnothrissa)* are favoured. Prey of 40% and more the size of the attacking tigerfish are taken from the side and then swallowed whole, usually head first (Balon, 1971).

The nomenclature of the genus has been problematic for many years. In 1757 Hasselquist described the taxon *Salmo dentex* of the Nile River which is now known as *Alestes dentex*. In 1775 Forsskal also described *Salmo dentex* as the nilotic fish of Hasselquist. Lacepede (1803) indicated that *S. dentex* of Hasselquist and Forsskal is identical to *Characinus dentex*. Geoffrey Saint Hilaire (1809) found differences between *S. dentex* of Hasselquist and Forsskal's *S. dentex.* In 1817 Cuvier created a new genus *Hydrocynus* with Forsskal's *S. dentex* as footnote. Cuvier renamed Forsskal's *S. dentex* to *H. forskalii*. This species was described as a fish with dark lateral lines, upper jaw covering the lower jaw and the presence of an adipose fin. Ruppell in 1829 renamed the tigerfish to *H. dentex.* The description of the tigerfish as seen by Boulenger in 1907 was: teeth in single rows, medium sized maxilla which moves under the second infra-orbital, elongated body, big supra orbital bone, fontanelles present in fingerlings, frontal posterior totally separated, huge air bladder with a posterior room three times the length of the anterior room and a strong ossified scull. In 1966 - 1969 Roberts (1974) claimed that *Hydrocynus* is related to *Alestes* on the following grounds: jaw and teeth structure, teeth replacement, posttemporal fossae and the presence of an orbito-sfenoid process. He used no osteological definitions for his classification. In this description he refers to *Hydrocynus* as having most probably *Alestes*-type ancestors. Examples of the fish described are still available in the British Museum, Museum National d'Histoire Naturelle, Paris, Musee Royal de l'Afrique Centrale, Institut Royal des Sceinces Naturelles, Brussels, Rijksmuseum van Natuurlijke Historie, Leiden and the California Academy of Sciences.

When the osteology of *Hydrocynus* is taken into account some special features unique to this genus need mentioning. The exoccipitals in *H. forskahlii* (Cuvier 1819) have an uneven pattern and up to a body length of 120 mm SL the capsule is mostly blown up which is characteristic of all characins. The genus *Hydrocynus* has a

bigger conformation than most other characiformes, thus the smaller declining blowing-up is a negative allometric phenomenon (Brewster, 1986). Brewster (1986) stated that most likely *H. vittatus* is a synomym of *H. forskahlii*. A study on the morphlogical comparison of *H. forskahlii* and *H. vittatus* indicated that these two sympatric species are distinct. The major differences between the two species as reported by Paugy and Guegan (1989) are in the proportions of the body and head with *H. forskahlii* being a more slender species with relatively shorter head and a dorsal fin which is more advanced on the body. Investigation of the monogeneans (gill parasites) which parasitise both species revealed the specific differences of the hosts. The species *H. vittatus* formerly synonymised with *H. forskahlii* was re-instated by Paugy and Guegan (1989).

These descriptions, ecological notes and distributions on the Characidae species from southern Africa were obtained from available information. The most economically important and well known Characidae species of this region is *H. vittatus,* and although no special attention was given to this species, most of the elaborative notes, descriptions and research by scientists concentrated on this tigerfish species and less on the economically unimportant species such as *R. maunensis* and *M. acutidens*.

The literature survey indicated that clear and full descriptions on distribution, taxonomy and ecology for the family Characidae from southern Africa exist. A genetic study of representatives from the family Characidae should therefore contribute to information on a different level such as relationships within and between populations. Since there are more than one method of establishing such relationships, some background information on the systematics and different techniques will be described from the literature for genetic characterisation of populations or species in the next section.

1.3 SYSTEMATICS AND TECHNIQUES FOR PHYLOGENETIC RELATIONSHIPS

Biological classification is one of the most ancient of scientific disciplines, considered by most to be founded by Aristotle (Sundberg & Pleijel, 1994). To be able to classify biological organisms and taxa in accordance with their evolutionary history, one must first construct the phylogeny. Dobzhansky (1935) declared that classification of organisms that existed before the advent of revolutionary theories has undergone surprisingly little change in the times following it. Systematics however, has recently undergone major transformation with more emphasis on having classifications accurately reflecting patterns of phylogenetic relationships. A classification aiming to represent evolutionary history must logically start with a reconstruction of phylogenetic relationships (Sundberg & Pleijel, 1994).

Morphological, biochemical and molecular data can for example be analysed with the use of cladistic methodology and a variety of mathematical methods that are available (Felsenstein, 1981; Swofford, 1985). Systematics are closely linked to evolution with no clear-cut boundary. While the evolutionary geneticist investigates the mechanisms of evolution, the systematist on the other hand uses information for the accurate placement of populations, species or taxa by keeping evolution and interrelationships in mind (Thorpe and Solé-Cava, 1994).

Morphology has not always been a useful guide for identifying phylogenetic relationships at lower taxonomic levels. In many cases, too little morphological differences and convergence are present between unrelated species. Although morphological traits are heritable, it is generally of little use for inferring the genetic structure of populations. With the advent of new technologies such as molecular techniques it is possible to indicate genetic distinctness between species although groupings were morphologically similar. The opposite is also true where morphologically or behaviourally distinct taxa were proved to be genetically undifferentiated (Swofford, 1985).

One major problem in the past was to obtain information on gene frequencies from natural populations. The use of molecular methods in population genetics and systematics began with the discovery of the structure of DNA in 1953 by Watson & Crick. Molecules have provided characters for phylogenetic reconstruction. The most commonly used molecular tools can be divided into two families. The first is a measure of similarity between organisms extracted from physical and chemical interactions between molecules by such methods as isozyme electrophoresis (Avise, 1974; Murphy *et al.* 1990), DNA hybridization, immunological distances, Random fragment length polymorphisms (RFLP) (Moritz and Hillis, 1990) and random amplification of polymorphic DNA (RAPD) (Hoetzel, 1992). In the second family the structure of the molecule is identified, from which a comparative approach allows phylogenetic investigations (Ferguson, 1995). These include nucleic and amino-acid sequences (Hillis & Moritz, 1990) and to a certain extent, karyological data.

With the development of isozyme electrophoresis it was possible to obtain gene frequency data from natural populations. Data in many cases confirmed or refuted old theories. It however produced new genetic paradigms such as genetic variation levels, population structure and differentiation dynamics of populations. Biochemical studies using the electrophoresis of proteins that are primary gene products have the advantage that the molecule is of known genetic derivation, normally a direct product of a single gene locus and that in almost all cases the molecular structure is solely genetically determined and likely to be free from environmental modification. Therefore, between related populations or species, differences in the structure of particular proteins are likely to be genetic (Utter *et. al.*, 1974; Thorpe and Solé-Cava, 1994).

Discrete characters have to be homologous, independent and variable. Using RFLP these authors argue that the assumption of character independence is violated. Data from RAPD studies are also not independent and may not be homologous. Data from random amplification of DNA are useful for typing but should be used with caution for phylogenetic purposes.

The structures of sequences are not directly observed but revealed by a factor, for example electric field, restriction enzyme and mix of probes that is more integrated. This factor can produce artificial homoplasy.

In the second family the structure of the molecule is identified, from which a comparative approach allows phylogenetic investigations (Ferguson, 1995). These include nucleic and amino-acid sequences (Hillis *et al.,* 1990) and to a certain extent, karyological data.

mt-DNA Sequence data is a very powerful tool for taxonomic and evolutionary studies as it was hoped that some insight will be gained from using molecular tools. Problems of character adaptation and convergengce, which can obscure the morphological analysis, are much reduced when using sequences of marker genes. These marker genes are not those controlling the development or the morphology of a species, but rather those encoding enzymes or rRNA (Avise, 1994; Hillis *et al.,* 1996). Sequences of mitochondrial genes (cytochrome-b gene) are often being employed to reconstruct the evolutionary past of vertebrates. In these genes deletions, inversions and insertions are of minor importance (Meyer *et al.,* 1990; Zhu *et al.,* 1994; Lydeard *et al.,* 1995).

This study therefore contributed to catalogue information on different data sets to confirm or contradict the speciation within the family Characidae from southern Africa. In spite of the new wave of molecular techniques that are becoming more and more useful for phylogenetic comparisons, Thorpe and Solé-Cava (1994) believed that allozyme analysis should be used as the basic method for systematics, principally at the species level. It is significantly cheaper to perform and much more robust without having to concentrate on the technique as such. This study mainly concentrated on obtaining data using allozyme analysis (Chapters Two and Three) whereas Chapter Four consists of a combination of both allozyme and DNA sequence data. In Chapter Five some morphological characters are analysed. The main objective of these studies is to accumulate genetic distance data to estimate

phylogenetic relationships between various species. Numerous tree building techniques are available and the usual method is to construct a phenetic dendrogram. Sometimes these alternative methodologies for contructing dendrograms can lead to trees which differ slightly or substantial in detail (Sundberg *et al.,* 1990)

The untapped and unmapped genes of the life forms on this planet will provide insight into our own make-up and adaptability. As far as the economically relevant species such as the tigerfish are concerned, evolution is now human driven with the goal of meeting desired qualities. Evolution in species of no economic relevance, for example some of the *Brycinus* species, is being constrained by the fast disappearance of all but the most uneconomical environments. Many species are suffering lowered numbers and a consequent inability to come up new strains able to meet changing conditions. Simpson (1991) has two simple choices. It can be put in bottles and kept on the shelf for future requirement or it can be used immediately to encourage and maintain the natural genetic diversity. Biotechnology can help meet both extremes and the range of options between while it is up to the professional biologists to inform. It is hoped that this study will contribute to such knowledge. As a result of replacing categories and types with phylogenetically defined taxon names, nomenclatural stability will increase, and the classifications will contain the information most biologists find interesting and useful, namely evolutionary relationships (Sundberg & Pleijel, 1994).

As the systematics in the family Characidae are only reliant on morphological traits, the determination of the genetic variation, population structure and phylogenetic relationships using different molecular techniques led to the formulation of the different aims for this study.

1.4 AIMS OF THE STUDY:

1. The first aim was to establish whether samples, from which genes are being compared, are from the same freely interbreeding population or gene pool or from different gene pools. For this study *H. vittatus* individuals from two different locations were sampled. *Hydrocynus vittatus* from the Upper Zambezi River displays an unique colouration which is not present in specimens from the Olifants River (Kruger National Park). This part of the study aimed to provide information on the amount and pattern of genetic variation within and between these two morphologically different populations and to determine the amount of genetic differentation between the populations. This study provided the first genetic variability data on the family and genus (Chapter 2).

2. Secondly the study had to determine how different, or how closely related the comparative gene pools are and what genes do they share. This part of the study was performed on *B. lateralis* specimens from the Upper Zambezi and Quando Rivers. The genetic variability were compared between the two populations to provide valuable information on the genetic variation, gene flow and possible inbreeding in sub-populations and the effect of population subdivision (Chapter 3).

3. The third aim was to support or contradict the current classification of representatives of the family Characidae from southern Africa with regard to systematics and phylogenies. No comparative genetic data for the genera and species were available. Both allozyme and cytochrome b-data were analysed comparatively in this part of the study. Representatives from the characin family from West Africa included *Brycinus schoutedeni, Brycinus longipinnis, Brycinus kingsleyae, Brycinus taeniurus, Distichodontidus hypostomatus* and *Distichodontidus notospilus* whereas *Brycinus affinis* from East Africa were included firstly to distinguish between the three families, Hepsetidae, Characidae and Distichodontidae and secondly to support the different genera of the Characidae family from southern Africa (Chapter 4).

4. The fourth aim was to compare a limited number of Characidae species with limited morphological characters to possibly aid in supporting the genetic relationships as found in the previous chapters, especially within the *Brycinus* genus and other Characidae species from southern Africa (Chapter 5).

5. Finally, the technical knowledge gained from this study was applied by establishing a routine service for the identification of different tissues on a species level for forensic purposes, as a service to consumers and the meat industries. Another application gained from this study is the genetic characterization of farm animal genetic resources that is currently regarded by the FAO (Food and Agricultural Organisation of the United Nations) as being the focal-point for the SADC (Southern African Development Community) region (Chapter 6).

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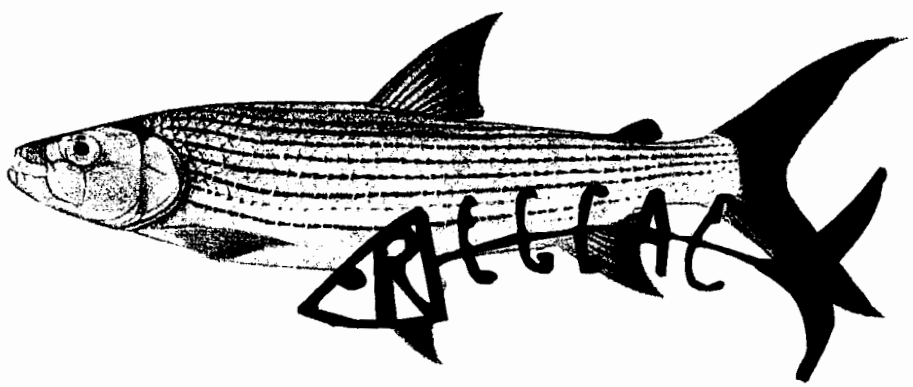
CHAPTER 2

Allozyme variation in two populations of *Hydrocynus vittatus* (Pisces, Characidae)



CHAPTER 2

Allozyme variation in two populations of *Hydrocynus vittatus* (Pisces, Characidae)

2.1 INTRODUCTION

This part of the study aimed to establish the gene pool status of similar interbreeding populations. The amount and pattern of genetic variation within and between two morphologically distinct populations as well as the amount of genetic differentiation between the populations can provide the first genetic variability data on this family and genus.

The scientific name of the tigerfish from southern African freshwaters was, for many years, known as *Hydrocynus vittatus*. The name means "striped water dog". The application of the genus name *Hydrocynus* to the African tigerfish has been a subject of controversy (Weitzman and Fink, 1983; Brewster, 1986). With a revision of the genus of tigerfish species from Africa, Brewster (1986) concluded that the southern African species was the same species as *H. forskahlii* (Cuvier), until then known only from the Nilo-Sudanic region (West and North Africa). More recently, Paugy and Guégan (1989) re-investigated the taxonomy of certain tigerfish species. Because of major morphological differences and supportive parasitological evidence, the latter authors re-instated *H. vittatus*. They concluded that *H. vittatus* occurs in southern Africa, whereas two other species, *H. forskahlii* and *H. brevis* occur

further north in the Nilo-Sudanic region. The ecological differences between the species is further manifested in their morphology and ecology (Skelton, 1990).

Hydrocynus is endemic to Africa and belongs to the Characidae which is one of the largest families of freshwater fishes found in Africa and the neotropics (Kenmuir, 1972; Skelton, 1993). *Hydrocynus vittatus* has an extensive distribution in Africa, from the Nile and Congo Rivers to rivers of West Africa through to the Zambezi, Okavango, Limpopo and the Pongola Rivers in the south (Jubb, 1967; Gaigher, 1970). It is notably absent from the Kunene, Kafue, and the Upper Save-Runde Rivers and Lake Malawi (Skelton, 1993). In most of these open water systems, the tigerfish represents the major piscivorous fish and mainly feeds on cichlid fishes, *Hepsetus odoe* and small characids (Winemiller and Kelso-Winemiller, 1994).

In southern Africa, tigerfish are found in the northern (upper), central (middle) and southern (lower) regions of the Zambezi River and associated flood plains (Winemiller & Kelso-Winemiller, 1994). South from the Zambezi, *H. vittatus* is mainly restricted to certain rivers in the Kruger National Park, the lower reaches of the Pongola River System in northern Kwa-Zulu Natal (Pienaar, 1978), the Komati River and Umbeluzi, Swaziland. This species supports important commercial and recreational fisheries in southern Africa, especially in the Zambezi, Okavango and Chobe Rivers and Lake Kariba (Winemiller and Kelso-Winemiller, 1994). It has a well deserved reputation as the most sought-after sport fish on the African continent. In South Africa increasing numbers of dams, weirs and reduced flow conditions in relevant rivers effectively prevent the annual migration of tigerfish to their spawning areas. This leads to the establishment of isolated populations in such rivers (Van Loggerenberg, 1983).

Tigerfish from the Upper Zambezi River display an unique colouration (Steyn *et al.*, 1995). Spectacular colour differences exists between mature males and females with the males having very prominent yellow ventral and caudal fins. The ventral section of the caudal fin has a bright red colouration. Mature females from the Upper Zambezi River have orange fins, that resembles the colouration of both

sexes from the Kruger National Park. With the recent possibility to exploit the commercial potential of *H. vittatus* by artificial spawning (Steyn *et al.*, 1995), the importance of fundamental knowledge of the genetic structure of the species became evident. A literature survey revealed that genetic variability in tigerfish has not been studied previously.

The use of molecular methods to study genetic variation of natural populations has provided considerable insight into population genetics and evolutionary processes (Grant and Leslie, 1993). Research on DNA markers is proceeding in several international laboratories and it is hoped that DNA-based genetic data continue to become available and at the same time more affordable. Isozyme analysis has been the standard molecular technique in fish population genetic research for three reasons, firstly it is inexpensive, secondly the method allows for quick processing times and the third asset is that isozyme data often constitute the largest existing genetic data set for many organisms, both within and between species.

This study therefore aims to provide information on the amount and pattern of genetic variation within and between two morphologically different populations of tigerfish, and to determine the amount of genetic differentiation between these populations by applying enzyme gel-electrophoresis.

2.2 MATERIAL AND METHODS

Heart, liver, muscle and testis samples were obtained from 35 and 40 sexually mature individuals from the Upper Zambezi River (24°18'S; 17°28'E) and the Olifants River System (31°07'S; 24°27'E) respectively. These populations will be referred to as ZAMBEZI and OLIFANTS henceforth. Samples were stored in liquid nitrogen and transported to the laboratory. Tissues were analyzed by horizontal starch gel-electrophoresis using TC (Whitt, 1970), RW (Ridgway *et al.*, 1970) and MF (Markert and Faulhaber, 1965) buffer systems and 13% gels as applied by Van der Bank *et al.* (1992). Samples were analyzed for activity at 15 enzymes, comprising 25 loci. Gel

banding patterns were interpreted according to the method used by Van der Bank *et al.* (1989) and the locus nomenclature used was as described by Shaklee *et al.* (1990).

TABLE 1: Locus abbreviations, enzyme commission numbers (E. C. No.), tissues and buffers giving the best results for each isozyme.

ENZYME	LOCUS	E.C. No.	TISSUE	BUFFER
Alcohol dehydrogenase	*ADH-1	1.1.1.1	M	MF
	*ADH-2		L	MF
Esterase	ES-1 to –4	3.1.1.-	L	RW
Glyceraldehyde-3-phosphate dehydrogenase	*GAPD-1, -2	1.2.1.12	L	RW,TC
Guanine deaminase	*GDA	3.5.4.3	T	MF
Glycerol-3-phosphate dehydrogenase	*G3PDH	1.1.1.8	L	TC
Isocitrate dehydrogenase	IDH	1.1.1.42	L	TC
L-Lactate dehydrogenase	*LDH-1	1.1.1.27	H,L,M,T	MF
	LDH-2		H,M	MF
	LDH-3		H,L	MF
Malate dehydrogenase	MDH-1	1.1.1.37	M	MF
	*MDH-2		M	MF
	*MDH-3		L	MF
Malic enzyme	*MEP	1.1.1.38	M	MF
Mannose-6-phosphate isomerase	*MPI	5.3.1.8	L	MF
6-Phosphogluconate dehydrogenase	PGD-1	1.1.1.44	L	TC
	*PGD-2		L	TC
Phosphoglucomutase	PGM	5.4.2.2	L,M	RW
Purine-nucleoside phosphorylase	*NP	2.4.2.1	T	MF
Superoxide dehydrogenase	*SDH	1.1.1.14	L	TC
Superoxide dismutase	*SOD	1.15.1.1	L	TC

*, Monomorphic loci; H, heart; L, liver; M, muscle; T, testis; MF, a continuous Tris, boric acid, EDTA buffer (pH 8.6) described by Market and Faulhaber (1965); RW, a discontinuous Tris, citric acid (gel pH 8.7), lithium hydroxide, boric acid (electrode pH 8.0) buffer system (Ridgeway *et al.*, 1970); TC, a continuous Tris, citric acid (pH 6.9) buffer system (Whitt, 1970).

Average heterozygosity (*H*) was calculated, using the formula of Nei (1975). The G-test for goodness-of-fit was used to test deviations of allele frequencies from expected Hardy-Weinberg proportions for polymorphic loci (Sokal and Rohlf, 1969), and the likelihood-ratio statistic was used to test the equality of allelic frequencies

among samples within and between populations (Smouse and Ward, 1978). Genetic distance (*D*) was calculated from allele frequencies using Nei's (1972; 1978) formulae. Different fixation indices were used to analyze genetic differentiation between populations (Wright, 1978), using BIOSYS-1 (Swofford and Selander, 1981), where F_{IT} and F_{IS} are the fixation indices of individuals compared with the total population and its subpopulations respectively and F_{ST} measures the amount of differentiation among subpopulations compared with the limiting amount under complete fixation.

2.3 RESULTS

Twenty-five enzyme coding loci provided interpretable results. Locus abbreviations, enzyme commission numbers, tissues and buffers giving the best results are listed in Table 1. Products of the following loci migrated cathodally: *ADH-2*, *GAPD-2*, *G3PDH*, *LDH-3*, *MDH-3*, *PGD-2* and *SDH*. In addition to these loci, staining was done for *AK*, *CK*, *GPI*, *HK* and *PEP* but neither of these systems showed sufficient activity or resolution to score it satisfactorily.

The heterozygotes at the *LDH* locus were five-banded, the allozyme products for *IDH*, *MDH* and *PGD* produced triple banded heterozygotes as expected for dimeric enzymes, and double banded heterozygotes for *ES* (Figure 1) and *PGM* as expected for monomeric enzymes. These allozyme phenotypes were therefore in agreement with the quartenary structure of the corresponding enzymes (Ward 1977). All four *ES* loci were best resolved with à-naphthyl acetate as staining substrate using liver tissue. No intraspecific genetic variation was found at the two *ADH* loci (Figure 2) and because no heterozygotes were observed at these loci, the subunit structure of this enzyme could not be established.

Liver tissue showed two monomorphic zones of *GAPD* activity. This is in accordance with that of cichlid fish where two loci were detected in muscle tissue (Cruz *et al.*,

1982). One monomorphic locus each was observed for *GDA*, *NP*, *G3PDH*, *MPI*, *SDH* and *SOD* in liver tissue and for *MEP* in muscle tissue. According to McAndrew and Majumdar (1983) and Basiao and Taniguchi (1984) two *G3PDH* loci occur in cichlids. In this study only one locus for *G3PDH* was observed and three *LDH* loci were best resolved in heart tissue. McAndrew and Majumdar (1983) found high activities in skeletal muscle, heart and liver of *LDH-1*, *-2* and *-3* respectively. Two *MDH* loci were observed in muscle (Figure 3) and one locus (*MDH-3*) in liver tissue in this study. The three loci for *MDH* is in accordance with results by Basiao and Taniguchi (1984) who observed three *MDH* loci, one being muscle specific, one being liver specific and a third being heart specific in tilapias. Two *PGD* loci were expressed in liver tissue. In cichlids only one locus was expressed for *PGD* (Cruz *et al.*, 1982).

Allele frequencies (see appendix 1 for input file for BIOSYS), coefficients for heterozygosity deficiency or excess (*d*), observed number of heterozygotes, Chi-square (X^2) values and individual heterozygosity values (*h*) are presented in Table 2. Loci where significant ($P<0.05$) deviations of allele frequencies from expected Hardy-Weinberg proportions occurred, are also listed. The degree of freedom for polymorphic loci was one in all cases.

The ZAMBEZI population indicated three heterozygous loci (*ES-1, -3, -4*) of which the allele frequencies approximated Hardy-Weinberg expectations. Deviations of allele frequencies occurred at *IDH* and *LDH-3* (Table 2) and no heterozygotes were observed at the latter locus. The OLIFANTS population showed close approximation of allele frequencies to Hardy-Weinberg proportions at four of the nine polymorphic loci (Table 2). Deficiencies of heterozygotes occurred at the *ES-1*, *-4*, *IDH*, *PGD-1* and *PGM* enzyme coding loci for OLIFANTS. The mean number of alleles per locus (*A*) was 1.20 (±0.08), *h* values ranged from 0.028 to 0.202 and the *H* value was 1.9% (±0.009) for ZAMBEZI. OLIFANTS had a *A* of 1.36 (±0.10), *h* values ranged from 0.025 to 0.399 and *H* was 4.6% (±0.021).

TABLE 2: Relative mobility (RM) of alleles, relative frequencies of polymorphic loci, observed (OBS) and expected (EXP) number of heterozygotes, coefficients for heterozygosity deficient (*d*) or excess, X^2 values and individual heterozygosities (*h*) for two populations of *Hydrocynus vittatus*.

Locus	RM	Upper Zambezi River Population (N=35)						Olifants River (Kruger National Park) Population (N=10)					
		Frequencies	OBS	EXP	*d*	X^2	*h*	Frequencies	OBS	EXP	*d*	X^2	*h*
ES-1	100 105	0.957 0.043	3	2.87	0.045	0.070	0.082	0.725 0.275	0	15.95	-1.000	40.000*	0.399
ES-2	90 95	1.000						0.988 0.012	1	0.99	0.013	0.006	0.025
ES-3	70 85	0.971 0.029	2	1.94	0.029	0.030	0.056	0.975 0.025	2	1.95	0.026	0.026	0.049
ES-4	60 55	0.986 0.014	1	0.99	0.014	0.007	0.028	0.975 0.025	0	1.95	-1.000	40.000*	0.049
IDH	100 95	0.886 0.114	2	7.09	-0.718	18.030*	0.202	0.975 0.025	0	1.95	-1.000	40.000*	0.049
LDH-2	65 50	1.000						0.975 0.025	2	1.95	0.026	0.026	0.049
LDH-3	-100 -95	0.943 0.057	0	3.77	-1.000	35.000*	0.108	1.000					
MDH-1	100 105	1.000						0.988 0.012	1	0.99	0.013	0.006	0.025
PGD-1	100 95	1.000						0.925 0.075	0	5.55	-1.000	40.000*	0.139
PGM	90 85	1.000						0.750 0.250	8	15.00	-0.467	8.710*	0.375
Average heterozygosity = 0.019 (± 0.009)								Average heterozygosity = 0.046 (± 0.021)					

* = Loci where significant ($P < 0.05$) deviations of alleles from expected Hardy-Weinberg proportions occurred.

Population differences were examined by calculating fixation indices for each locus as well as the mean value across all loci. The mean F_{IS} and F_{IT} values across all loci were 0.646 and 0.673 respectively. Wright's (1978) measure of differentiation, F_{ST}, between the populations studied was 0.076 and the loci that contributed most to population differences were *ES-1* (F_{ST}=0.101) and *PGM* (F_{ST}=0.143). Nei's (1972; 1978) genetic distances were both 0.005 between the two populations studied.

2.4 DISCUSSION

Genetic variation - ZAMBEZI displayed 20% polymorphism (at *ES-1*, *-3*, *-4*, *IDH* and *LDH-3*), whereas OLIFANTS displayed a much higher percentage (36%) at *ES-1* to *-4*, *IDH*, *LDH-2*, *MDH-1*, *PGD-1* and *PGM*. The two populations showed close similarity in genetic variability with *A* values of 1.20 and 1.36 for ZAMBEZI and OLIFANTS respectively. Deviations of allele frequencies from expected Hardy-Weinberg proportions in ZAMBEZI occurred at two of the five polymorphic loci, and for OLIFANTS at five of the nine polymorphic loci (Table 2). Such deviations can be due to non-random mating, gene flow, mutations, genetic drift and selection (Soltis and Soltis, 1988). Non-random mating and gene flow can therefore be excluded since these processes generally affect all loci. Heterozygote deficiencies occurred at all the loci which deviated in allele frequencies from expected Hardy-Weinberg proportions for both populations studied. The observed heterozygote deficiencies (Table 2) may be due to mutations and/or natural selection.

The estimate of *H* for ZAMBEZI was 1.9% (±0.009) whereas OLIFANTS had a much higher value (4.6%, ±0.021). Previous studies reported *H* values ranging from 4.7 to 31.6% (Avise and Aquadro, 1982). These authors compared the electrophoretical characters of more than 77 freshwater fish species and estimated H to average 5.4% for panmictic populations. The *H* value obtained for OLIFANTS compares favourably with estimates reported by the above authors. Although it was thought OLIFANTS was more isolated (Van Loggerenberg, 1988), this population has more genetic variation than ZAMBEZI. The *H* value for ZAMBEZI is low if compared to *H* for other fish species from the same geographical area. Average heterozygosity values ranged from 1.3 to 4.7% in a study of fifteen southern African cichlids, and 8.5% for *Synodontis leopardinus* (Van der Bank *et al.*, 1989; Van der Bank, 1993).

The higher level of genetic variation in OLIFANTS can be attributed to random sampling. During winter tigerfish migrate to Mozambique to escape the relatively colder winter conditions of the Kruger National Park. During spring, before flooding,

an upstream migration occurs probably to search for new breeding grounds and to expand their range. Samples were collected when the sexually active fish congregated below a barrier near the joining of the Letaba and Olifants Rivers in the Kruger National Park. Therefore, it is conceivable that a random sample of the total gene pool of this population was taken to effectively eliminate sample error. On the other hand, the lower level of heterozygosity in ZAMBEZI may be due to inbreeding. Inbreeding can occur due to the isolation of populations. Isolated populations can form since the tigerfish is a significant predator at the interface between river backwaters and channel habitats and food resource competition exists due to a high degree of habitat partitioning between larger size classes of the species (Badenhuizen, 1966; Winemiller and Kelso-Winemiller, 1994). Freshwater fish is often topographically subdivided into smaller breeding populations and small or isolated breeding populations tend to lose variation as a result of selection and inbreeding (Utter *et al.*, 1973; Grant and Stahl, 1988). Isolation of ZAMBEZI also occurred by the Victoria Falls, which forms a major barrier and prevent fish from migrating. Colour variation of the mature male tigerfish from the Upper Zambezi River have not been found in the tigerfish from Lake Kariba. This probably indicate that tigerfish do not survive the drop over the Victoria Falls, giving rise to a distinct gene pool above the falls (Steyn *et al.*, 1995). Adult fish, which display distinct territoriality, were sampled with artificial lures in the separate upper part of the Zambezi River at the Caprivi Strip, Namibia.

Genetic differentiation - The genetic distance was calculated between the two populations studied to determine if genetic different species are involved, since ZAMBEZI differs morphologically from OLIFANTS. The morphological differences are described in the introduction. Genetic distance values between congeneric species range between 0.03 and 0.61 with an average of 0.3 (Shaklee *et al.*, 1982). The *D* value (0.005) obtained for tigerfish in this study does not fit in this range. This indicates that conspecific populations (and not congeneric species as hypothesized) are involved. This is also reflected by the F-statistics. The population structure, as determined with the hierarchical F-statistics, gives the total inbreeding coefficient

estimate (F_{IT}) as 0.67 for the tigerfish studied. Therefore, the inbreeding in the individuals relative to the total populations is quite large and can be due to random genetic drift among the subpopulations studied. Values of F_{IS} in most natural populations are typically close to zero, that indicate random mating within subpopulations (Nei, 1986). The high F_{IS} value of 0.63 calculated in the present study is indicative of effective barriers to gene flow between the populations. No gene flow is possible between the two populations due to geographical constraints. The low fixation index (F_{ST}= 0.076), which is the amount of inbreeding due solely to population subdivision, also shows that there is low genetic differentiation between the two populations.

To conclude, the study indicated that ZAMBEZI and OLIFANTS are conspecific populations and in addition sufficient intraspecific genetic variability exists to distinguish the two populations. Individuals from OLIFANTS, with higher heterozygosity, should be used in artificial propagation programmes. Artificial breeding can lead to the establishment of domesticated broodstock which in turn may resolve the problems associated with the artificial production of wild tigerfish (Steyn and Van Vuren, 1991; Steyn, 1993; Steyn *et al.*, 1995). Restocking of tigerfish propagated by an artificial breeding programme is important for conservation and recreational fishing purposes.

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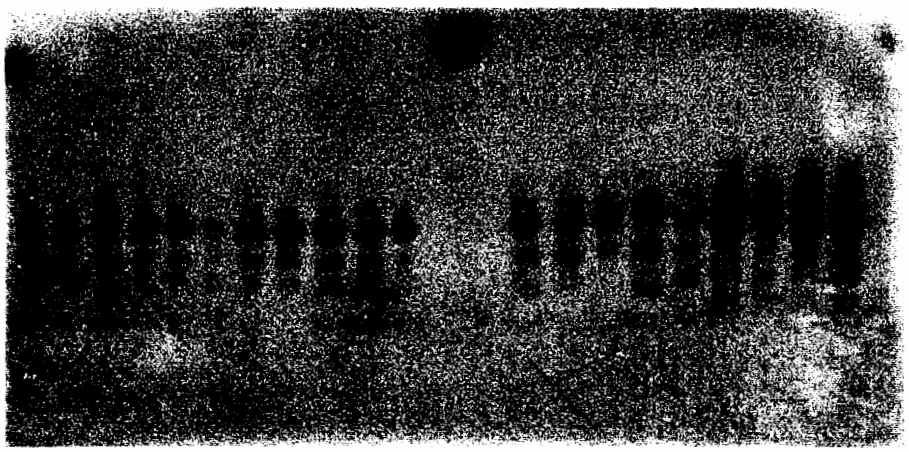
FIGURE 1. Esterase loci as expressed in liver tissue

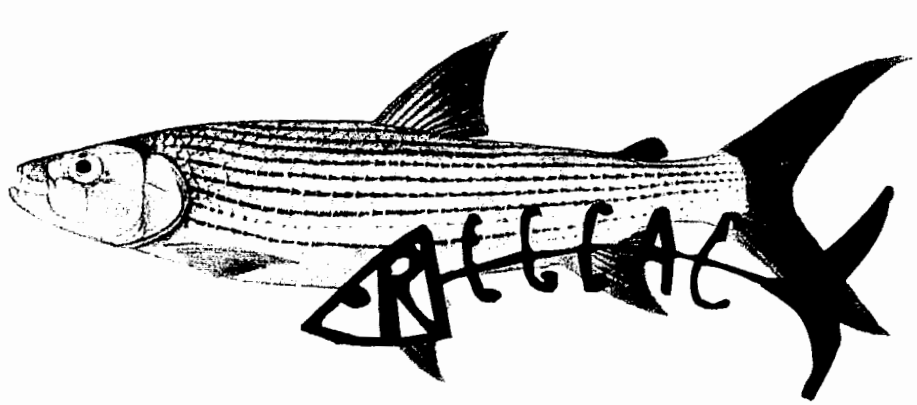
FIGURE 2. ADH locus as expressed in liver tissue

FIGURE 3. MDH loci in muscle tissue



CHAPTER 3

Allozyme variation in two populations of *Brycinus lateralis* (Pisces, Characidae)



CHAPTER 3

Allozyme variation in two populations of *Brycinus lateralis* (Pisces, Characidae)

3.1 INTRODUCTION

To determine how different, or how closely related the comparative gene pools are and what genes are shared, a study was compiled using *B. lateralis* specimens from two separate river systems.

The Characidae is a large family of African and South American freshwater fishes. All species in this family can be distinguished by having sharp teeth and a small adipose fin. Of the 18 genera and over 100 known African characin species, only 5 genera and 6 species occur in southern Africa (Skelton, 1993). The genus *Brycinus* shows typically large eyes, bony cheeks, sharp-pointed multicuspid teeth, large silvery scales, short dorsal fins and long-based, sexually dimorphic anal fins. *Brycinus lateralis* is a small to moderate sized shoaling species (101-150mm standard length), that are swift swimmers, usually in clear, slow flowing or quiet, well vegetated waters. They occur in the Zambezi, Okavango, Cunene and Buzi River Systems, and in the St. Lucia Catchment area of KwaZulu-Natal. The species is favourite bait among anglers for catching tigerfish and largemouth bream (*Serranochromis angusticeps*), and they have some commercial value for subsistence fisheries (Skelton, 1993).

A literature survey revealed that the distribution of *B. lateralis* is still unclear. Isolated populations occur throughout eastern and southern Africa in streams, which are isolated from the main Zambezi River. In contrast, *B. imberi* is encountered mainly in the Zambezi River, which suggest that this species was preceded by *B. lateralis*

during early migrations southward from the Zaire System. Both species, therefore, occurred sympatrically at one point in time, but continuous invasions of *B. imberi* resulted in the displacement of *B. lateralis* (Bell-Cross & Minshull, 1988). It appears that only isolated populations of *B. lateralis* survived due to the formation of physical barriers to movement from the main river system. On the other hand, a sudden replacement of an original species, *B. imberi* by *B. lateralis* was observed in Lake Kariba (Balon, 1971). It seems that individual specimens of *B. lateralis* driven over the edge of the Victoria Falls created a prosperous population, since this species was never before found in the Middle or Lower Zambezi River. This space was solely occupied by *B. imberi*. It is evident that *B. lateralis*, as well as *B. imberi* plays an important part in the ecology of the Zambezi River (Balon, 1971). In a previous study on tigerfish, results indicated lower genetic variability in a population sampled from the Upper Zambezi River compared to one from the Olifants River (Kotze *et al*, 1998). The low average heterozygosity value obtained in the former population was attributed to isolation. The major goal of this study was to determine if other Characidae species from Namibia also have low genetic variability due to isolation. To achieve this goal, two *B. lateralis* populations, one from the Upper Zambezi River and another from the Cuando River were sampled and the genetic variability compared. A study to determine the genetic variability within *B. lateralis* could provide valuable information on gene flow, possible inbreeding in subpopulations and the effect of population subdivision.

3.2 MATERIAL AND METHODS

A total of 63 *B. lateralis* individuals were obtained from the Upper Zambezi River (17°28'S; 24°18'E) and 60 from the Cuando River (18°27'S; 23°42'E). These populations will be referred to as ZAMBEZI and CUANDO henceforth. Fishes were stored in liquid nitrogen and transported to the laboratory. Whole fishes were homogenised and analysed by horizontal starch gel-electrophoresis using buffer systems and 13% gels as applied by Kotzé *et al.* (1998) and general proteins were analysed by horizontal polyacrylamide gel-electrophoresis as described by Gahne *et al.* (1977). Samples

were analysed for activity at 20 proteins. Gel banding patterns were interpreted according to Van der Bank *et al.* (1992) and the locus nomenclature used was as described by Shaklee *et al.* (1990). The BIOSYS-1 programme (Swofford and Selander, 1981) was used to calculate average heterozygosity (H) values using the formula of Nei (1975), allele frequencies deviating from expected Hardy-Weinberg proportions (Nei, 1975) and the likelihood-ratio statistic were compared to test the equality of allelic frequencies among samples within and between populations (Sokal & Rohlf, 1969). Nei's genetic distance (Nei, 1972) as well as Wright's fixation indices (Wright, 1978) was calculated.

3.3 RESULTS

Genetic variability determined at 12 protein systems, comprised 21 protein-coding loci in both populations (Table 1). Products of five loci, AAT-2, ADH, GPI-2, LDH-2 and MDH-3 migrated anodal. In addition to these loci, I have stained for AK, CK, G3PDH, GDA, HK, IDH, NP and SDH. These enzyme systems did not show sufficient activity or resolution to be scored satisfactorily.

ZAMBEZI displayed 57.1% polymorphism at loci and CUANDO 61.9%, with average number of alleles per locus ranging between 1.7 and 1.9. Allele frequencies for polymorphic loci, observed and expected heterozygosity (H_o and H_e) estimates, x^2-test values for allele frequencies deviating significantly ($P<0.05$) from expected Hardy-Weinberg proportions and the degrees of freedom for both populations are presented in Table 2. Twelve loci in ZAMBEZI and 14 loci in CUANDO revealed allele frequencies which deviated from expected Hardy-Weinberg proportions because a deficit of heterozygotes occurred at all loci (Table 2). No heterozygotes were observed at the AAT-1, GPI-1 and PGD loci for ZAMBEZI. For CUANDO, no heterozygotes were observed at the ADH, GPI-1, LDH-2, MDH-2, MPI, PROT-3 and -4 protein coding loci. Only alternative homozygotes were observed at these loci. Double-banded heterozygotes were observed at ES-1, -2, PGM, MEP, MPI, PROT-1, -2 and -3, which correspond to the monomeric structure of these loci. In Figure 1 and 2, allele variation at ES-1, -2 and PROT-1, -3 and -4 are indicated to

TABLE 1. Proteins and enzymes, locus abbreviations, enzyme commission numbers (E.C. No.) and buffers giving the best results for each protein

ENZYME	LOCUS	E.C. No.	BUFFER
Aspartate aminotransferase	*AAT-1*	2.6.1.1	MF
	**AAT-2*		
Alcohol dehydrogenase	*ADH*	1.1.1.1	MF
Esterase	*ES-1*	3.1.1.-	RW
	ES-2		
Glucose-6-phosphate isomerase	*GPI-1*	5.3.1.9	RW
	GPI-2		
L-Lactate dehydrogenase	*LDH-1*	1.1.1.27	MF
	LDH-2		
Malate dehydrogenase	*MDH-1*	1.1.1.37	MF
	MDH-2		
	**MDH-3*		
Malic enzyme	*MEP*	1.1.1.40	MF
Mannose-6-phosphate isomerase	*MPI*	5.3.1.8	MF
6-Phosphogluconate dehydrogenase	*PGD*	1.1.1.44	TC
Phosphoglucomutase	*PGM*	5.4.2.2	RW
Superoxide dismutase	**SOD*	1.15.1.1	MF
General protein	*PROT-1*		TS
	PROT-2		
	PROT-3		
	PROT-4		

*, Monomorphic loci;
MF, a continuous Tris, boric acid, EDTA buffer (pH=8.6)(Markert & Faulhaber, 1965); RW, a discontinuous Tris, citric acid, (gel pH=8.7)(Ridgway *et al.*, 1970); Lithium hydroxide, boric acid (electrode pH=8.0) buffer system (Whitt, 1970); TC, a continuous Tris, citric acid (pH=6.9) buffer system (Whitt, 1970); TS, a discontinuous Tris, sulphate (gel pH=9.0), Tris, borate (electrode pH=9.0) buffer system (Gahne *et al*, 1977).

demonstrate heterozygote deficiencies. The dimeric structure of the ADH and SOD loci could not be confirmed because no heterozygotes were observed, whereas GPI displayed a dimeric structure in seven *B. lateralis* individuals from ZAMBEZI. The tetrameric structure of LDH was reflected in two individuals from ZAMBEZI, which displayed five banded heterozygotes. The H value was 0.177 (±0.038) for ZAMBEZI and 0.217 (±0.046) for CUANDO; the unbiased genetic distance (*D*) (Nei, 1972) was 0.143 and the measure of differentiation (Wright, 1978) between populations was 0.214. The loci that contributed most to population differences were GPI-1 and PGM. The fixation index values (F_{ST}) were 0.406 and 0.460 respectively at these loci.

TABLE 2. Number of animals (N), relative frequencies of polymorphic loci, observed (H_O) and expected (H_E) number of heterozygotes, x^2 values and degrees of freedom (DF) for two populations of *Brycinus lateralis*.

UPPER ZAMBEZI RIVER POPULATION							CUANDO RIVER POPULATION					
LOCUS	N	ALLELE FREQUENCIES	H_O	H_E	X^2	DF	N	ALLELE FREQUENCIES	H_O	H_E	X^2	DF
AAT-1	21	A=0.857 B=0.143	0	5.14	21.00	1	52	A=1.000				
ADH	18	A=1.000					52	A=0.904 B=0.096	0	9.04	52.00	1
ES-1	51	A=1.000					32	A=0.469 B=0.531	4	15.94	17.95	1
ES-2	52	A=0.167 B=0.722 C=0.111	13	23.67	27.10	3	59	A=0.186 B=0.619 C=0.195	23	32.13	11.07	3
GPI-1	54	A=0.778 B=0.222	0	18.67	54.00	1	49	A=0.143 B=0.857	0	12.00	49.00	1
GPI-2	52	A=0.760 B=0.240	7	18.99	20.73	1	49	A=1.000				
LDH-1	19	A=0.211 B=0.789	2	6.32	8.87	1	42	A=1.000				
LDH-2	40	A=0.875 B=0.125	2	8.75	23.80	1	21	A=0.952 B=0.048	0	1.91	21.00	1
MDH-1	45	A=1.000					51	A=0.912 B=0.088	1	8.21	39.33	1
MDH-2	51	A=1.000					42	A=0.857 B=0.143	0	10.29	42.00	1
MEP	40	A=1.000					37	A=0.838 B=0.162	6	10.05	6.02	1
MPI	33	A=0.182 B=0.773 C=0.045	3	12.14	34.68	3	36	A=0.111 B=0.889	0	7.11	36.00	1
PGM	28	A=0.196 B=0.804	3	8.84	12.22	1	43	A=0.093 B=0.058 C=0.779 D=0.070	7	16.17	51.88	6
PGD	52	A=0.885 B=0.115	0	10.62	52.00	1	17	A=0.412 B=0.265 C=0.324	1	11.15	27.83	3
PRT-1	51	A=0.863 B=0.137	4	12.08	22.81	1	59	A=0.669 B=0.331	1	26.11	54.57	1
PRT-2	51	A=0.431 B=0.569	2	25.02	43.17	1	59	A=1.000				
PRT-3	42	A=0.917 B=0.083	1	6.42	29.93	1	59	A=0.441 B=0.559	0	29.09	59.00	1
PRT-4	41	A=1.000					59	A=0.915 B=0.085	0	9.15	59.00	1

3.4 DISCUSSION

The accuracy of polymorphism and heterozygosity estimates depend on the number and type of loci examined, sample size, population size, degree of migrations between different populations, population bottlenecks, phyletic age and the variability of the environment (Kirpichnikov *et al.*, 1990). Fourteen loci are commonly taken as the minimum number of loci for population genetic studies (Altukhov, 1981). It was possible to analyse 21 loci to estimate genetic variability, and compare the two populations (Table 1). Although more tissue types can increase the number of potential polymorphic enzyme coding loci that could be detected, the method used for the preparation of the samples in this study (i.e., whole fish were minced and organs were not removed for analysis due to small size), has restricted the number of loci found (Nevo, 1978). For example, activity was observed at AK, G3PDH, GDA, HK, IDH and SDH, but the concentration of these enzymes were not sufficient to score them satisfactorily in the samples analysed.

Table 2 indicates that deviations of allele frequencies from expected Hardy-Weinberg proportions occurred at all polymorphic loci in both populations. Examples of the polymorphic loci ES-1, -2, PROT-1, -3 and -4 (Figures 1 and 2) are included. Various factors can shift the equilibrium and disrupt the stability of a population, giving rise to change in the genetic structure, as ideal Hardy-Weinberg populations do not actually occur in nature (Altukhov, 1981). Significant deviations may occur due to natural selection, crossing and linking, inbreeding, sampling error, population bottlenecks and random genetic drift. Small sample size could account for allele frequency deviations at the AAT-1 and LDH-1 loci in ZAMBEZI and for LDH-2 and PGD in CUANDO (Table 2). Deviations of allele frequencies from expected Hardy-Weinberg proportions were due to a deficit of heterozygotes at all polymorphic loci in both populations (Table 2, Figures 1 and 2). For ZAMBEZI the biggest heterozygote deficiency occurred at AAT-1, GPI-1 and PGD, whereas the biggest heterozygote deficiency in CUANDO occurred at ADH, GPI-1, LDH-2, MDH-2, MPI, PROT-3 and -4. The deficiency of observed heterozygotes can probably be attributed to isolation

and the subsequent establishment of founder populations. Such founder populations can produce a larger number of progeny with certain genotypes than others (Altukhov, 1981). The establishment of isolated *B. lateralis* populations in streams (and not in the main river) was referred to in the introduction.

Average heterozygosity values were 17.7% for ZAMBEZI and 21.7% for CUANDO. These values are relatively high when compared to that of relatives from the same family. Estimated H values for tigerfish from the Upper Zambezi and Olifants Rivers were 1.9% and 4.6% respectively (Kotze *et al.*, 1998). Reported H values for other bonefishes from southern Africa ranged from 1.3% to 4.7% in a study of fifteen southern African cichlids sampled from the same geographical area (Van der Bank *et al.,* 1989) to as high as 8.5% for *Synodontis leopardinus* (Van der Bank, 1993). The heterozygosity estimates for the two *B. lateralis* populations are also much higher than the average value of 0.054 calculated for 77 vertebrate species (Avise & Aquadro, 1982) and 0.051 for 51 teleost species (Nevo, 1978). The high amount of genetic variation could be essential to allow these fish to adapt to environmental changes associated with isolation, as mentioned above. According to a hypothesis, some species experiencing their environment as coarse grained, reflect adaptation to a wider niche. Such species tend to have more genetic variability (Lawson *et al,* 1989). This theory may also explain the establishment of prosperous populations of *B. lateralis* in a short time in Lake Kariba (Balon, 1971). The breeding biology of *B. lateralis* allows spawning on submerged flora, irrespective of the variation of the water level.

Genetic distance (D) values between congeneric species range between 0.03 and 0.61 with an average of 0.30 (Shaklee *et al.,* 1982), while genetic identity (I) values range below 0.85 (Thorpe and Sole-Cava, 1994). Conspecific populations have relatively small allele frequency differences (at only a few loci) with I values above 0.95 (D=0.05). Therefore, the estimate of D=0.14 between the two *B. lateralis* populations indicates relatively high genetic differentiation that can occur between sub-populations of the same species (Nei and Roychoudhury, 1974). Theoretically,

the divergence between two populations can be the result of mutation, geographical and reproductive isolation, natural and or artificial selection and genetic drift (Nei, 1986). In this case, isolation can possibly explain the relatively high genetic differentiation. The fixation index (F_{ST} = 0.214), indicating the amount of inbreeding due solely to population subdivision, shows that non-random mating occurred within the sub-populations. A possible explanation can be that both sexes were not equally represented to decrease the effective population size. The total inbreeding coefficient (F_{IT}) of 0.83 confirms that inbreeding in the individuals relative to the total populations is quite large and this can be due to random genetic drift among the sub-populations studied. In most natural populations F_{IS} values are typically close to zero, which indicates random mating within sub-populations. The value for F_{IS} is high (0.78), indicating the separation of the two *B. lateralis* populations by barriers, which do not allow gene flow. This is in agreement with ecological findings, since gene flow is restricted because the Upper Zambezi River forms a separate River System from the Cuando River. In the short term there may be barriers between the Zambezi and Kwando Rivers, but probably not in the longer term.

In conclusion, the *two B. lateralis* populations from the Upper Zambezi and Cuando Rivers had high average heterozygosity values compared to another species from the same family, and other freshwater fish from the same geographical area. This is expected according to the ecological theory postulating that species not adapted to their environment need more genetic variation (Lawson *et al.,* 1989). The relatively high genetic variation obtained for *B. lateralis* in this study, can allow for better adaptation to a new environment. Furthermore, the results indicate that a high degree of random genetic drift occurred among the two populations and that gene flow within and between populations was restricted by barriers isolating the populations.

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FIGURE 1. Allele variation at the ES-1 and ES-2 loci indicating heterozygote deficiencies.

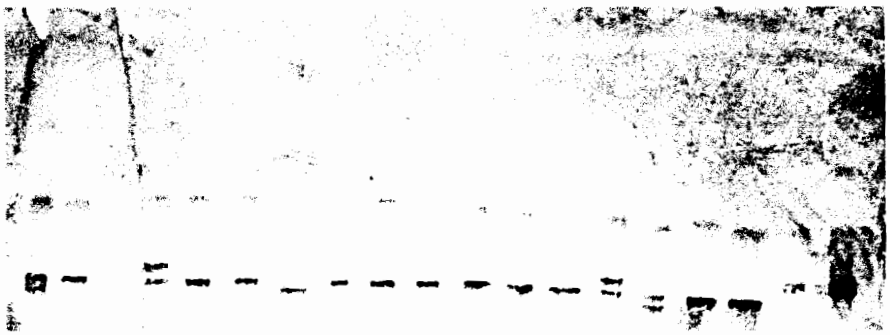
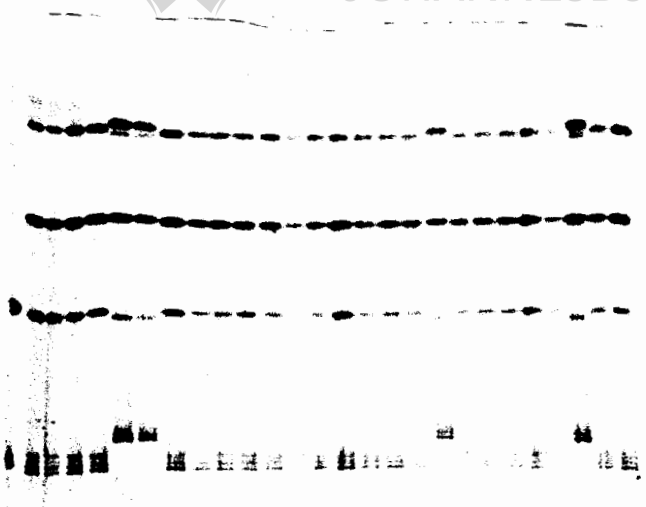
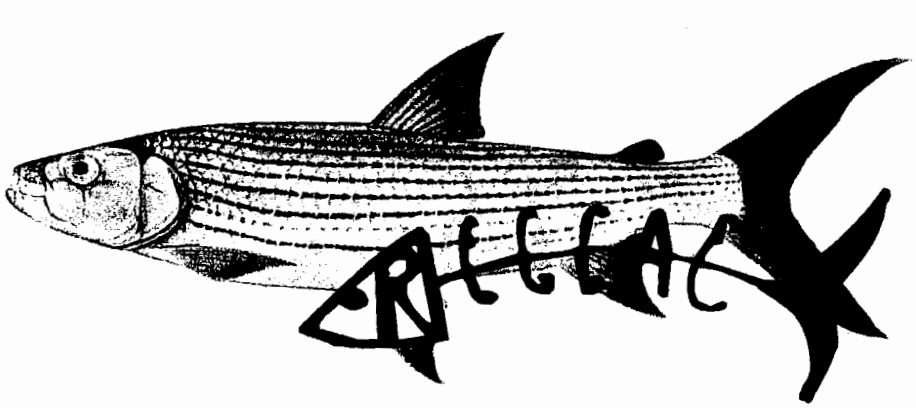


FIGURE 2. Allele variation at the PROT-1, -3 and –4 loci indicating heterozygote deficiencies.



CHAPTER 4

Systematic status and phylogenetic relationships of southern African Characidae species: results of allozyme and mtDNA studies



CHAPTER 4

Systematic status and phylogenetic relationships of southern African Characidae species: results of allozyme and mtDNA studies

4.1 INTRODUCTION

The current classification of the southern African family Characidae with genera has to be supported or contradicted. Since no genetic data for the genera and species were available, this chapter contributes to determine the systematic status and phylogenetic relationships of the southern African Characidae species using both allozyme and mtDNA data sets.

The characids are part of a large freshwater fish order found throughout Africa, South and Central America. Their families are easily distinguished from similar shaped cyprinids by having sharp teeth and a small adipose fin (Brewster, 1986). The Characidae family in southern Africa is represented by five genera (Skelton, 1993), viz. *Brycinus* Valenciennes, 1849, *Micralestes* Boulenger, 1899, *Rhabdalestes* Hoedeman, 1951, *Hemigrammoptersius* Pellegrin, 1926 and *Hydrocynus* Cuvier, 1816. *Brycinus* is represented by two species in southern Africa, i.e. *B. imberi* and *B. lateralis*. Before 1978 both *Brycinus* species, together with *B. affinis* (see section 2.1), belonged to the genus *Alestes* Müller and Troschel, 1844. The genus *Micralestes* is represented by the silver robber, *M. acutidens*, and *Rhabdalestes* are small slender characids of which only one species *R. maunensis* occurs in southern Africa. The most well known genus of the family Characidae is *Hydrocynus*, which is endemic to

Africa (Brewster, 1986). Tigerfishes are large, specialised predators regarded by many as the finest freshwater game fishes in the world (Schultz, 2001). *Hydrocynus vittatus* was formerly also known as *Salmo dentex, Alestes dentex, Characinus dentex, H. dentex* and *H. forskahlii*; the name *H. vittatus* was re-instated in 1989 (Paugy and Guegan, 1989; Skelton, 1990).

Weitzman and Fink noted in 1983 that the classification of the characids is problematic and clearly unsatisfactory and needs further phylogenetic analysis. Presently this is the case. The usefulness of allozyme studies for defining taxonomic borders and for inferring evolutionary relationships has been reported on other species by several authors (e.g. Van der Bank et al., 1998). Cytochrome b studies are representing another powerful tool for systematic and evolutionary analyses (e.g. Kocher and Stepien, 1997). In the present contribution both above-mentioned genetic data sets were analysed comparatively with regard to the systematics and phylogeny of representatives of the family Characidae from southern Africa. Other African species were included for outgroup comparisons.

4.2 MATERIAL AND METHODS

4.2.1 Allozyme analysis

Sixty-nine individuals comprising two families, five genera and species were sampled in southern and eastern Africa. Genera, species, localities, authorities and numbers sampled are listed in Table 1. To compare the taxonomic status of the *Brycinus* species, a sister-group, sampled in Kenya, East Africa, namely *B. affinis* was included. The African pike, *Hepsetus odoe*, was selected as outgroup taxon for this study, because it was once classified under the Characidae (Bell-Cross and Minshull, 1988; Merron *et al.*, 1990). It is now the sole representative of the family Hepsetidae.

TABLE 1. Species, localities, authorities and numbers (N) sampled

SPECIES	LOCALITY; AUTHORITY	N Allozymes	N Cytochrome b
	<u>Southern Africa</u>		
H. odoe	Makwena, Okavango Delta, Botswana, 19°07'30"S, 22°22'E;	6	
	Katima Malilo, Upper Zambezi, Namibia, 17°32'40"S, 24°31'41"E; (Bloch, 1974)	7	5
H. vittatus	Katima Malilo; Castelnau, 1861	7	
R. maunensis	Katima Malilo; (Fowler, 1935)	15	5
M. acutidens	Katima Malilo; (Peters, 1852)	10	5
B. imberi	Kruger National Park, Convergence of Letaba and Olifants Rivers, South Africa, 23°59'24"S, 31°49'33"E; (Peters, 1852)	6	6
B. lateralis	Katima Malilo; (Boulenger, 1900)	9	8
	<u>East Africa</u>		
B. affinis	Baomo, Tana River, Kenya, 0°05'N, 35°31'E; (Günther, 1894)	9	8
	<u>Gabon, West Africa</u>		
B. schoutedeni	M'Passa, Ivindo River, 0°27'N, 12°48'E; Békoyo (Franceville), Ogowe Basin, 1°38'N, 13°35'E; (Boulenger,1912)		2
B. longipinnis	Mikassa (Franceville), Ogowe Basin, 1°38'N, 13°35'E; (Günther, 1864)		1
B. kingsleyae	Ntsiétsie, 0°34'N, 12°52'E and Loa Loa, 0°31'N, 12°49'E, Ivindo rivers; (Günther, 1896)		2
B. taenlurus	Ntsiétsie; (Günther, 1867)		1
D. hypostomatus	Loa Loa; Pellegrin, 1900		2
D. notospilus	Ebeigne, Ntem Basin, 1°29'N, 11°36'E and Loa Loa, Ivindo Rivers; Günther, 1867		2

Hemigrammopetersius barnardi (Herre, 1936) is the only southern African representative of this genus. It was not included in this study because it occurs in Mozambique and could not be obtained.

Muscle and liver samples of large individuals, or whole fish for smaller species, were stored in liquid nitrogen and transported to the laboratory. Reference material was donated to the South African Institute for Aquatic Biodiversity in Grahamstown. Muscle samples were also stored in 70% ethanol for mtDNA analyses.

Tissue extracts were prepared from muscle, liver or whole fish and analysed by starch gel electrophoresis (12% gels) as described in Van der Bank *et al.* (1992). The following buffer systems were used: MF – a continuous Tris, boric acid, EDTA buffer, pH 8.6 (Markert and Faulhaber, 1965); RW – a discontinuous Tris, citric acid (gel pH 8.7), lithium hydroxide, boric acid (electrode pH 8.0) buffer (Ridgway *et al.*, 1970); TC – a continuous Tris, citric acid (pH 6.9) buffer system (Whitt, 1970) and A – a continuous Tris, EDTA, borate (pH 8.6) buffer system (Goncharenco *et al.*, 1992). Polyacrylamide gel electrophoresis (PAGE) following the method of Gahne *et al.* (1977) was used to separate the general proteins. Locus nomenclature, numbers, abbreviations and allelic designations followed the recommendations of Shaklee *et al.* (1990). Table 2 presents a list of protein and enzyme systems studied, enzyme commission (EC) numbers and buffer systems used. Loci were numbered beginning at the anodal end of the gel, and cathodally migrating allozymes were designated by a minus sign.

Allozyme data were analysed with the BIOSYS-2 computer program (Swofford *et al.*, 1997). (See Appendix 2 for input file). Gene frequencies were calculated and Nei's (1978) unbiased genetic distance (*D*) values were then used to construct dendrograms by means of UPGMA and neighbor-joining cluster algorithms. The DISPAN computer program (Ota, 1993) was used for neighbor-joining analyses.

TABLE 2. Enzymes and proteins, enzyme commission numbers (E.C. No.), locus abbreviations and buffers giving the best results are listed. See Material and Methods for abbreviations of buffers used

ENZYMES AND PROTEINS	E.C. NO.	LOCUS	BUFFER SYSTEM
Aspartate aminotransferase	2.6.1.1	SAat	A
		MAat	
Alcohol dehydrogenase	1.1.1.1	Adh	MF
Adenylate kinase	2.7.4.3	Ak	A
Creatine kinase	2.7.3.2	Ck	RW
Esterase	3.1.1.1	Est-1	RW
		Est-2	
Glyceraldehyde phosphate	1.2.1.12	Gapdh	TC
Glycerol-3-phosphate dehydrogenase	1.1.1.8	G3pdh	A
Glucose–6-phosphate isomerase	5.3.1.9	Gpi-A	RW
		Gpi-B	
Isocitrate ddehydrogenase	1.1.1.42	Sidhp-1	TC
		Sidhp-2	
$_L$-Lactate dehydrogenase	1.1.1.27	Ldh-A	MF
		Ldh-B	
Malate dehydrogenase	1.1.1.37	SMdh-1	TC
		SMdh-2	
Malate dehydrogenase (NADP$^+$)	1.1.1.40	sMdhp	MF
Mannose phosphate isomerase	5.3.1.8	Mpi	MF
Phosphogluconate dehydrogenase	1.1.1.44	Pgdh	MF
Phosphoglucomutase	2.7.5.1	Pgm	RW
Superoxide dismutase	1.15.1.1	SSod	RW
General protein		Prot-1	PAGE
		Prot-2	
		Prot-3	
		Prot-4	

TABLE 3. Gene frequencies and relative mobilities (RM) of alleles at variable loci in seven characin species

Species:	*H.odoe*	*H. vittatus*	*R. mauriensis*	*M. aouitidens*	*B. kriberi*	*B. lateralis*	*B. affinis*
Locus							
RM							
sAat							
105			1.000	0.500		1.000	
100		0.429		0.500	1.000		1.000
90	1.000						
85		0.571					
mAat							
-100		1.000	1.000	1.000	1.000	0.222	1.000
-105	1.000					0.778	
Adh							
-105	1.000					1.000	1.000
-100			1.000				
-95		1.000		1.000	1.000		
Ak							
75			1.000	1.000	1.000		1.000
70						1.000	
60	1.000	1.000					
Ck							
100	1.000						
95		1.000					
80				1.000	1.000		
75			1.000			0.625	0.929
70						0.375	0.071
Est-1							
110				1.000			
105	1.000	1.000					
100			1.000		1.000	0.938	1.000
90						0.062	
Est-2							
120	1.000	1.000					
110			0.033		1.000		
100			0.967	1.000		0.938	1.000
90						0.062	
Gapdh							
110		1.000			1.000		
100			1.000	1.000		1.000	1.000
95	1.000						
G3pdh							
100	1.000						
95		1.000		1.000	1.000	1.000	1.000
90			1.000				
Gpi-A							
110			0.100	0.600			
100		0.929	0.900	0.400	1.000	1.000	1.000
95	1.000						
85		0.071					
Gpi-B							
115						1.000	
110		1.000	0.100	0.250			1.000
105	1.000		0.200	0.700	1.000		
100			0.700	0.050			
sldh							
110						1.000	
100	1.000	1.000					1.000
85			1.000	1.000	1.000		
Ldh-A							
110		1.000	1.000	1.000		0.722	

100	1.000				0.333	0.278	0.600
95							0.400
90					0.667		
Ldh-B							
110	1.000						
105					1.000	1.000	0.800
100		1.000	1.000	1.000			0.200
sMdh-1							
110	1.000						
100		1.000	1.000	1.000	1.000	1.000	
80							1.000
sMdh-2							
100	1.000	1.000	1.000	1.000	1.000	0.938	1.000
90						0.062	
sMdhp							
130	0.333		1.000				
120		1.000		0.600		1.000	
110				0.400	1.000		1.000
100	0.667						
Mpi							
100			1.000				
95				1.000			
90					1.000		1.000
85		1.000				1.000	
80							
70	1.000						
Pgdh							
120		1.000					
110			1.000	1.000	1.000		1.000
105						1.000	
100	1.000						
Pgm							
120	1.000						0.500
110					1.000		0.500
100		1.000	1.000	1.000		1.000	
Prot-1							
105	1.000			1.000		1.000	
100		1.000	1.000		1.000		1.000
Prot-2							
125	1.000						
105		1.000		1.000	1.000		1.000
100			1.000			1.000	
Prot-3							
115	1.000						
105		1.000					
100			1.000	1.000	1.000	1.000	.000
Prot-4							
110	1.000						
100		1.000		1.000		1.000	
95					1.000		1.000
90			1.000				

TABLE 4. Matrix of Nei's (1978) unbiased genetic distances between Characidae species and *H. odoe*.

SPECIES	*H. odoe*	*H. vittatus*	*R. maunensis*	*M. acutidens*	*B. imberi*	*B. lateralis*
H.vittatus	1.404					
R. maunensis	2.656	1.000				
M. acutidens	1.859	0.727	0.448			
B. imberi	1.995	0.858	0.784	0.602		
B. lateralis	1.566	0.994	0.663	0.630	1.071	
B. affinis	1.547	0.915	0.678	0.680	0.373	0.789

4.2.2 Cytochrome-b analysis

Forty-seven individuals comprising three families, five genera and 12 species were sampled in southern, eastern and western Africa. Species, localities and numbers sampled are listed in Table 1.

Variations in partial sequences of the cytochrome b gene (cyt-b) were analysed by PCR and direct automated sequencing of the amplified products. Total genomic DNA was isolated from ethanol preserved muscle tissue (20 mg) by phenol/chloroform extraction (Hillis *et al.*, 1990). Cyt-b fragments (306bp) were amplified using primers L14841 and H15149 (Kocher *et al.*, 1989). PCR reactions were performed in 50μl reaction mixtures containing 1x reaction buffer (Proof*Sprinter* kit, Hybaid), 3.5 mM $MgCl_2$, 1.2 μM of each primer, about 40-90 ng total DNA, 0.4 mM of each dNTP (dNTP-Mix, Hybaid) and 1.5 units Tag/Pwo polymerase mixture (Proof*Sprinter*, Hybaid). The thermal profile was: 1. 94°C/2 min (1 cycle); 2. 94°C/20 sec; 56°C/90 sec; 72°C/60 sec (35 cycles); 3. 72°C/15 min (1 cycle). Amplified PCR products were purified by electrophoresis on 1.5% QualexGold agarose gels (Hybaid) and recovered using GFX PCR gel extraction kits (Amersham Pharmacia Biotech Inc). The purified double-stranded mtDNA products were used directly in

dideoxy-termination sequencing reactions using the BigDye Terminator Cycle Sequencing Mix (Applied Biosystems). Cycle sequencing reactions were performed in 20 μl reaction volumes containing 3 μl BigDye, 5μl 2.5x sequencing buffer (Applied Biosystems), 10 μl of the amplified products (200-350 ng) and 2 μl primer (1 μM). The thermal profile was: 96°C/30 sec; 55°C/15 sec; 60°C/4 min (30 cycles). After removal of unincorporated dye terminators (DyeEx, Qiagen) sequencing reactions were denatured and the samples were run on an ABI Prism 377 automated sequencer (Applied Biosystems).

Sequences were further processed and aligned using ABI Prism Sequence Navigator, version 1.0.1 (Perkin Elmer). Unique mtDNA haplotypes were analysed by the PHYLIP software package version 3.57 (Felsenstein, 1995) and by MEGA, version 2.1 (Kumar *et al.*, submitted). Phenetic analyses of sequence data were performed using Kimura's genetic distance estimates (Kimura, 1980) and the neighbor-joining method (Saitou and Nei, 1987). Bootstrapping comprised 1000 replicates. The Seqboot, DNAdist, Neighbor and Consense programs of PHYLIP were used. Cytochrome-b sequences (N=20) of the species studied here were subsequently deposited in GenBank.

4.3 RESULTS

4.3.1 Allozyme analysis

Gene products of 35 loci were detected of which 25 yielded interpretable results. Gene frequencies at variable loci are listed in Table 3. Figure 1 is a photo example of the Gpi-A and Gpi-B loci run on a starch gel (p95). Figure 2 indicates the different mobilites of the general proteins on the polyacrylamide gels (p95). All loci were considered polymorphic with at most six alleles at Mpi, except for Sod, which appeared monoallelic. In the genus *Brycinus*, *B. lateralis* owns the most (11 or 44%) private alleles, followed by *B. imberi* (6) and *B. affinis* (3). The latter two species share five additional alleles at corresponding loci in comparison to *B. lateralis* and *B. affinis*.

Only seven private alleles are shared between *R. maunensis, M. acutidens* and *B. lateralis.*

Genetic differences between species were estimated using Nei's genetic distances (Nei, 1978) calculated from allele frequency data (Table 4). The smallest *D* value was obtained between *B. affinis* and *B. imberi* (0.373), whereas the largest value occurred between *R. maunensis* and *H. odoe* (2.265). Within the genus *Brycinus,* the calculated *D* value between *B. lateralis* and both other species of the genus appeared to be approximately 2.5 times larger than the one calculated between *B.affinis* and *B. imberi.* The average distance between the species was 1.059.

UPGMA clustered all the Characidae species in a single clade with *H. odoe* in a sister group relationship. *Hepsetus odoe* represented the most widely divergent species. Within the Characidae *Hydrocynus vittatus* appeared at the most basal position of this family. All the trees obtained were similar (cophenetic correlation value = 97.4%). Overall relationships between the Characidae species can be seen from the neighbor-joining tree constructed from Nei's *D* values (Figure 3).

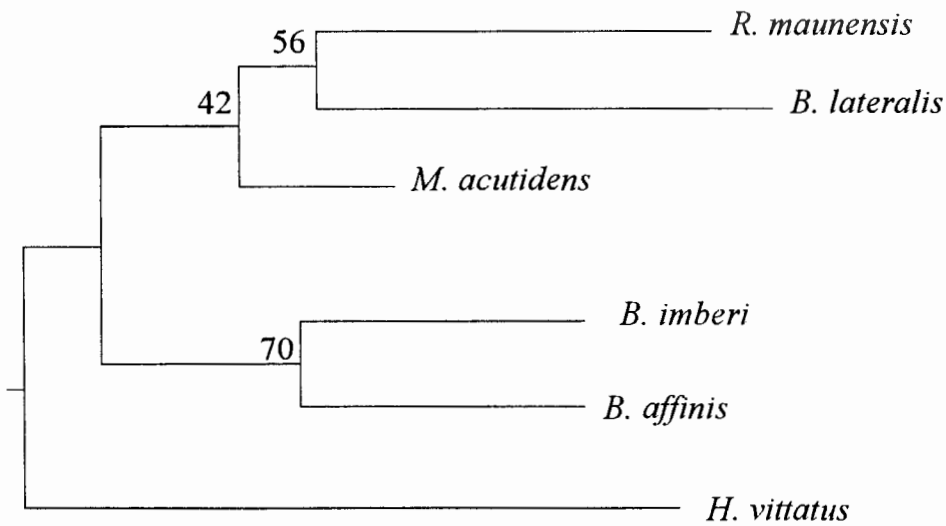


FIGURE 3. Neighbor-joining tree for Characidae species constructed from Nei's (1978) genetic distances (Table 4). The number at each node indicates percentage recovery of the particular node (1000 bootstrap replicates).

Rhabdalestes maunensis, M. acutidens and *B. lateralis* are clustered together (bootstrap value = 42%), with *B. imberi* and *B. affinis* forming a separate clade (bootstrap value = 70%). All five taxa are clearly separated from *Hydrocynus vittatus*, which again represents the most basal species in the Characidae. However, the relationships between *B. lateralis* and the other Characidae species are only weakly supported by bootstrapping.

4.3.2 Cytochrome-b analysis

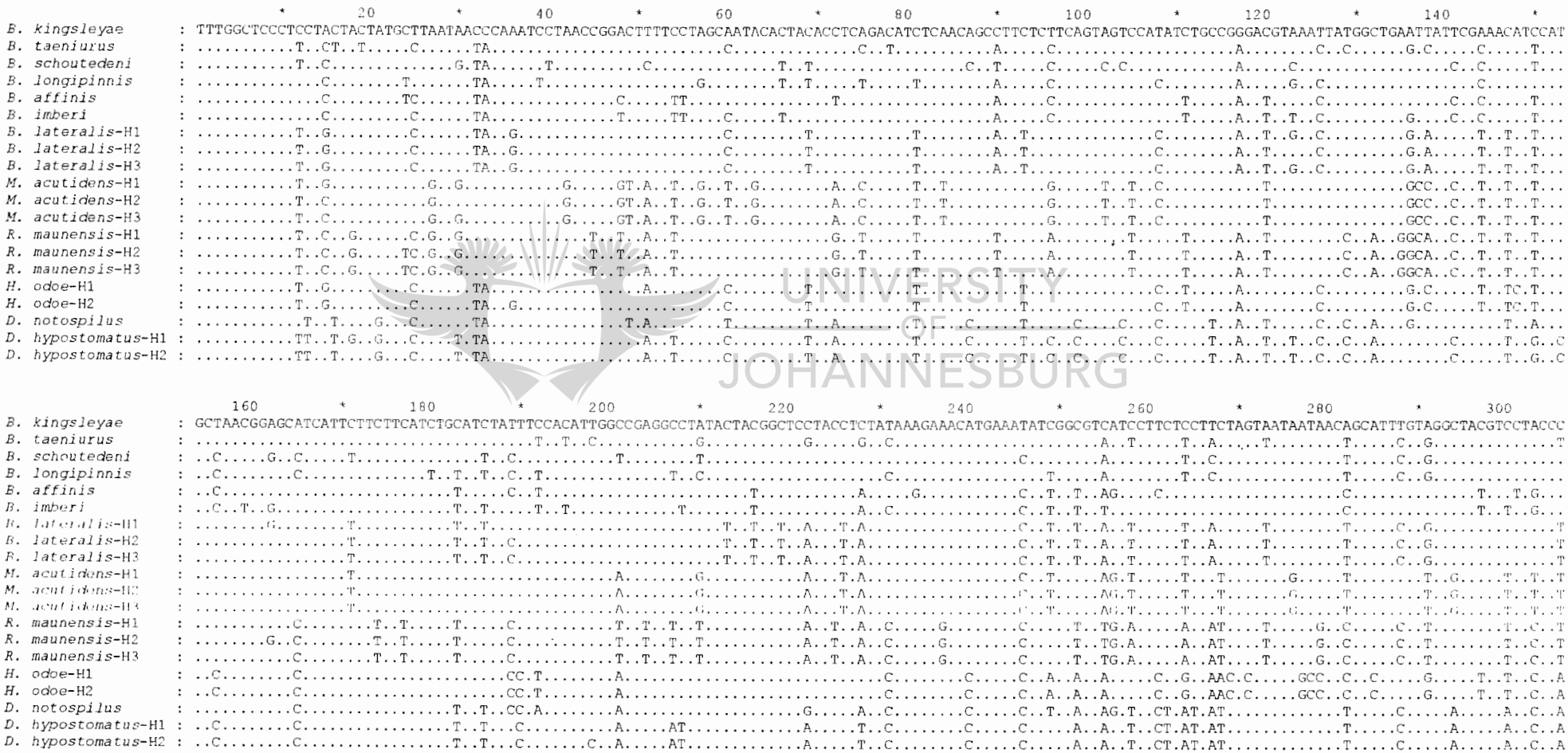
Variations in mitochondrial DNA sequences were studied from a total of 47 specimens comprising 12 African characiform taxa. Aligned sequences for different cytochrome-b haplotypes of each species are shown in Table 5.

The sequenced mtDNA region contained 306 nucleotides that code for 102 amino acids of an anterior part of the cytochrome b (amino acid positions 33 to 134). Eleven amino acid differences were found among all the specimens examined. As expected, the majority of variation was found at third codon positions. Despite sequence variability among different haplotypes, no significant differences were noted in base compositions across sequences or taxa (chi-square tests of homogeneity of base frequencies). Substitutions were identified at 120 of the 306 sites (39%) of which 96 (80%) occurred at third positions of the codons.

Overall, 20 different mtDNA haplotypes were detected among the 47 individual samples sequenced. Corrected Kimura's genetic distances (Kimura, 1980) between different haplotypes ranged between 0.003 and 0.262. Among closely related taxa (up to 13% genetic divergence) transitions dominated over transversions more than 4 fold (Figure 4). However, comparisons between more distantly related taxa revealed evidence of substitutional saturation. Above a *D* value of 18% the transition/transversion ratio dropped from 4:1 to 2:1 (Figure 4). Because of this effect, only transversional substitutions were considered in subsequent phylogenetic reconstructions.

Table 5

Aligned sequences of 20 different cytochrome-b haplotypes from 12 African characiform taxa. Nucleotide substitutions are represented; H = haplotype



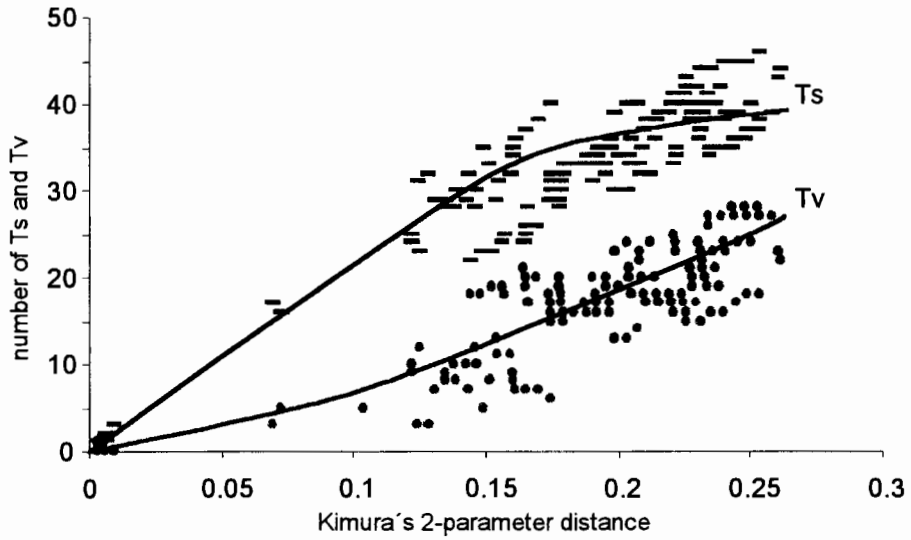


FIGURE 4. Plot of the number of transitions (Ts) and transversions (Tv) in pair-wise sequence comparisons against Kimura's 2-parameter distances (1980) for partial cytochrome-b sequences of 12 characiform taxa.

Figure 5 presents a neighbor-joining phylogeny based upon Kimura's 2-parameter model, rooted by *Distichodus*. Only transversional substitutions are considered. A total of five monophyletic clades are evident: *Brycinus, Micralestes, Rhabdalestes, Hepsetus* and *Distichodon*; all of them are supported by high bootstrap and interior branch test values. Within the *Brycinus* clade two mtDNA lineages are distinguished. In the first lineage *B. affinis* and *B. imberi* are clustering with West African species like *B. schoutedeni, B. longipinnis* and *B. kingsleyae*. The second lineage comprises *B. lateralis* and another West African species, *B. taeniurus*. The cytochrome-b phylogeny thus supports the validity and monophyly of the genus *Brycinus* and both other genera (*Micralestes, Rhabdalestes*) of the family Characidae. Moreover, the validity of all three characiform families (Characidae, Distichodontidae and Hepsetidae) is also well supported by bootstrapping.

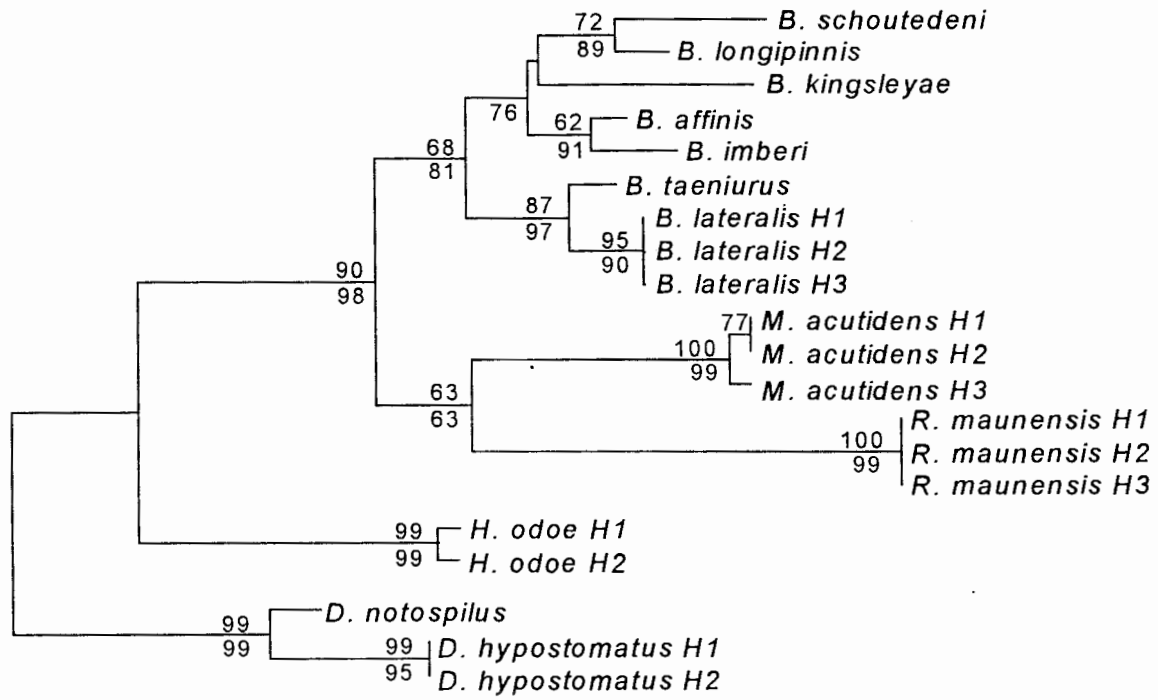


FIGURE 5. Neighbor-joining phylogeny of the 20 different mtDNA haplotypes identified within the sequenced part of the cytochrome b gene. Genetic distances between taxonomic units were calculated using Kimura's (1980) two-parameter model. The numbers at each node indicate percentage recovery of the particular node (1000 replicates). Above branches: bootstrap values, below branches: interior branch test values. Only transversional substitutions are considered.

4.4 DISCUSSION

The results of the present study provide clear evidence of substantial genetic differentiation between southern African characins and confirm the validity of the presently known systematics of these taxa. However, allozyme data indicated unexpected large genetic differences between the species involved, in particular among the species of the genus *Brycinus*. Fixed gene differences were encountered at 15 (60%) of the loci analysed between *B. lateralis* and *B. imberi*, whereas only seven (Adh, Ck, Est-2, Gapdh, Gpi-B, sIddh and sMdh-1) fixed allozyme differences

contributed to genotypic differences between *B. imberi* and *B. affinis* (Table 3). The latter two species share most loci. Consequently, genetic distance estimates (Nei, 1978) revealed similar results.

Overall, large estimates of *D* values were obtained in the present study, ranging from 0.373 to 2.656 (average 1.059). According to Shaklee *et al.* (1982), these estimates may be used to characterize different hierarchical taxonomic levels. Between pairs of conspecific fish populations *D* values generally range from 0.002 to 0.070 (average: 0.05). Species of the same genus are distinguished by *D* values ranging from 0.03 to 0.61 (average: 0.30), whereas *D* values between genera of the same family are considered to range from 0.58 to 1.21. According to this division, *D* values between *Hepsetus* and the genera of the family Characidae (*D*: 1.547 - 2.656) clearly support the placement of this species in a separate family (Table 3). *D* values between *Hydrocynus* and the other Characidae genera ranged between 0.727 and 1.000, thus confirming a separate generic rank in the Characidae. Similar results were obtained for *Rhabdalestes* (0.448 - 2.656; average: 1.038) and *Micralestes* (0.630 - 1.859; average: 0.824). Within the species of the genus *Brycinus* the smallest *D* value was observed between *B. imberi* and *B. affinis* (0.373). This value falls within the range for species of the same genus. However, quite large distances separated *B. lateralis* and *B. imberi* (1.071), and *B. lateralis* and *B. affinis* (0.789), respectively. According to Shaklee *et al.* (1982), these values are generally characterising different generic ranks. *Brycinus lateralis* clearly falls outside its generic grouping with respect to the observed genetic differences as proposed by Shaklee *et al.* (1982). However, genetic boundaries between different hierarchical taxonomic units are often fluent.

Similar relationships were found by phylogenetic analyses of allozyme data. UPGMA trees (not included here) suggest a sister group relationship between *H. odoe* and the species of the family Characidae. Fixed allele differences occurred between *H. odoe* and the ingroup species at 12 loci (sAat, Ck, Gapdh, G3pdh, Gpi-A, Ldh-B, sMdh-1, Mpi, Pgdh and Prot-2-4). These genetic markers contribute to its wide separation from the other Characidae species; the new classification of *H. odoe* in a

separate family within the characins is thus greatly supported by allozyme data. Moreover, UPGMA and neighbor-joining analyses coincidentally suggest a basal position of *H. vittatus* within the Characidae family. However, genetic relationships among the remaining species and genera could not be resolved (Figure 3). In particular, the branching position of *B. lateralis* remained questionable.

In contrast to the results obtained by allozyme studies, cytochrome b analyses clearly support the presently known systematics of all the taxa involved. The three families currently recognised in southern Africa (Distichodontidae, Characidae and Hepsetidae (Skelton, 1993)) are well supported by phylogenetic reconstructions (Figure 5). This also concerns the monophyly of the three Characidae genera, *Micralestes*, *Rhabdalestes* and *Brycinus*. Bootstrap and interior branch test values of neighbor joining analyses reached 68/81% to 100/99%, respectively. Moreover, these values, in particular for the validity of the genus *Brycinus*, considerably increased after removal of the four West African *Brycinus* species from the phylogenetic analyses (bootstrap value: 84%; interior branch test value: 89%). In accordance with the allozyme data, molecular analyses also support a close relationship between *B. affinis* and *B. imberi*, and further indicate the presence of two different mtDNA lineages within the genus *Brycinus*. For future studies it would be of special interest to include *Alestes* species and to evaluate their relationships in particular with regard to the genus *Brycinus*.

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FIGURE 1. Starch gel of GPI-A and GPI-B indicating allele mobility differences between *H. odoe* (H.o), *H. vittatus* (H.v.), *R. maunensis* (R.m.), *M. acutidens* (M.a.), *B. imberi* (B.i.), *B. affinis* (B.a.) and *B. lateralis* (B.l.)

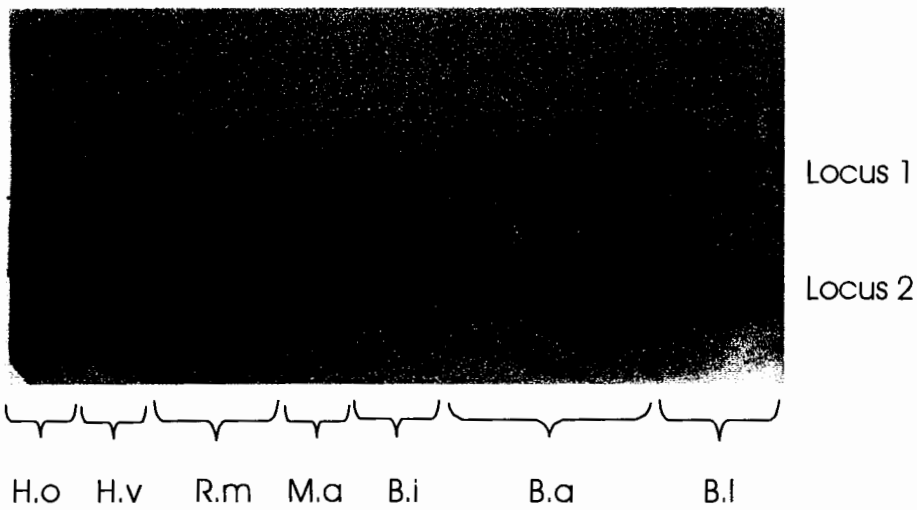
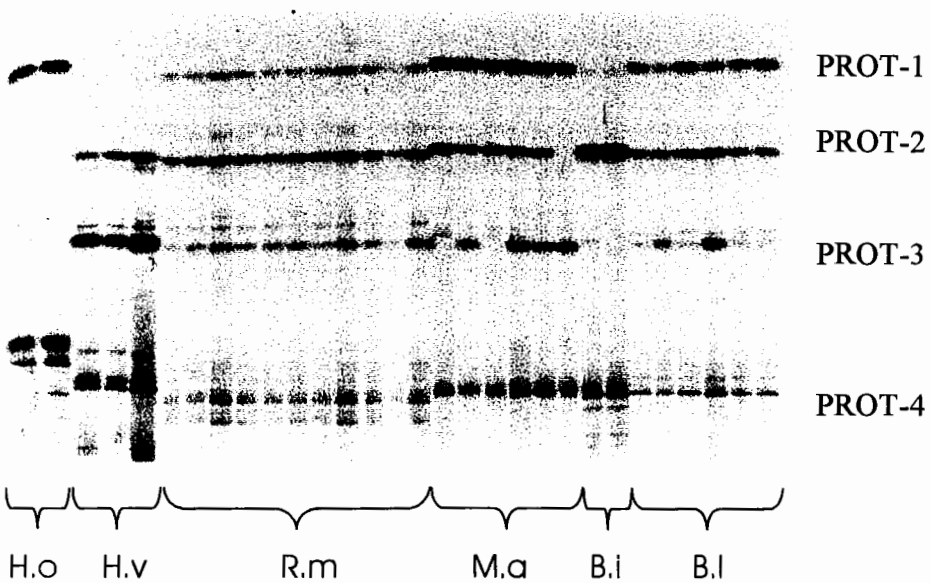
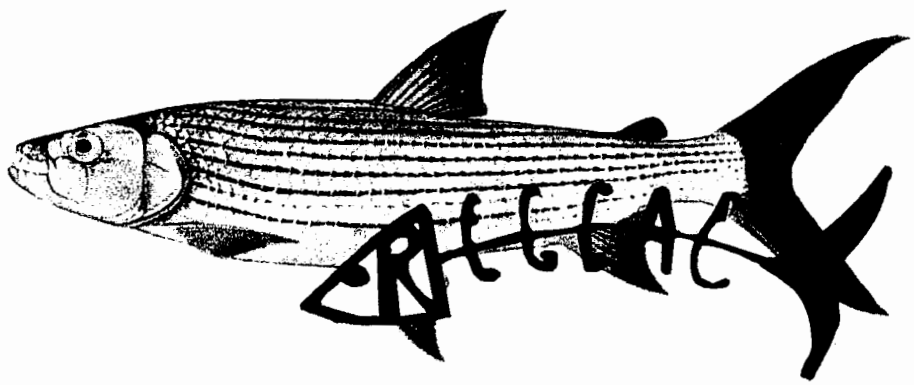


FIGURE 2. Allele mobility differences of general proteins between *H. odoe* (H.o), *H. vittatus* (H.v.), *R. maunensis* (R.m.), *M. acutidens* (M.a.), *B. imberi* (B.i.) and *B. lateralis* (B.l.)



CHAPTER 5

Morphological analysis of representatives of the family Characidae from southern Africa



CHAPTER 5

Morphological analysis of representatives of the family Characidae from southern Africa

5.1 INTRODUCTION

From the previous chapter, it is clear that there was genetic distinctness between the species and the different characin families using molecular techniques. This chapter will aim to indicate whether some of the groupings are morphologically similar. Only limited characters from a few Characidae species could be obtained for this comparative study. Although the information in this chapter is very basic and the contribution short, it can possibly aid in supporting the genetic relationships of the different species within the family Characidae and also support the southern African characin families.

At the lower taxonomic level morphology has not always been such a useful guide for identifying phylogenetic relationships as many sibling species show few morphological differences as well as morphological convergence among unrelated species. In contrast, many morphologically similar groups have been shown to be genetically distinct species with molecular methods (Sundberg and Pleijel, 1994). Examples of morphologically cryptic species in fish can be found in a wide group range (see Van der Bank and Kramer, 1996). In other cases, morphologically or behaviourally distinct taxa have proved to be genetically undifferentiated (Berlocher, 1981). For example, little genetic divergence was detected between morphologically different species of pupfish (see Van der Bank and Kramer, 1996) and among trophically specialised forms of fishes that had been regarded as distinct species (Graves and Rosenblatt, 1980).

Although the use of morphological variation is hampered by the inability to resolve the relationship between phenotype and genotype for polygeneic characters, this chapter will aid to provide some data on the morphological characters.

5.2 MATERIAL AND METHODS

The morphological data was obtained from Jubb, 1967, Brewster, 1986, Bell-Cross and Minshull, 1988 and Skelton, 1993. Useful morphological characters to distinguish between and to determine affinities were dorsal and anal fin rays, scale counts along the lateral line, teeth and fish length. The data was transformed to produce a cladogram for phylogenetic analysis (PAUP, Version 2.4, Swofford, 1985).

The different characters are listed in Table 1. Only data from the southern African species was analysed. The species included *B. lateralis, B. imberi, M. acutidens, R. maunensis and H. vittatus* with *H. odoe* as the outgroup for the family Characidae.

TABLE 1. Morphological characters for six characin species

Character	*B. lateralis*	*B. imberi*	*M. acutidens*	*R. maunensis*	*H. vittatus*	*H. odoe*
Fin spines:						
Dorsal	Dii 8	Dii 8	Dii 8	Dii 7-9	Dii 8-9	Dii 7,9
Anal	Aii-iii	Aiii	Aiii	Aiii	Aiii	Aii
Rays	14-16	15-16	14-16	16-19	10-13	9
Lateral line scales	23-29	30-33	23-28	33-36	43-48	49-58
Caudal peduncle scales	9-12	10-14	9-11	12-14	15-16	24
Teeth	16 above 10 under	16 above 10 under	4-6 above 10 under	4-6 above 8 under	12 above 15 + under	4 above 30+ under
Length	180mm	140mm	80mm	60mm	700mm male 500mm female	470mm male 500mm female

5.3 RESULTS

The cladogram obtained (consistency index of 83.3%) using dorsal and anal fin rays, scale counts along the lateral line and around the peduncle, the number of teeth and the length is illustrated in Figure 1. The clade containing the Characidae species was separated from the outgroup, *H. odoe*. *Hydrocynus vittatus* separated basally from this cluster. *Brycinus imberi* and *B. lateralis* clustered with *M. acutidens* and *R. maunensis*.

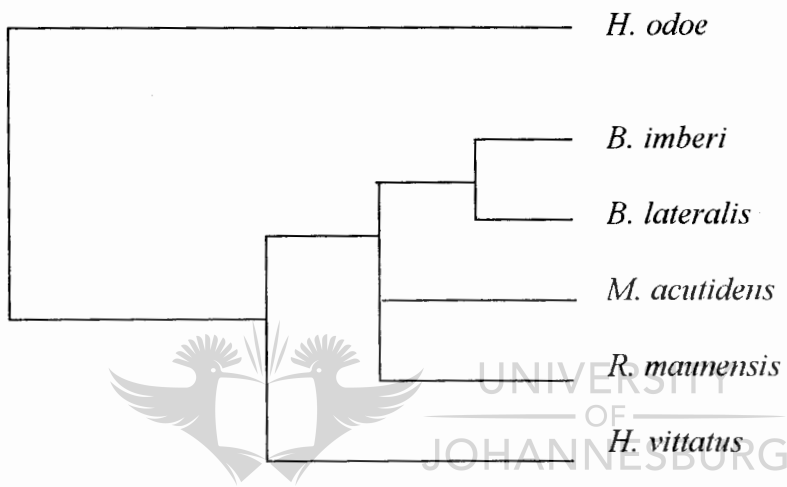


FIGURE 1. Cladogram of the family Characidae constructed from available meristic and morphologic characters (consistency index 83.3%)

5.4 DISCUSSION

The more ancient method for the systematist to infer phylogenetic relationships from studies of phenotypic divergence can be subjective and unstable. In many cases only selected or alternative characters are studied. As indicated previously, only a few morphological characters were available to compare the different species with and therefore it can be possible that the conclusions can be partially subjective and unstable. However, the results indicate that the grouping of the two *Brycinus* species are congruent with findings from the previous chapter. The cladogram also supports the allozyme and mtDNA data sets with distinct differences between the two families

Hepsetidae and Characidae. In conlcusion, the morphological similarities between the species were useful as quantitative characters to separate the two families and to indicate closeness within the two *Brycinus* species.

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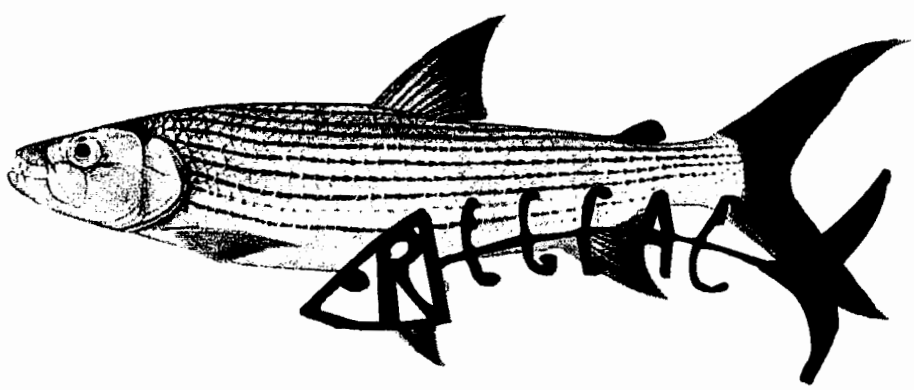
CHAPTER 6

Industrial application of biochemical and genetic tools for species identification and genetic characterization



CHAPTER 6

Industrial application of biochemical and genetic tools for species identification and genetic characterisation

6.1 INTRODUCTION

There is a growing need worldwide for the application of molecular techniques to determine the origin of species and to genetic characterise animals in the industry. These molecular techniques have a broad range of applications with regard to the management and conservation of organisms. This chapter consists of two sections. The first part will concentrate on species identification while the second part will focus on genetic characterisation of animals.

The applications of species identification techniques are divided into four general categories. Firstly, compliance enforcement, which often depends on genetic identification techniques to enable officials to identify the species to which regulations pertain. Secondly, quality control applications, to allow for the testing of products to guard against fraudulent substitution with less valuable species, which is particularly pertinent since processing often obliterates identifiable features. Thirdly, a variety of applications to ecological and life-history studies and conservation management are reported and here genetic identification techniques of species, especially those with cryptic life-cycle stages, are an indispensable tool for scientists, conservationists and managers. The last application of genetic techniques is the sourcing of population origin.

Through the knowledge gained from procedures applied in Chapters 2, 3 and 4, it was possible to establish a species identification service by applying the

biochemical techniques used to distinguish between different species and taxa. The service is rendered at the Animal Genetics Section situated at the Agricultural Research Council's Animal Improvement Institute, Irene. The service is available to consumers, the different white meat (chicken and turkey) and red meat (beef, pork, mutton, ostrich, horse, etc.) industries and most important to the South African Police Service for forensic analysis in stock theft and poaching cases. These activities will be discussed in the first part of this chapter.

The second part of this chapter will focus specifically on genetic characterisation and understanding animal genetic resources. Little information exists on performance, production, adaptive qualities and molecular biology of most animals. The Food and Agricultural Organisation (FAO) of the United Nations has the mandate to help safeguard food security worldwide with the Global Strategy for the Management of Farm Animal Genetic Resources. The data and knowledge gained and applied in Chapters 2, 3 and 4 will build the foundation for the better use, development and maintenance of domestic animal diversity.

6.2 TISSUE SPECIES IDENTIFICATION

With the use of phylogenetics, it was always hoped to discover mutable, heritable and taxon-specific genetic characters to be able to delineate and understand the phylogenetic relationships between taxa (De Quieroz and Gauthier, 1992). When characters can be genetically detectable, it can be applied to the identification of species. These biochemical and molecular methods of species identification have been applied in many diverse fields such as medical (Van Belkum et al., 1998), veterinary diognostics (see Taylor, et al., 1996), food science and safety (Meyer and Candrian, 1996, Hunt et al., 1997), forensic science (Bartlett and Davidson, 1992) and conservation (Haig, 1998). This part of the chapter aims to indicate the application of species identification of some of the four areas mentioned. These include compliance enforcement and commercial quality control, both of which can be forensic applications.

For distinct taxa, the species concept is the subject of much debate, particularly in the fish environment (Avise, 1994). The phylogenetic species concept that considers a phylogenetic species as "an irreducible cluster of organisms possessing at least one diagnostic character" (Baum, 1992) provides parameters within which to operate and is applicable at several levels in the phylogenetic hierarchy, including the sub-species level. In the meat industry specifically, the second act on Foodstuffs, Cosmetics and Disinfectants of 1972 (Act no 54 of 1972, South Africa) the word "species" refers to a particular animal species, including game or bird species used as a foodstuff.

This level of identification can be used to determine the origin of the species, not only in blood but also in meat and other tissues as was concluded from Chapter Two. In Chapter Four, it was found that each species has a species-specific protein profile. This was observed in the general proteins in particular. It was further found that it is possible to distinguish not only between different fish species, but also between the different farm animal and game species.

A considerable interest in the species identification of blood, meat and meat products in South Africa developed that coincided with activities in other countries. It seems that there will always be a clear and growing need to be able to identify the origin of species to monitor and regulate activities in the field, to inspect and certify products in the wholesale and retail trade and to protect consumers.

The international trade in meat (fish, poultry, farm animal and game meats) continues to grow. Most often meats are not declared or have been the subject of a false declaration. There is a steady increase in marketing processed products instead of intact meat. In the processed food industry such marketing practices make it difficult to identify the species involved as the external features by which species are usually identified are commonly removed or obscured by processing. This resulted in an increase in the frequency and extent of substitution of one species for another in the meat markets and led to falsely labeled products. In the aforementioned Act, the regulations also state that the minimum percentage total

meat and maximum percentage fat must now be stated on the label. Manufacturers and butchers may produce any product they wish, providing that they label it correctly and truthfully.

Certain meat products and species have a higher value than closely related congeners and typically, many of the identifying morphological features of these species are lost during processing and packaging. This provides the opportunity for fraudulent substitution of high value products with lower value products and or the mislabeling of such products. An example is where cheaper smoked trout (*Oncorhynchus mykiss*) is difficult to distinguish by taste and appearance alone from the more expensive smoked salmon (*Salmo solar*) for which it has been substituted (Carrera *et al.*, 1996; Carrera *et al.*, 1998).

These problems are not confined to fish species alone. In the red meat industry an increasing substitution of different species occur. Some examples are beef being substituted with horse meat and mutton being substituted with beef. Horse meat is cheaper and it is commonly used for pet food and not for human consumption in South Africa. Different religious groups such as the jewish and muslims do not consume pork. Muslims also regard cattle as sacred. In most cases these species are being substituted especially in sausages and patties. The species identification of game is also increasingly important. With the modern techniques of drying and packaging of meat the external features are obscured. Examples of biltong (a dried meat product made from different game species) being substituted with another species are quite common. When disputes arise, the inability to identify products morphologically on species level can destroy any case. Hence the need for forensic genetic species identification methods that can be employed to distinguich between different meat and processed products.

Species testing methods can include chemical, immunological and electrophoretic techniques (Protz, 1983). The drawback of chemical methods is that the presence of compounds or markers not species unique is measured, that little distinction can be made between markers and other compounds of similar reactivity and that the

methods are too cumbersome and time consuming for routine use.

Immunological methods are based on extremely specific antigen-antibody interactions with possible cross-reactions between species. This makes the method unreliable. Protein-based methods are generally regarded as less robust than DNA based methods, but it nevertheless still have several benefits and are still widely applied. The analysis of general muscle and blood proteins by horizontal slab polyacrylamide electrophoresis has proven to be a simple and powerful method for species identification (Yman, 1987; Protz, 1983; Kurth and Shaw, 1983). This makes it a valuable analytical tool in species testing programmes. Expertise and evidentiary techniques used can withstand legal challenges in courts (Slattery and Sinclair, 1987).

The gel casting method of Gahne *et al.* (1977) as was utilised in Chapters 2, 3 and 4 was applied to improve resolution and simplify the analysis of samples. Thin layer polyacrylamide gels are used to resolve total protein extracts from muscle or blood samples. Total protein extracts of the appropriate tissues are made in a suitable buffer, the crude extract is electrophoresed on thin layer polyacrylamide gels and visualised by applying an appropriate protein stain (Shaklee and Keenan, 1986). The migration of these proteins to their respective positions on the gel separates them into species-specific banding patterns that can be regarded as phenotypic expressions of genetic polymorphisms and that consistently differentiate the species concerned (Figure 1).

It is essential to utilize reference or control samples representative of the species and type on each gel. Either a muscle or blood control must be used as reference material for identifying unknowns. A genetic profile register was established for southern Africa to allow better monitoring and tracking of species, meat and meat products for species idenification.

Samples are prepared in an alkaline buffer prior to the electrophoresis procedure. Following centrifugation, the supernatant is stored until analysis or in a biological bank as reference samples.

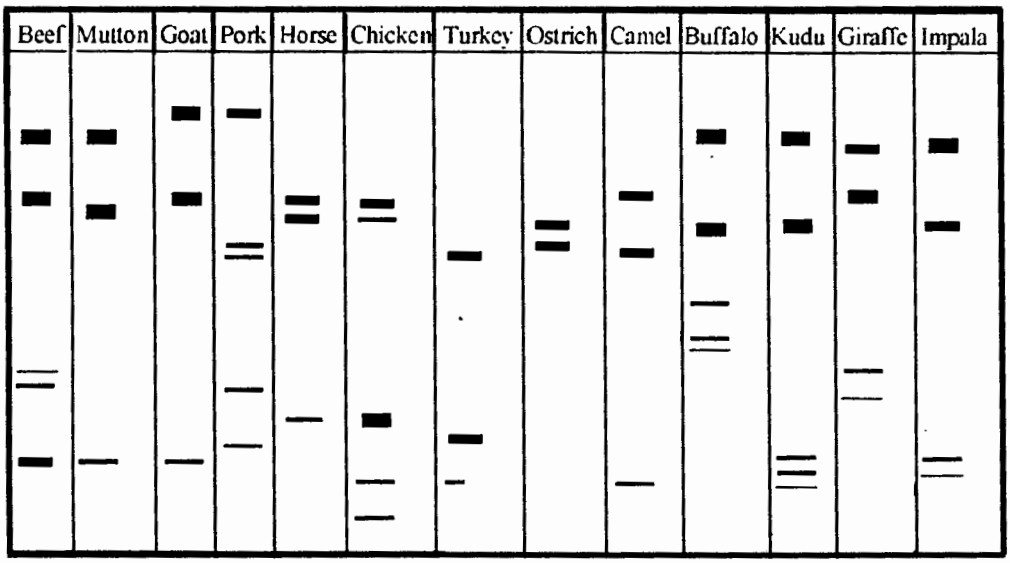


FIGURE 1. General protein profiles of some domestic and game species using polyacrylamide gel electrophoresis

Repetitive analysis indicated reproducible mobilities and these could be recorded for each species. This data is truly characteristic of the species and even closely related species can be distinguished. Each species has a distinct protein profile with characteristic concentration and mobilities of proteins through the gel. It is also possible to distinguish between mixed meat products (Kim and Shelef, 1986). Different cuts of meat in single species, for example beef or springbok were tested and these gave identical banding configurations. The results corresponded with other studies (Kim and Shelaf, 1986).

In some cases, regulation of the species origin in products can have a positive outcome, for example a contribution was made in the pie industry where the monitoring of species contents led to the development of a new product on the market. It is now possible to buy a meat pie containing 100% ostrich meat.

It is evident that, with the increasing substitution of different species in meat and meat products, there is a formidable task to quantitatively establish the origin of the

protein. It is also increasingly important to identify species for the prevention of stock theft and poaching and to render a service to the South African Police Service. With these electrophoretic techniques it is possible to identify species origin of tissues and mixtures and to provide comprehensive information. In forensic cases, the possibility of being able to identify the origin of the species for example of blood or meat found at the crime scene or on the accused can lead to convictions. With the increase in stock theft and poaching the need for a species identification service to solve cases is becoming even more important.

6.3 GENETIC CHARACTERISATION

Another application of molecular and biochemical techniques is genetic characterisation. Characterisation is usually required at four levels. The first is a baseline survey of the resources, monitoring the population status, comparative evaluation and lastly a comparative molecular description where molecular markers can be used to establish significant genetic diversity in order to better target conservation actions. These actions are not only applicable to important or nearly extinct species, but are now included in a global research project for the Measurement of Domestic Animal Diversity (MoDAD). MoDAD uses molecular techniques to establish the extent of diversity within and between species by quantifying the genetic distance between populations based on differences in their genetic make-up. MoDAD is a programme of the FAO (Food and Agricultural Organisation) of the United Nations with the mandate to help safeguard food security worldwide.

Gaining on the practical experiences and knowledge such as the determination of the genetic variation, population structure, genetic distances and phylogenetic relationships within the family Chracidae as were illustrated in Chapters 2, 3 and 4, it was possible to establish a genetic characterisation service laboratory for indigenous farm animals in southern Africa. This service is now regarded by the FAO

as the focal point for genetic characterisation projects and training in the southern African Developing Community (SADC) region.

The primary aim of this service to study the genetic diversity, is to understand the extent of differentiation of populations within species. Population specific genetic markers or alelles can be generated using a range of methods available for detection of polymorphic loci. These polymorphic genetic markers are extremely useful for a number of applications such as measurement of the amount of genetic diversity in species, discrimination between individuals, strains or species, identification of markers linked to economically useful traits as well as analyses of animal kinship relationships, behavioral and population ecology.

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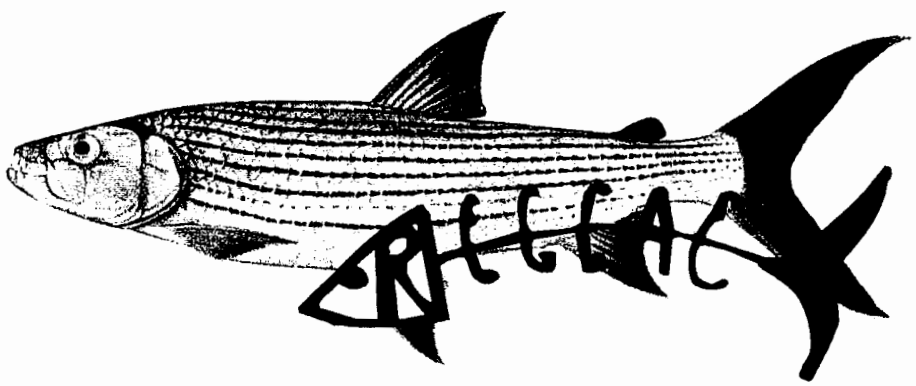
SUMMARY



SUMMARY

The thesis is made up of a series of publications on research work that was structured to facilitate a systematic evaluation of biochemical and genetic technology with potential application in other species. While this approach may not flow as effectively as a more singular study, it has facilitated a link between two primary objectives that deal with five specific aims, namely:

A) To investigate and establish genetic variation / distances / phylogenetic relationships and to test and refine technology with potential industrial application;

- To investigate and establish genetic distances / variation between two morphologically different populations of *Hydrocynus vittatus* from the Upper Zambezi River and the Olifants River in the Kruger National Park;
- To obtain information on the genetic variation, gene flow and possible inbreeding in two comparative gene pools of *Brycinus lateralis* from the Zambezi and Quando Rivers;
- To investigate the current classification of representatives from the family Characidae with regard to systematics and phylogenetics, and to develop comparative data on the respective species;
- To compare a limited number of Characidae species to support morphological relationships;

B) To investigate the industrial application of the technology developed during the initial research phase of this thesis with regard to other species.

The first study to investigate and establish genetic distances / variation in *H. vittatus* from the Zambezi and Olifants River Systems indicated that sufficient genetic variation existed to distinguish between the two popualtions. The Olifants population

had a higher degree of heterozygosity, a characteristic that could have application in breeding programs to establish viable populations for restocking for conservation and recreational purposes.

The second part of the study as discussed in Chapter 3, indicated that the two comparable populations of *B. lateralis* had a higher degree of heterozygosity / genetic variation than other freshwater fish from the same geographical area. This supports the theory on the higher degree of variation within introduced species to enable such species to adapt more effectively. This variation could also have application in the identification of within species ecotypes.

The results obtained in Chapter 4 to verify the current classification of representatives from the family Characidae from southern Africa provided clear evidence of substantial differentiation between southern African characins and thereby affirming the currently known systematics of these taxa. This in turn, verifies the technology and its possible aplication in other species.

In comparing a limited number of Characidae species using a combination of morphological and genetic data, it was also indicated that morpholical similarities between species were useful as quantitative characters to separate the two families (Characidae and Hepsetidae) and to verify the close relationship (distance) between the two *Brycinus* species.

The first phase of the study not only provided more information on freshwater fish species, with potential application in breeding for both conservation and utilisation purposes, but also tested, refined and developed procedures with potential application in other species and related fields. All too often, studies end with the publication of a few articles and a thesis that is then shelved for later reference without any consideration of using the information and technology in related fields where a broader spectrum of the industry could benefit. In this study, opportunity existed for fairly extensive industrial application and concurrent and parallel

application for the southern African region that led to the enhancement and development of technology to support the following:

- Law enforcement – the identification of species
- Quality control – verification of products
- Genetic distancing studies (characterisation of farm animal breeds)
- Screening of populations for origin.

The application of the biochemical and genetic tools used in this study contributed to the establishment of a species identification service, the only laboratory of its kind in Africa. This laboratory renders a service to the industries involved in the importation of meat and meat products and to the law enforcement sector with forensic services in species and individual identification (poaching and stock theft). It has also been possible to provide training for researchers and technologists from a number of countries in the Southern African Development Community (SADC) region and thereby creating links for future collaborative work.

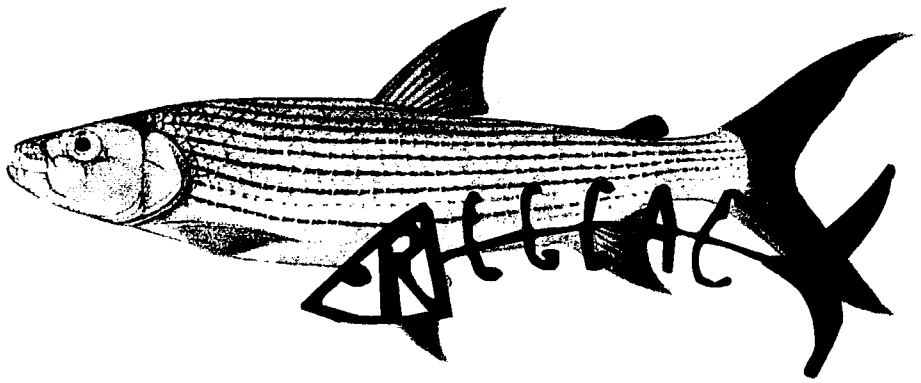
The second and possible the most rewarding has been the contribution to the genetic characterisation of farm animal genetic resources (FanGR). This has enabled South Africa and other SADC member countries to meet a commitment to the United Nations Convention on Biodiversity to characterise the FanGR in the region and to develop programs for the conservation and sustainable use of the breeds in question. It will also enhance the quality and accuracy of country reports being prepared for the FAO-driven first Global Report on the State of the World Animal Genetic Resources. The technology has also enabled researchers and developers to add value to identified breeds by labeling them as pure – a prerequisite for the effective marketing of FanGR.

APPENDICES

(The raw data is included in the appendices to be available to other researchers and for possible further analysis for future publication purposes.)

APPENDIX 1

APPENDIX 2



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NEXT

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NEXT																										
TIE	TIERVIS																									
0001	DS2	DD	AA	OO	OO	CC	BB	BB	AA	OO	BB	BB	BB	BB	BB	AA	CC	BB	AA	AA	EE	CC	OO	OO	OO	OO
0002	DS2	DD	AA	OO	OO	CC	BB	BB	AA	OO	BB	BB	BB	BB	BB	AA	CC	BB	AA	AA	EE	CC	OO	OO	OO	OO
0003	DS2	DD	AA	OO	OO	CC	BB	OO	AA	OO	BB	BB	BB	BB	BB	AA	CC	BB	AA	AA	EE	CC	OO	OO	OO	OO
0004	DS2	DD	AA	OO	OO	CC	BB	OO	OO	OO	BB	BB	OO	BB	OO	AA	CC	BB	AA	AA	EE	CC	OO	OO	OO	OO
0005	DS2	BB	AA	CC	AA	OO	OO	BB	AA	AA	BB	OO	BB	BB	OO	AA	CC	BB	AA	OO	EE	CC	BB	BB	BB	BB
0006	DS2	BB	AA	CC	AA	OO	OO	BB	AA	AA	BB	OO	BB	BB	OO	AA	CC	BB	AA	OO	EE	CC	OO	BB	BB	BB
0007	DS2	BB	AA	CC	AA	OO	OO	BB	AA	AA	OO	OO	BE	BD	OO	AA	CC	BB	AA	OO	EE	CC	OO	BB	BB	BB
NEXT																										
RMA	RHABDALESTES MAUNENSIS																									
0001	DS2	AA	AA	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0002	DS2	AA	AA	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	AB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0003	DS2	AA	AA	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0004	DS2	AA	AA	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0005	DS2	OO	AA	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0006	DS2	OO	OO	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0007	DS2	OO	OO	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0008	DS2	OO	OO	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0009	DS2	OO	OO	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0010	DS2	OO	OO	BB	AA	OO	OO	CC	CC	BB	AA	OO	DD	BB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	CC	DD
0011	DS2	OO	OO	BB	AA	OO	OO	CC	BC	BB	AA	OO	BD	AB	OO	AA	CC	BB	AA	OO	AA	CC	BB	CC	OO	OO
0012	DS2	AA	AA	OO	OO	AA	DD	CC	CC	OO	AA	CC	CC	BB	CC	AA	CC	BB	AA	BB	AA	CC	OO	OO	OO	OO
0013	DS2	AA	AA	OO	OO	AA	DD	CC	CC	OO	AA	CC	CC	BB	OO	AA	CC	BB	AA	BB	AA	CC	OO	OO	OO	OO
0014	DS2	AA	AA	OO	OO	AA	DD	CC	CC	OO	AA	CC	BC	BB	OO	AA	CC	BB	AA	BB	AA	CC	OO	OO	OO	OO
0015	DS2	AA	AA	OO	OO	AA	DD	CC	CC	OO	AA	CC	BC	AB	OO	AA	CC	BB	AA	BB	AA	CC	OO	OO	OO	OO

NEXT
MAC MICRALESTES ACUTIDENS

0001	DJ1	AA	AA	OO	OO	AA	CC	OO	CC	OO	CC	BB	BB	AB	CC	AA	CC	BB	AA	BB	BB	CC	OO	OO	OO	OO
0002	DJ1	AA	AA	OO	OO	AA	CC	OO	CC	OO	CC	BB	BB	AB	CC	AA	CC	BB	AA	BB	BB	CC	OO	OO	OO	OO
0003	DJ1	AA	AA	OO	OO	AA	CC	OO	OO	OO	CC	BB	BC	AB	CC	AA	CC	BB	AA	BB	BB	CC	OO	OO	OO	OO
0004	DJ1	AA	AA	OO	OO	AA	OO	OO	OO	OO	CC	BB	CC	BB	OO	AA	CC	BB	AA	BB	BB	CC	OO	OO	OO	OO
0005	DJ1	BB	AA	BB	AA	OO	OO	AA	CC	BB	BB	OO	CC	AA	OO	AA	CC	BB	AA	OO	BB	CC	AA	BB	CC	BB
0006	DJ1	BB	AA	BB	AA	OO	OO	AA	CC	BB	BB	OO	CC	AB	OO	AA	CC	BB	AA	OO	BB	CC	AA	AA	AA	AA
0007	DJ1	BB	AA	BB	AA	OO	OO	AA	CC	BB	BB	OO	CC	AA	OO	AA	CC	BB	AA	OO	BB	CC	AA	BB	CC	BB
0008	DJ1	BB	AA	BB	AA	OO	OO	AA	CC	BB	BB	OO	CC	AA	OO	AA	CC	BB	AA	OO	BB	CC	AA	BB	CC	BB
0009	DJ1	OO	AA	OO	AA	OO	OO	AA	CC	BB	BB	OO	CC	AA	OO	AA	CC	BB	AA	OO	BB	CC	AA	BB	CC	BB
0010	DJ1	OO	AA	OO	AA	OO	OO	OO	OO	BB	BB	OO	CD	BB	OO	AA	CC	BB	AA	OO	BB	CC	AA	OO	OO	BB

NEXT
BIM BRYCINUS IMBERI

0001	DJ2	BB	AA	OO	OO	AA	CC	CC	BB	OO	CC	BB	CC	BB	CC	DD	BB	BB	AA	BB	CC	BB	OO	OO	OO	OO
0002	DJ2	BB	AA	OO	OO	AA	CC	CC	BB	OO	CC	BB	CC	BB	CC	DD	BB	BB	AA	BB	CC	BB	OO	OO	OO	OO
0003	DJ2	OO	AA	OO	OO	AA	CC	CC	BB	OO	CC	BB	CC	OO	CC	DD	BB	BB	AA	BB	CC	BB	OO	OO	OO	OO
0004	DJ2	OO	AA	OO	OO	AA	OO	CC	BB	OO	CC	BB	CC	OO	OO	DD	BB	BB	AA	BB	CC	BB	OO	OO	OO	OO
0005	DJ2	BB	AA	CC	AA	OO	OO	CC	BB	AA	CC	OO	CC	BB	OO	BB	BB	BB	AA	OO	CC	BB	BB	BB	CC	CC
0006	DJ2	OO	AA	CC	AA	OO	OO	OO	BB	AA	CC	OO	CC	BB	OO	BB	BB	BB	AA	OO	CC	BB	BB	BB	OO	CC

NEXT
BLA BRYCINUS LATERALIS

0001	TD1	AA	BB	AA	AA	OO	OO	CC	CC	BB	BB	OO	AA	BB	OO	AA	BB	BB	AA	OO	DD	CC	AA	CC	CC	BB
0002	TD1	AA	BB	AA	AA	OO	OO	CC	CC	BB	BB	OO	AA	BB	OO	AA	BB	BB	AA	OO	DD	CC	AA	CC	CC	BB
0003	TD1	AA	BB	AA	AA	OO	OO	CC	CC	BB	BB	OO	AA	BB	OO	AA	BB	BB	AA	OO	DD	CC	AA	CC	CC	BB
0004	TD1	AA	BB	AA	AA	OO	OO	CC	CC	BB	BB	OO	AA	BB	OO	AA	BB	BB	AA	OO	DD	CC	AA	CC	CC	BB
0005	TD1	AA	BB	AA	AA	OO	OO	CD	CD	BB	BB	OO	AA	BB	OO	AA	BB	BB	AA	OO	DD	CC	AA	CC	CC	BB
0006	TD1	AA	BB	OO	OO	BB	DD	CC	CC	OO	BB	BB	AA	BB	AA	AB	BB	BB	AB	CC	DD	CC	OO	OO	OO	OO
0007	TD1	AA	BB	OO	OO	BB	DD	CC	CC	OO	BB	BB	AA	BB	AA	AB	BB	BB	AA	CC	DD	CC	OO	OO	OO	OO
0008	TD1	AA	AA	OO	OO	BB	DE	CC	CC	OO	BB	BB	AA	BB	AA	AB	BB	BB	AA	CC	DD	CC	OO	OO	OO	OO
0009	TD1	AA	AA	OO	OO	BB	EE	OO	OO	OO	BB	BB	AA	OO	AA	BB	BB	BB	OO	CC	DD	CC	OO	OO	OO	OO

NEXT
BAF BRYCINUS AFFINUS

0001	TD2	BB	AA	AA	OO	AA	DD	CC	OO	OO	OO	BB	BB	BB	OO	OO	OO	CC	AA	BB	OO	OO	BB	BB	OO	CC
0002	TD2	BB	AA	AA	OO	AA	DD	CC	OO	OO	OO	BB	BB	BB	OO	OO	OO	CC	AB	BB	OO	OO	BB	BB	OO	CC
0003	TD2	BB	AA	AA	OO	AA	DD	CC	OO	OO	CC	BB	BB	BB	BB	BC	BB	CC	AA	BB	CC	AB	BB	BB	OO	CC
0004	TD2	BB	AA	AA	OO	AA	DD	OO	OO	OO	CC	BB	BB	BB	BB	BB	BB	CC	AA	BB	CC	AB	BB	BB	OO	CC
0005	TD2	BB	AA	AA	OO	AA	DD	OO	OO	OO	OO	BB	BB	BB	OO	OO	OO	CC	AA	BB	OO	BB	BB	BB	OO	CC
0006	TD2	BB	AA	OO	OO	AA	DD	OO	OO	OO	OO	BB	BB	BB	OO	OO	OO	CC	AA	BB	OO	BB	BB	BB	OO	CC
0007	TD2	BB	AA	OO	OO	AA	DE	OO	OO	OO	CC	BB	BB	BB	BB	CC	BB	CC	AA	BB	CC	AB	BB	BB	OO	CC
0008	TD2	BB	AA	OO	OO	AA	OO	OO	OO	OO	CC	BB	BB	BB	BB	BB	CC	CC	AA	BB	CC	BB	BB	BB	OO	CC
0008	TD2	BB	AA	OO	OO	AA	OO	OO	OO	OO	CC	BB	BB	OO	OO	BC	BB	CC	AA	BB	CC	AA	BB	BB	OO	CC

END