Population genetics of *Pseudobarbus phlegethon*, *Barbus calidus* and *Barbus erubescens* (Teleostei: Cyprinidae) of the Olifants River System, Western Cape Province

by

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Declaration: I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

ABSTRACT

Endemic redfin minnows of the Olifants River System are under threat of extinction, mainly because of alien predatory fish, water extraction and pollution. The taxonomic position and biogeography of redfins in relation to other barbs are uncertain. Enzyme electrophoresis was employed to assess genetic differentiation within and among certain *Barbus* and *Pseudobarbus* taxa. Fourteen enzymes were routinely recorded from muscle tissue extracts, yielding a differential number of active loci. The latter is due to different ploidy levels. Polyploid species showed fewer loci than the tetraploid or hexaploid number of loci expected just after the polyploidy event. *Barbus aenus* (hexaploid) expressed 6.2 % of the duplicated loci expected after the hexaploidy event. The tetraploid *Pseudobarbus* and serrated *Barbus* spp., expressed 37.5 % and 31.2 % of the duplicated loci expected after the tetraploidy event, respectively. This suggests that the ploidy event in *Barbus aenus* is more ancient than in the above-mentioned tetraploid barbs. The similar amount of diploidization in *Pseudobarbus* and tetraploid serrated *Barbus* spp. investigated, suggests a shared ploidy event between these two lineages. The number of active loci is apparently not a good indication of the ploidy level of African barbs, as hexaploid *Barbus aenus* expressed less active loci than the tetraploid barbs investigated. Initial screening of allozyme loci yielded fixed allele differences at 22 loci, but polymorphism only at seven. This suggests more potential for delineating species boundaries than for assessing gene flow and genetic diversity of populations. Genetic differentiation within and among populations of *Pseudobarbus phlegethon*, *Barbus calidus* and *Barbus erubescens* were investigated by screening 27 allozyme loci. A clear divergence between *Pseudobarbus phlegethon* populations from the Olifants and Doring Rivers was found (Nei's unbiased genetic distance = 0.355; F-statistic for subpopulation against the total = 0.877). Preference and adaptation for mountain stream habitat might explain the past isolation and

subsequent divergence of *Pseudobarbus phlegethon* populations between these two rivers. *Barbus calidus* was genetically homogenous over its distribution (Nei's unbiased genetic distance = 0-0.009; F-statistic for subpopulation against the total = 0.135). It is proposed that *Barbus calidus* is better adapted than *Pseudobarbus phlegethon* to disperse through the main stream. The genetic divergence between *Barbus calidus* and *Barbus erubescens* (Nei's unbiased genetic distance = 0.063) is characteristic of conspecific populations. There seems to have been a loss of genetic diversity in redfin populations of the Doring River tributaries (heterozygosity = 0-0.01), compared to the redfin populations of the Olifants River tributaries (heterozygosity = 0.01- 0.04). From phylogenetic analysis of allozyme characters, it seems as if the serrated tetraploid barbs from South Africa is the sister-group of *Pseudobarbus*, whilst *Barbus anoplus* was rejected as a sister-species for the latter. *Barbus calidus*, *Barbus erubescens* and *Barbus serra* were found to be closely related to each other. The ancestor of the redfins seems to have been present in the Cape Fold Mountains since at least the late-Tertiary. On the basis of distributional and allozyme information, conservation units reflecting historical divergence, historical gene flow and current gene flow were identified as Evolutionarily Significant Units, Historical Management Units and Current Management Units respectively. The Olifants and Doring River populations of *Pseudobarbus phlegethon* should be recognized as two distinct Evolutionarily Significant Units. *Barbus calidus* forms a separate Evolutionarily Significant Unit from *Barbus erubescens*. Twelve Historical Management Units and nineteen Current Management Units were recognized for redfins of the Olifants River System. The size of Current Management Units should be expanded and secured to prevent loss of genetic diversity. It is recommended that a recovery program of redfins should establish new populations of at least *Barbus erubescens* and Doring River *Pseudobarbus phlegethon* Evolutionarily Significant Unit. Centrarchids should be irradicated, as they are the main reason for the decline of redfins.

UITTREKSEL

Die endemiese rooivlerkies van die Olifantsriviersisteem word bedreig deur uitsterwing, hoofsaaklik as gevolg van uitheemse roofvisse, water onttrekking en besoedeling. Die taksonomiese posisie en biogeografie van rooivlerkies in verhouding met ander barbusse is onseker. Ensiemelektroferese is gebruik om genetiese differensiasie binne en tussen sekere *Barbus* en *Pseudobarbus* taksa te bepaal. Veertien ensieme, wat verskillende hoeveelhede aktiewe lokusse gelewer het, is op roetine basis waargeneem uit spierweefselekstrakte. Die verskillende hoeveelhede aktiewe lokusse wat waargeneem is, is as gevolg van verskillende ploied vlakke. Poliploïede spesies het minder lokusse getoon as wat verwag sou word net na die tetraploïede of heksaploïede gebeurtenisse. *Barbus aenus* (heksaploïed) het 6.2% van die gedupliseerde lokusse, wat verwag sou word na die heksaploiede gebeurtenis, uitgedruk. Die tetraploïede *Pseudobarbus* en saagagtige *Barbus* spp. het 37.5% en 31.2% respektiewelik van die gedupliseerde lokusse, wat verwag sou word na die tetraploïede gebeurtenis, uitgedruk. Dit dui daarop dat die ploïede gebeurtenis in *Barbus aenus* meer histories as in die bogenoemde tetraploïede barbusse. Die soortgelyke hoeveelheid diploïedisasie in *Pseudobarbus* en tetraploïede saagagtige *Barbus* spp. wat ondersoek is, dui op 'n moontlike gesamentlike ploïede gebeurtenis tussen hierdie twee evolusionêre lyne. Die aantal aktiewe lokusse blyk nie 'n goeie aanduiding van die ploïed vlakke van Afrika barbusse te wees nie, aangesien die heksaploïede *Barbus aenus* minder lokusse as die tetraploïede barbusse wat ondersoek is, getoon het. Aanvanklike analisering van allosiem lokusse het vaste alleel verskille in 22 lokusse opgelewer, maar slegs sewe het polimorfisme getoon. Dit dui op moontlike beter potensiaal om spesie-grense vas te stel, eerder as die bepaling van genevloei of genetiese diversiteit van populasies. Genetiese differensiasie binne en tussen populasies van *Pseudobarbus phlegethon*, *Barbus calidus* en *Barbus erubescens* is ondersoek deur 27

allosiem lokusse te analiseer. 'n Duidelike divergensie tussen *Pseudobarbus phlegethon* populasies van die Olifants- en Doring- riviere is gevind (Nei se onbevooroordeelde genetiese afstand = 0.355; F-statistiek vir subpopulasie teen die totaal = 0.877). Voorkeur en aanpassing vir bergstroom habitat, mag moontlik die historiese isolasie en daaropvolgende divergensie van *Pseudobarbus phlegethon* populasies tussen hierdie twee riviere verduidelik. *Barbus calidus* was geneties homogeen oor sy verspreiding (Nei se onbevooroordeelde genetiese afstand = 0-0.009; F-statistiek vir subpopulasie teen die totaal = 0.135). Dit word voorgestel dat *Barbus calidus* beter aangepas is as *Pseudobarbus phlegethon* om deur die hoofstroom te versprei. Die genetiese divergensie tussen *Barbus calidus* en *Barbus erubescens* (Nei se onbevooroordeelde genetiese afstand = 0.063) is kenmerkend van spesie-spesifieke populasies. Dit lyk of daar 'n verlies aan genetiese diversiteit in rooivlerkie populasies vanaf die Doringrivier sytakke was (heterosigositeit = 0-0.01), in vergelyking met rooivlerkie populasies vanaf die Olifantsrivier sytakke (heterosigositeit = 0.01- 0.04). Uit die filogenetiese analise van allosiem kenmerke, blyk dit dat die saagagtige tetraploiede barbusse van Suid-Afrika die sistergroep van *Pseudobarbus* is, terwyl *Barbus anoplus* verwerp word as sisterspesie vir laasgenoemde. Dit is gevind dat *Barbus calidus*, *Barbus erubescens* en *Barbus serra* baie naverwant aan mekaar is. Dit lyk asof die voorvader van die rooivlerkies sedert ten minste die laat-Tersiêr in die Kaapse Plooiberge aanwesig was. Bewaringseenhede wat historiese divergensie, historiese genevloei en huidige genevloei voorstel, is geidentifiseer respektiewelik as Evolusionêre Beduidende Eenhede, Historiese Bestuurseenhede en Huidige Bestuurseenhede. Dit is gedoen op grond van verspreidings- en allosieminformasie. Die Olifants en Doring populasies van *Pseudobarbus phlegethon* moet as twee unieke Evolusionêre Beduidende Eenhede erken word. *Barbus calidus* en *Barbus erubescens* vorm aparte Evolusionêre Beduidende Eenhede. Twaalf Historiese Bestuurseenhede en negentien Huidige Bestuurseenhede is geidentifiseer vir rooivlerkies van die Oifantsriviersisteem. Die

grootte van Huidige Bestuurseenhede moet vergroot en beveilig word om verlies van genetiese diversiteit te vermy. Dit word voorgestel dat 'n herstelprogram van rooivlerkies moet poog om nuwe populasies, van ten minste *Barbus erubescens* en Doringrivier *Pseudobarbus phlegethon* Evolusionêre Beduidende Eenhede, te vestig. Baars en blouwang sonvis moet uitgeroei word, aangesien hulle die hoofoorsaak is vir die afname van rooivlerkies.

Dedication: In memory of my grandfathers, J.F.A. Swartz and S.W. Vorster.

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CHAPTER 1

GENERAL INTRODUCTION

The Olifants River System of the Western Cape Province, South Africa, has been described as a natural aquarium (Scott, 1982), having the highest endemicity of fish south of the Zambezi River System (Gaigher, 1973a). It has also been described as the "Nile of South Africa" and serves one of the largest irrigation agricultural complexes in southern Africa (Burman, 1970). Unfortunately, the entire endemic fish assemblage of the Olifants River System, consisting of six cyprinids and two austroglanids, is in danger of extinction (Skelton, 1987; Baillie & Groombridge, 1996). This is due to unsustainable water extraction, habitat degradation, pollution and the introduction of alien fish (Skelton, 1987; Impson & Hamman, in prep.). The introduction of North-American Black Bass species to improve the sportfishing potential of the Olifants River System (Harrison, 1950), in particular has had a catastrophic impact on the indigenous fish (Brooks, 1949), and severely reduced and fragmented native fish populations (Gaigher 1973b; Bills, 1999).

Conservation agencies face the challenge to conserve genetic diversity within populations and genetic integrity of unique lineages of the Cape fishes amidst the above-mentioned adversities. Cape Nature Conservation (CNC), which is the statutory provincial conservation agency for the Western Cape, took the initiative to support population genetic studies like the present one, because information is needed urgently in order to prioritize conservation efforts and effectively manage native fish populations of the Olifants River System (Impson, pers. comm.). As part of this initiative, it was decided to investigate the genetic differentiation and diversity of populations of three cyprinid minnows endemic to the Olifants River System,

namely the fiery redfin (*Pseudobarbus phlegethon*), Clanwilliam redfin (*Barbus calidus*) and the Twee River redfin (*Barbus erubescens*).

The Olifants River System consists of two main drainage systems, namely the Olifants and Doring Rivers. The upper reaches of the Olifants River drain tributaries of the western Cedarberg Mountains, from the Koue Bokkeveld Valley to Clanwilliam. The Doring River drains the tributaries on the eastern side of the Cedarberg Mountains and joins the Olifants River north of Clanwilliam. The above-mentioned redfins are endemic to these eastern and western flowing tributary streams draining the Cedarberg Moutains, but this reflects only a fraction of their former distribution, as they once also occurred in the main stream of the Olifants River (Harrison, 1938; Barnard, 1943). Fragmentation of redfin populations can cause loss of genetic diversity. In theory, populations with greater genetic diversity will be able to adapt better to stress factors like habitat alteration and the introduction of alien parasites (Leberg & Vrijenhoek, 1994), and it will also play a role in the fitness of populations (Quattro & Vrijenhoek, 1989). There are concerns, for these reasons, about small size of some redfin populations in the Olifants River System (Impson, pers. comm.), and they are considered to be in danger of extinction (Skelton, 1987; Baillie & Groombridge, 1996).

Apart from concerns about their restricted and fragmented distribution, declining numbers, and isolation in tributaries of the Olifants River System, these redfins were also chosen because they occur together with several other native fishes in the tributary streams. Effective management of these tributary streams should thus benefit all the other indigenous fish. Most of these streams are relatively undisturbed as compared to the main stream and they are often the only areas where specialist main stream native fish are still able to breed successfully.

Boulenger (1911) classified African barbs into four broad categories based on scale radii and the shape of the last unbranced dorsal ray. *Pseudobarbus phlegethon*, *Barbus calidus* and *B. erubescens* have radiating scales, which distinguishes them from large hexaploid barbs having scales with parallel striae (Skelton, 1976; Oellermann & Skelton, 1990). Barbs with radiating scale striae are subdivided into three categories, namely those with a (a) bony and smooth, (b) bony and serrated, or (c) soft and unserrated last unbranched dorsal fin-ray (Boulenger, 1911). *Pseudobarbus* spp. fall within the last category (Skelton, 1988). *Barbus calidus* and *B. erubescens* (i.e., "serrated redfins") fall within Boulenger's (1911) third category (category b above). The outgroup relationships of these two sister species, *B. calidus* and *B. erubescens*, are unknown (Skelton, 1986). When *Pseudobarbus* was described by Skelton (1988), *B. calidus* and *B. erubescens* were not included on the basis of having a serrated bony dorsal ray, despite the presence of red fins. Other minnows falling within Boulenger's (1911) third category, like *B. hospes*, *B. trevelyani* and *B. argenteus*, were named as possible close relatives of the "serrated redfins" (Skelton, 1986). Skelton (1976), however, suggested a possible close relationship between the sister species *Barbus serra* and *B. andrewi* and the "serrated redfins". This is because they are all "serrated barbs", having radiating scales and more anal fin-rays (except for *B. serra*) than the characteristic five for other barbs. *Barbus serra* and *B. andrewi* also have close geographic distributions to the "serrated redfins".

The idea of a close relationship among the "serrated redfins" and the two large serrated barbs (*B. serra* and *B. andrewi*), was strengthened when it was found that they all have a chromosome number of 100 which is characteristic of tetraploid species (Naran, 1997). Skelton (1980) suggested that *B. anoplus* is the sister group of *Pseudobarbus*, but Naran (1997) dismissed this. She suggested the South African tetraploid serrated *Barbus* spp. as the outgroup (consisting of *B. calidus*, *B. erubescens*, *B. serra*, *B. andrewi*, *B. hospes* and *B.*

trevelyani). This is surprising, as there is morphological evidence against this association (Skelton, 1988) and it does not agree with Boulenger's (1911) categories. She also proposed on the basis of a phylogenetic analysis of morphological, osteological and meristic characters, that the tetraploidy event might be shared between *Pseudobarbus* and South African tetraploid serrated *Barbus*. These issues remain largely unresolved.

The origin of *Pseudobarbus* and South African tetraploid serrated *Barbus* is generally uncertain (Skelton 1986, 1994; Naran, 1997). There are two main ideas on the biogeography of the minnows of the Cape Ichthyofauna. The first suggests that cyprinids of the Cape ichthyofauna have originated, mostly in a dispersalist fashion, from relatively recent (Pliocene to Pleistocene) invasions from the north (Barnard, 1943; Jubb, 1964; Jubb & Farquharson, 1965; Gaigher & Pot, 1973; Mulder, 1989). The second suggests that redfins are remnants of the early Tertiary and that they speciated largely through vicariance with dispersal playing a lesser role (Skelton, 1980, 1986, 1994).

Pseudobarbus phlegethon, *Barbus calidus* and *B. erubescens* are tetraploid (Naran, 1997). Naran (1997) found that small *Barbus* spp. of South Africa are either diploid or tetraploid and that all the redfin species (*Pseudobarbus*, *Barbus calidus* and *Barbus erubescens*) are tetraploid. Large South African *Barbus* spp. are either tetraploid or hexaploid (Oellermann & Skelton, 1990; Naran, 1997). Golubtsov & Krysanov (1993) and Naran (1997) observed an absence of multivalents in meiotic spreads of chromosomes of tetraploid and hexaploid African *Barbus* spp., as well as *Pseudobarbus*. This possibly indicates that ploidy events happened a long time ago in these taxa (Golubtsov & Krysanov, 1993; Naran, 1997).

Although some systematic work has been done on redfins (e.g. Barnard, 1943; Skelton, 1980, 1988), almost nothing is known about their genetic structure. Genetic investigations by Bloomer & Impson (in press) and Waters & Cambray (1997), showed that much genetic divergence exists within some of the currently recognised fish species of the Western Cape. No previous work has been done on the genetic structure of fish in the Olifants River System. Such studies would greatly benefit conservation management of this river system, and improve knowledge on the biogeography of this region. There is a need to assess the impact of habitat fragmentation on redfins of the Olifants River System by investigating genetic diversity of the mentioned taxa (Impson, pers. comm.). There is also a need to assess the divergence between redfin lineages, particularly the apparently close relationship between *B. calidus* and *B. erubescens* (Skelton, 1974). Concerns about the small size of some redfin populations have to be addressed by assessing the genetic diversity of these populations. A population genetic study of *B. calidus* and *P. phlegethon* will offer the opportunity to relate their genetic structure to their differential morphology and niche preference, as these two taxa occur mostly sympatrically. *B. calidus* and *P. phlegethon* seems to occupy different ecological niches in the Cedarberg mountain streams (Bills, 1999), with *B. calidus* more common in both lower and headwater sections of the tributary streams, whilst *P. phlegethon* concentrate in the mid-to-lower sections of these streams. Differential niche preference, behaviour and body form between *B. calidus* and *P. phlegethon* (Bills, 1999; Skelton, 1980, 1988) may have played an important role in dispersability and consequently their genetic structure before recent fragmentation.

Allozyme electrophoresis was chosen as molecular tool to analyze the genetic structure of redfin populations. Since the development of gel electrophoresis (Smithies, 1955; Hunter & Markert, 1957) and its application in studies of genetic variation in populations (Hubby &

Lewontin, 1966; Lewontin & Hubby, 1966), allozyme electrophoresis became the predominant tool used in studying populations genetics. Allozyme electrophoresis is still the most widely used method in answering conservation genetic problems (Leberg, 1996) and has a widespread use in conservation management (Allendorf & Waples, 1996; Leberg, 1996; Vrijenhoek, 1996).

Protein electrophoresis involves the migration of proteins through a support medium under the influence of an electric field (Grant & Robinson, 1989). The structure, shape and charge of the protein, the electric field, the nature of the support medium and the buffer solution, all affect the protein mobility. Enzymes consisting of different primary amino acid sequences, but catalyse the same reaction using the same substrate, are called isozymes (Markert & Moller, 1959). A subset of isozymes is called allozymes. These are enzymes encoded by different alleles at the same locus (Grant & Robinson, 1989). The separation of alleles during electrophoresis can be interpreted as enzyme structural differences. These structural differences are assumed to be due to underlying genetic variation. This assumption can be wrong, however, in certain cases if post-translational modification, intra-cistronic recombination, or non-genetic variation occur (reviewed by Murphy et al., 1996). The most widely used support medium for electrophoresis, is starch gel (Murphy et al., 1996). Numerous buffer solutions have been developed which can be applied to optimally resolve different enzymes (see Markert & Faulhaber, 1965; Ridgeway et al., 1970; Shaw & Prasad, 1970; Whitt, 1970; Harris & Hopkinson, 1976, Murphy et al., 1996). Detailed methodological descriptions of allozyme electrophoresis, including histochemical staining methods are available (eg. Shaw & Prasad 1970; Harris & Hopkinson, 1976; Grant & Robinson, 1989; Murphy et al., 1996).

A disadvantage of allozyme electrophoresis is its inability to detect most of the variation occurring in the genome even though it may be possible to screen up to 300 loci in an organism (Wright et al., 1983; Morizot & Siciliano, 1984; Manchenko, 1994). According to Ward et al. (1992) and Leberg (1996), most allozyme studies do not even come close to this number of loci, and usually less than 40 are investigated. Archie (1985) sets the latter number as a minimum to have good confidence in heterozygosity estimates. According to Murphy et al. (1996), however, this is a problem associated with most molecular techniques.

Allozyme electrophoresis will be used in the present study mainly because of its potential to estimate genetic diversity (e.g. Quattro & Vrijenhoek, 1989; Leary et al., 1993) and to determine population structuring (e.g. Smith et al., 1983; Hanzawa et al., 1988; Naish et al., 1993). This method has been used to estimate time since divergence between taxa (Sarich, 1977; e.g. Dowling & Moore, 1985) and will used in the present study to test conflicting hypothesis on the biogeography of redfins. Allozyme electrophoresis can also be applied to systematics (e.g. Dimmick & Lawson; 1991; Karakousis et al., 1995; Machordom et al., 1995; Quattro et al., 1996) and will be employed in the present study to investigate the uncertain phylogenetic position of redfins in relation to other barbs. It will also be used to distinguish between ploidy levels (e.g. Agnèse et al. 1990; Berrebi et al., 1990) and to determine the relative amount of diploidization after a ploidy event (e.g. Agnèse et al. 1990; Berrebi et al., 1990) in South African barbs.

The first objective of this thesis was to assess allozyme loci that can be used in routine analyses of *Barbus* and *Pseudobarbus*. This was done in Chapter 2 with emphasis on the number of active loci that can be resolved for each enzyme, specifically in relation to the ploidy level of the taxon involved. Inferences on the amount of diploidization after the ploidy

event and the remoteness of the ploidy event in *Barbus* and *Pseudobarbus* spp. were made by investigating representatives of *Pseudobarbus*, serrated tetraploid *Barbus* spp., a diploid *Barbus* sp. and a hexaploid *Barbus* sp. from South Africa. The suitability of allozyme electrophoresis for the aims of the present thesis is scrutenized in this chapter.

Chapter 3 focusses on aspects of population genetics, biogeography and phylogenetics of *B. calidus*, *B. erubescens* and *P. phlegeton* of the Olifants River System. The following key questions concerning these redfins are addressed:

1) Is there gene flow among populations at present, or had there been in the past?
2) Have the populations lost genetic diversity because of fragmentation caused by bass?
3) Can the influence on genetic diversity and on gene flow patterns of recent fragmentation, as opposed to historical events, be distinguished from each other?
4) Have the differential morphology (Skelton, 1980, 1988) and differential niche preference of *B. calidus* and *P. phlegeton* (Bills, 1999) affected dispersability and hence gene flow in these two taxa?
5) Which hypotheses on the biogeography of the Cape Ichthyofauna (Barnard, 1943; Jubb, 1964; Jubb & Farquharson, 1965; Gaigher & Pot, 1973; Mulder, 1989; Skelton, 1980, 1986, 1994) is consistent with enzyme evolution rates among redfins?

The apparently close relationship between *B. calidus* and *B. erubescens* was investigated to better understand the event that led to their speciation. The *B. calidus* and *B. erubescens* lineage was also related to members of *Barbus* and *Pseudobarbus* to investigate their uncertain phylogenetic relationship with other barbs. *B. anoplus* was included as an outgoup to test its relationship to *Pseudobarbus*.

In Chapter 4 the implications of the findings of earlier chapters for conservation management of redfins in the Olifants River System, is discussed. The following questions were posed:

1) Why have the redfins been declining?

2) How many redfin populations are left?

3) Which streams should be declared as sanctuaries for the conservation of redfins?

4) Is there additional suitable habitat that can be rehabilitated for redfins in the future?

5) Are there unique lineages in need of protection and what are the priorities in conserving redfins?

6) How can conservation agencies effectively manage and conserve genetic diversity and unique lineages of redfins of the Olifants River System?

To answer these questions, Evolutionarily Significant Units (ESU's) and Management Units (MU's) of Olifants River redfins are identified to assist conservation agencies in prioritizing their efforts and to effectively manage these taxa. Threats to the survival of redfin populations in the Olifants River System were assessed. Recommendations were made to help conservation agencies to conserve genetic diversity and the integrity of unique lineages of redfins of the Olifants River System.

CHAPTER 2

PROCEDURES FOR ANALYSES, AND LOCUS EXPRESSION OF ALLOZYMES IN THE GENERA *BARBUS* AND *PSEUDOBARBUS*.

2.1 INTRODUCTION

A differential number of allozyme loci may be resolved in taxa showing different ploidy levels (Allendorf & Thorgaard, 1984; Ferris, 1984). Amongst polyploid taxa of the same ploidy level, functional diploidization of loci or the occurrence of isoloci may account for differences in the number of loci resolved. South African *Barbus* spp. show three ploidy levels, namely diploid, tetraploid and hexaploid (Oellermann & Skelton, 1990; Naran, 1997), whilst all the *Pseudobarbus* spp. are tetraploid (Naran, 1997). Isoloci would be very common just after a ploidy event, before the loci have had a change to diverge, or before a null allele can become fixed in the duplicated locus or loci (Ohno, 1970a, 1970b; Allendorf & Thorgaard, 1984; Ferris, 1984). In time, however, silencing of duplicated loci will occur because of deleterious mutations (Ohno, 1970a). Diploidization of duplicated loci can cause loss of loci in any species at any time, and can lead to variation in the number of loci expressed in polyploid species (Ohno, 1970a, 1970b; Ferris & Whitt, 1977; Allendorf, 1978; Buth, 1983; Allendorf & Thorgaard, 1984; Ferris, 1984).

Apart from South African tetraploid barbs, European *Barbus* spp. and *Barbus callensis* from North Africa all seem to be tetraploid (Berrebi et al., 1990; Collares-Pereira & Madeira, 1990; Berrebi, 1995; Berrebi et al., 1995, Machordom et al., 1995). Agnèse et al. (1990) and Berrebi et al. (1990) investigated small and large barbs from West Africa electrophoretically and identified

the large barbs as tetraploid species from allozyme evidence. Most of the large barbs in these two studies were shown to be hexaploid, however, when they were karyotyped (Guégan et al., 1995). Guégan et al. (1995) also suggested that it might be possible to distinguish diploid barbs from polyploid barbs using allozyme electrophoresis, but that it is not possible to distinguish tetraploid species from hexaploid ones. This can be due to functional diploidization and the occurrence of isoloci, the existence of which is dependent on how historic the polyploid event was. Golubtsov & Krysanov (1993) and Naran (1997) observed an absence of multivalents in meiotic spreads of chromosomes of tetraploid and hexaploid African *Barbus* spp. and tetraploid *Pseudobarbus*, possibly indicating "distant" ploidy events in these taxa. Karyological studies also point to a possible Pan African lineage of hexaploid barbs (Oellermann & Skelton, 1990; Golubtsov & Krysanov, 1993; Guégan et al., 1995).

Representatives of all three ploidy levels found in African barbs are included in the present study. This is done to investigate the relationship between ploidy level and the number of allozyme loci that can be recorded, and to assess the amount of diploidization occurring in polyploid South African barbs (e.g. Agnèse et al., 1990; Berrebi et al., 1990). *Barbus anoplus* (chromosome number = 50) is included as a representative of diploid barbs, whilst *B. calidus*, *B. erubescens*, *B. serra*, *Pseudobarbus phlegethon* and *P. burchelli* are included as representatives of tetraploid barbs (chromosome number = 100) (Naran, 1997). *Barbus aenus*, found to be hexaploid with a chromosome number of 148 by Oellermann & Skelton (1990), was included in the present study as a representative of the hexaploid "yellow fishes" of southern Africa.

The objectives of the present chapter is to: (1) scrutinize the ability of allozyme electrophoresis to distinguish between ploidy levels (e.g. Agnèse et al., 1990; Berrebi et al., 1990) of South African

barbs; (2) scrutinize the suitability of this method for the aims of the present thesis; (3) determine the relative amount of diploidization that occurred in the above-mentioned polyploid taxa (Agnèse et al., 1990; Berrebi et al., 1990); (4) describe enzyme systems that can be routinely screened for the genera *Barbus* and *Pseudobarbus*. The latter is done to ensure that: (1) a population genetic study on *B. calidus*, *B. erubescens* and *P. phlegethon* can be done; (2) the age of ploidy events in South African *Barbus* and *Pseudobarbus* can be investigated; (3) the uncertain phylogenetic position of redfins in relation to other barbs can be resolved. The latter three aspects will be addressed in Chapter 3.

2.2 MATERIALS AND METHODS

2.2.1 Study Area

Fish were collected from three river systems within South Africa, namely the Olifants, Breede and Orange River Systems (Table 2.1; Fig. 2.1). Redfin and sawfin (*B. serra*) samples were collected in the Olifants River System draining the Cedarberg Mountains. The Cedarberg Mountains form part of the Cape Fold Mountains, and are associated with the Cape Floristic Region (smallest of the seven plant kingdoms of the world). The chubbyhead barb (*B. anoplus*) and Smallmouth yellowfish (*B. aenus*) were collected from the Sout and Kraai Rivers (Orange River System), respectively, on the interior plateau.

2.2.2 Collection procedures

Samples of seven recognized fish species from three river systems were obtained during 1997, 1998 and 1999 (Table 2.1). This was done using electrofishing, seine netting, snorkeling with a hand net, flyfishing and bait fishing. Netting proved the most successful method. Electrofishing in the low-conductivity water did not stun the cyprinid fish effectively. The small barbs were often easy to net, as they did not flee and they were also attracted to the disturbance made by the net.

2.2.3 Electrophoretic analyses

Muscle tissue samples were homogenized in 0.01 M tris buffer (pH 8) in plastic Eppendorf tubes, using a glass rod attached to a variable speed motor. Samples were then stored at -80°C. Only muscle tissue was used, as liver samples of the minnows were often too small. Allelic variation was examined on horizontal starch gel (13% hydrolyzed potato starch, Sigma Chemicals). Three electrophoretic buffer systems were used: i) a discontinuous tris-citrate-borate-lithium hydroxide buffer system with the gel buffer at pH 8.7 and the electrode buffer at pH 8.0 (Ridgeway et al., 1970); ii) a continuous tris-borate-EDTA buffer system with gel and electrode buffer at pH 8.6 (Markert & Faulhaber, 1965); and iii) a continuous tris-citrate buffer system with the gel and electrode buffer at pH 6.9 (Whitt, 1970).

Before electrophoresis, the samples were thawed and centrifuged for 5 min. Filter paper wicks (Whatman #3) were dipped into the supernatant of the samples and inserted into a slit made in the

gel. Red food dye was used as supernatant for "marker" wicks. Gels were run for between 3-5 hours at a constant current of 40 mA in a fridge kept at 4 °C. Gels were divided into three to four slices and stained for enzymatic activity using specific chemical reagents in agar overlays that were prepared according to Shaw & Prasad (1970), Harris & Hopkinson (1976) and Murphy et al. (1996).

Table 2.1. List of *Barbus* and *Pseudobarbus* spp. included in the allozyme electrophoretic analysis. Ploidy levels of the taxa as determined by Naran (1997)[1] and Oellermann & Skelton (1990)[2] are included. Additional voucher specimens collected for future identification of these taxa are shown in Table 3.2.

Sample number, tributary & population	Ploidy	N	Collection date	Collectors
OLIFANTS RIVER SYSTEM				
1) Thee *Barbus calidus*	Tetraploid[1]	20	24/03/1998	ES
8) Twee *Barbus erubescens*	Tetraploid[1]	5	29/03/1998	ES
22) Olifants Gorge *Barbus serra*	Tetraploid[1]	24	11/03/1998	ES, RB, DN, DI
2) Thee *Pseudobarbus phlegethon*	Tetraploid[1]	20	24/03/1998	ES
BREEDE RIVER SYSTEM				
20) Witte *Pseudobarbus burchelli*	Tetraploid[1]	5	21/3/1998	ES, RB, DN
ORANGE RIVER SYSTEM				
23) Kraai *Barbus aenus*	Hexaploid[2]	5	22-24/2/1999	RB
21) Sout *Barbus anoplus*	Diploid[1]	5	23/3/1997	ES

ES = E.R. Swartz; RB = I.R. Bills; DN = D. Naran; DI = N.D. Impson.

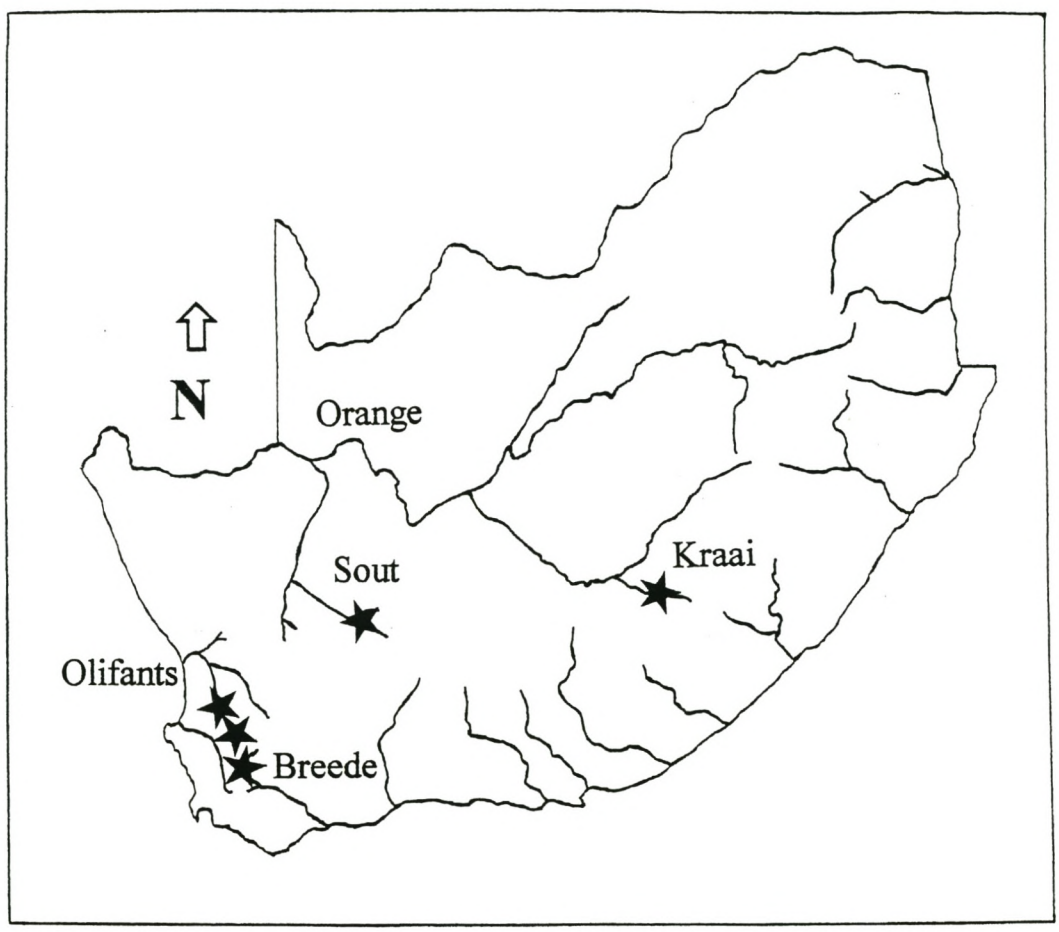

Fig. 2.1 Map of South Africa showing where the samples for the present study were collected (solid stars).

When more than one locus was found, they were sequentially numbered from the cathodal end (Shaklee et al., 1990). The most common allele was assigned a value of 100. Other alleles were scored relative to this 100 % allele. Electrophoretic homologies for some loci were reversed in certain species. In these cases, loci were numbered the same as corresponding homologous loci.

After preliminary screening of 22 enzymes, 14 were selected yielding interpretable bands that were routinely successfully stained for. *Pseudobarbus phlegethon*, *B. calidus*, *B. erubescens*, *P. burchelli*, *B. anoplus*, *B. serra* and *B. aenus* specimens (total N = 84), were analyzed for these 14 enzymes (Table 2.1). The percentage of loci expressed in the polyploid species, in relation to that expected after the polyploid event, was inferred from the number of active loci resolved and the occurrence of isoloci. The number of active loci observed in *B. anoplus* was assumed to reflect the ancestral diploid state (Naran, 1997).

The allozyme electrophoretic findings of the present study will be compared to allozyme studies done on other South African *Barbus* spp. (Mulder 1989; Engelbrecht & Van der Bank, 1994, 1996a, 1996b, 1997) as well as work done on West African, North African, European and Asian species of *Barbus* (Agnèse et al., 1990; Berrebi et al., 1990; Karakousis et al., 1995; Machordom et al., 1995).

2.3 RESULTS

The enzymes stained for and their abbreviations, the presumptive loci found, the enzyme commission numbers, buffer systems used and the intensity of the banding patterns under the specific stain are listed in Table 2.2. Relative mobility and frequency of alleles are given in Table 2.3. No "null alleles" as described by Grant & Robinson (1989) were observed. Silenced or "phantom" loci (Berrebi et al., 1990) were observed and will be discussed under each enzyme (Section 2.3.1-2.3.15). Although few individuals were investigated, polymorphic loci were observed in *B. calidus* (PGM-1 and PGM-2), *P. phlegethon* (GPI and PEP-LT-4), *P. burchelli* (EST-2, GPI and PGD-1), *B. anoplus* (PGD-1 and PGM-2) and *B. serra* (GPI and MDH-1). Specific observations for each enzyme follows.

Table 2.2 Summary of enzyme names, enzyme commission numbers, the presumptive loci found (locus abbreviations), buffer systems used and the intensity of the banding patterns observed in the electrophoretic analysis of *Barbus* and *Pseudobarbus* spp. The asterisk mark cases where it is difficult to assign enzyme commission numbers, because of multiple substrate affinities (Murphy et al., 1996). The double asterisk indicate stains (described in sections 2.3.6 and 2.3.8) yielding what was considered lactate dehydrogenase activity.

Enzyme	Enzyme commission number	Locus	Buffer	Intensity of the stain
Aspartate aminotransferase	2.6.1.1	AAT-1	TC	Medium
		AAT-1	TC	Medium
Adenylate kinase	2.7.4.3	AK	TC, RW, MF	High
Creatine kinase	2.7.3.2	CK-1	RW	Medium
		CK-2	RW	Medium
Esterase	3.1.1.1	EST-1	MF	High
		EST-2	MF	High
		EST-3	MF	Medium
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	GAPDH-1	RW	Low
		GAPDH-2	RW	Low
Glucose dehydrogenase **		GDH-1	TC, RW, MF	Low
		GDH-2	TC, RW, MF	Low

Table 2.2 continued

Glucose-6-phosphate	5.3.1.9	GPI-1	RW	High
isomerase		GPI-2	MF	Low
		GPI-3	MF	Low
		GPI-4	MF	Low
		GPI-5	MF	Low
		GPI-6	MF	Low
L-iditol dehydrogenase **		IDDH-1	TC, RW, MF	Low
		IDDH-2	TC, RW, MF	Low
Lactate dehydrogenase	1.1.1.27	LDH-1	TC, RW, MF	High
		LDH-2	TC, RW, MF	High
Malate dehydrogenase	1.1.1.37	MDH-1	MF	Medium
		MDH-2	MF	Medium
		MDH-3	MF	Medium
		MDH-4	MF	Medium
Malate dehydrogenase	1.1.1.40	MDHP-1	MF	Medium
(NADP+)		MDHP-2	MF	Medium
Octopine dehydrogenase	1.5.1.11	OPDH-1	TC	Low
		OPDH-2	TC	Low
Peptidases:				
Leucyl-glycyl-glycyl *		LGG	MF	Medium

Table 2.2 continued

Leucyl-tyrosine *		LT-1	MF	Low
		LT-2	MF	Medium
		LT-3	MF	Medium
		LT-4	MF	Medium
Phosphogluconate	1.1.1.44	PGDH-1	MF	Medium
dehydrogenase		PGDH-2	MF	Medium
Phosphoglucomutase	5.4.2.2	PGM-1	MF	High
		PGM-2	MF	High

RW = Discontinuous tris-citrate-borate-lithium hydroxide buffer system; gel buffer pH = 8.7; electrode buffer pH = 8.0 (Ridgeway *et al*, 1970)

MF = Continuous tris-borate-EDTA buffer system; pH = 8.6 (Markert & Faulhaber, 1965)

TC = Continuous tris-citrate buffer system; pH = 6.9 (Whitt, 1970)

Table 2.3. Summary of relative mobilities and allelic frequencies for samples of *Barbus* and *Pseudobarbus* investigated for 14 interpretable enzymes. Silenced loci are indicated as "NULL". Question marks refer to samples that were not resolvable. Sample numbers are explained at the bottom of the table. Locus abbreviations are explained in Table 2.2. Sample sizes are indicated next to the locus abbreviation.

Locus abbreviation		Populations (Sample 1-2 and 20-23)						
& relative mobility		1	2	8	20	21	22	23
AAT-1	N =	20	20	5	5	5	24	5
100		-	1.000	-	1.000	-	?	?
97		1.000	-	1.000	-	-	?	?
64		-	-	-	-	1.000	?	?
AAT-2	N =	20	20	5	5	5	24	5
100		-	1.000	-	1.000	-	?	?
75		1.000	-	1.000	-	-	?	?
Null		-	-	-	-	1.000	?	?
AK	N =	20	20	5	5	3	24	5
100		1.000	1.000	1.000	1.000	-	1.000	-
75		-	-	-	-	1.000	-	-
130		-	-	-	-	-	-	1.000

Table 2.3 continued

CK-1	N =	20	20	5	5	5	24	5
100		-	1.000	-	-	1.000	-	-
80		1.000	-	1.000	-	-	1.000	-
82		-	-	-	1.000	-	-	-
75		-	-	-	-	-	-	1.000
CK-2	N =	20	20	5	5	5	24	5
100		-	1.000	-	1.000	-	-	-
78		1.000	-	1.000	-	-	1.000	-
70		-	-	-	-	-	-	1.000
Null		-	-	-	-	1.000	-	-
EST-1	N =	20	20	5	5	5	24	5
100		1.000	-	1.000	1.000	-	1.000	-
97		-	1.000	-	-	-	-	-
90		-	-	-	-	1.000	-	1.000
EST-2	N =	20	20	5	5	5	24	5
100		1.000	1.000	1.000	0.800	-	1.000	-
90		-	-	-	0.200	-	-	-
93		-	-	-	-	1.000	-	1.000
EST-3	N =	20	20	5	5	5	24	5
100		?	?	?	?	?	1.000	?

Table 2.3 continued

GAPDH-1	N =	20	20	5	5	5	24	5
100		?	?	?	?	?	1.000	?
GAPDH-2	N =	20	20	5	5	5	24	5
100		?	?	?	?	?	1.000	?
GPI-1	N =	20	20	5	5	5	24	5
100		1.000	0.250	1.000	-	-	-	-
60		-	0.750	-	1.000	0.200	0.021	1.000
50		-	-	-	-	0.800	0.979	-
GPI-2	N =	20	20	5	5	5	24	5
100		?	?	?	?	?	1.000	?
GPI-3	N =	20	20	5	5	5	24	5
100		?	?	?	?	?	1.000	?
GPI-4	N =	20	20	5	5	5	24	5
100		?	?	?	?	?	1.000	?
GPI-5	N =	20	20	5	5	5	24	5
100		?	?	?	?	?	1.000	?
GPI-6	N =	20	20	5	5	5	24	5
100		?	?	?	?	?	1.000	?

Table 2.3 continued

LDH-1	N =	20	20	5	5	5	24	5
100		1.000	-	1.000	-	1.000	1.000	1.000
78		-	1.000	-	-	-	-	-
94		-	-	-	1.000	-	-	-
LDH-2	N =	20	20	5	5	5	24	5
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-1	N =	20	20	5	5	5	24	5
100		1.000	1.000	1.000	-	-	0.979	-
115		-	-	-	-	-	0.021	-
88		-	-	-	1.000	-	-	1.000
Null		-	-	-	-	1.000	-	-
MDH-2	N =	20	20	5	5	5	24	5
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-3	N =	20	20	5	5	5	24	5
100		-	-	-	1.000	-	-	-
88		1.000	1.000	1.000	-	1.000	-	-
105		-	-	-	-	-	1.000	-
Null		-	-	-	-	-	-	1.000

Table 2.3 continued

MDH-4	N =	20	20	5	5	5	24	5
100		-	1.000	-	-	-	-	-
65		-	-	-	1.000	-	-	-
Null		1.000	-	1.000	-	1.000	1.000	1.000
MDHP-1	N =	20	20	5	5	5	24	5
100		-	1.000	-	-	-	-	-
50		1.000	-	1.000	-	-	-	-
120		-	-	-	-	1.000	1.000	1.000
140		-	-	-	1.000	-	-	-
MDHP-2	N =	20	20	5	5	5	24	5
100		1.000	-	-	-	1.000	1.000	1.000
85		-	1.000	1.000	1.000	-	-	-
ODH-1	N =	20	20	5	5	5	24	5
100		1.000	1.000	1.000	1.000	1.000	?	?
ODH-2	N =	20	20	5	5	5	24	5
100		1.000	1.000	1.000	1.000	1.000	?	?

Table 2.3 continued

LGG	N =	20	20	5	5	5	24	5
100		-	1.000	-	-	-	-	-
50		1.000	-	1.000	-	-	1.000	-
70		-	-	-	-	1.000	-	1.000
90		-	-	-	1.000	-	-	-
LT-1	N =	20	20	5	5	5	24	5
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000
LT-2	N =	20	20	5	5	5	24	5
100		1.000	-	1.000	-	-	1.000	-
Null		-	1.000	-	1.000	1.000	-	1.000
LT-3	N =	20	20	5	5	5	24	5
100		1.000	-	1.000	-	-	1.000	-
Null		-	1.000	-	1.000	1.000	-	1.000
LT-4	N =	20	20	5	5	5	24	5
100		-	0.625	-	-	1.000	-	-
93		1.000	0.100	1.000	1.000	-	1.000	-
110		-	0.275	-	-	-	-	-
106		-	-	-	-	-	-	1.000

Table 2.3 continued

PGDH-1	N =	20	20	5	5	5	24	5
100		-	1.000	-	0.100	-	-	1.000
85		1.000	-	1.000	0.900	-	-	-
77		-	-	-	-	0.300	1.000	-
52		-	-	-	-	0.700	-	-
PGDH-2	N =	20	20	5	5	5	24	5
100		1.000	1.000	1.000	1.000	-	1.000	-
Null		-	-	-	-	1.000	-	1.000
PGM-1	N =	20	20	5	5	5	24	5
100		0.400	1.000	1.000	-	-	-	-
128		0.600	-	-	-	-	-	-
112		-	-	-	1.000	-	-	-
76		-	-	-	-	-	1.000	1.000
Null		-	-	-	-	1.000	-	-
PGM-2	N =	20	20	5	5	5	24	5
100		0.600	-	1.000	1.000	-	1.000	-
86		0.350	1.000	-	-	0.600	-	-
70		0.050	-	-	-	0.400	-	1.000

2.3.1 Aspartate aminotransferase (AAT/ASAT/GOT)

Aspartate aminotransferase was formerly known as glutamate-oxaloacetate transaminase (GOT). It is best resolved from extracts of fresh tissue and has a dimeric structure (Murphy et al., 1996). Soluble (supernatant or cytosolic) and mitochondrial forms of this enzyme are known (Harris and Hopkinson, 1976).

The bands were expressed in the top half on the anodal side of the gel in the present study. Two loci were resolved for aspartate aminotransferase in all the species in the present study, except for *B. aenus* where activity was absent. *Barbus serra* also showed two loci, but the expression was too weak for routine use.

Engelbrecht & Van der Bank (1996a, 1996b, 1997) also observed two loci in diploid South African barbs. Diploid barbs studied by Agnèse et al. (1990) and Berrebi et al. (1990) from West Africa, North Africa and Asia, revealed a single locus, except where Machordom et al. (1995) resolved a second locus for *B. apoensis*, believed to be the mitochondrial form of this enzyme. If the second locus found in South African diploid barbs is homologous to the mitochondrial form found by Machordom et al. (1995), then it seems as if South African barbs only express one supernatant locus.

The Iberian Peninsula and North African tetraploid barbs studied by Machordom et al. (1995) all revealed a single supernatant locus, but a mitochondrial locus was also resolved. They found that European tetraploid barbs have three loci - one mitochondrial and two supernatant. The studies done by Agnèse et al. (1990), Berrebi et al. (1990), Berrebi et al. (1995) and Karakousis et al.

(1995), which include European and North African tetraploid barbs, revealed two to three loci for this enzyme. In hexaploid African barbs examined by Agnèse et al. (1990) and Berrebi et al. (1990), only two loci were interpreted. Variation in the number of loci interpreted in these studies may be because the supernatant and mitochondrial forms that exist are not always recorded. Alternatively, it can be due to the polyploid species expressing the diploid state, either due to diploidization or the absence of divergence between loci (isoloci).

2.3.2 Adenylate kinase (AK)

According to Murphy et al. (1996) adenylate kinase has a monomeric structure. A single locus was observed for all the species in the present study. Bands were observed on the annodal side of the gel, close to the origin. A single locus was also observed in other diploid, tetraploid and hexaploid *Barbus* spp. in South Africa (Mulder, 1989; Engelbrecht & Van der Bank, 1994, 1996a, 1996b, 1997). This locus was monomorphic in all the latter cases and in the present study except where Mulder (1989) identified rare alleles occurring at low frequency for *B. kimberleyensis*, *B. polylepis* and *B. natalensis*. This enzyme thus seems to have low variability in South African *Barbus* and *Pseudobarbus spp*. examined to date.

Most of the diploid *Barbus* spp. studied elsewhere in Africa and in Asia (Agnèse et al., 1990; Berrebi et al., 1990), also revealed a single locus. The only exception is where Machordom et al. (1995) resolved two loci for *B. apoensis* (a diploid species from Saudi Arabia) whilst Berrebi et al. (1990) only found a single locus for this species.

The European and North African tetraploid and African hexaploid barbs studied by Agnèse et al. (1990), Berrebi et al. (1990), Berrebi et al. (1995) and Machordom et al. (1995), revealed at least two loci. The only exception was where Agnèse et al. (1990) and Berrebi et al. (1990) resolved one locus for *Barbus meridionalis* (tetraploid European barb); where-as Machordom et al. (1995) found two.

The detecting of a single locus in the polyploid South African barbs in the present study, and those investigated by Mulder (1989), can be explained by different lab conditions or differential tissue expression. Mulder (1986, 1989), however, did use a variety of tissues and three buffer systems (MF, TC and RW). The present study also used the three buffer systems mentioned, but only muscle tissue was screened. Alternatively, diploidization of the duplicate loci or isoloci could have occurred in the South African polyploid barbs.

2.3.3 Creatine kinase (CK)

According to Ward et al. (1992), several creatine kinase loci exist in vertebrate species. This enzyme is considered to be a dimer (Ward et al., 1992; Murphy et al., 1996), but according to Ferris & Whitt (1978), the skeletal muscle form of creatine kinase in teleost fish does not form the expected dimer in heterozygous individuals. Instead, the heterozygotes appear as just two bands.

Bands were observed on the bottom quarter of the gel on the annodal side. Two loci were observed in the muscle tissue of the tetraploid and hexaploid barbs in the present study. Two loci were also found in the hexaploid and tetraploid *Barbus* spp. studied by Mulder (1989). The

diploid small *Barbus* spp. studied by Engelbrecht & Van der Bank (1994, 1996a, 1996b, 1997) yielded a single locus for creatine kinase. The same was found for *B. anoplus* in the present study.

Work done by Berrebi et al. (1995), Karakousis et al. (1995) and Machordom et al. (1995) revealed a single creatine kinase locus. These studies were done on European, North African and Asian diploid, and European and North African tetraploid *Barbus* spp. The occurrence of two loci in the South African tetraploid *Barbus* spp., and a single in the North African and European species, possibly indicates diploidization or the occurrence of isoloci in the North African and European species.

2.3.4 Esterase (EST)

Esterase can be either a monomer or a dimer (Murphy et al., 1996). According to Ward et al. (1992), esterase is mostly a monomer in vertebrates. Another form called esterase-D in humans appears to be mostly a dimer in vertebrates. However, trimeric esterase has been identified in eutherian mammals (Searle, 1986) and was also found in pig liver (Inkerman et al., 1975). Esterase was found to be a monomer for the species investigated in the present study.

The banding pattern was observed in the middle of the gel. Two loci were observed for all the species in the present study, except in *B. serra*, where a third locus was found to be expressed between the other two loci. This third locus was also found in *B. calidus* and *B. erubescens*, but its activity was too weak for routine use. The second locus was polymorphic in *B. calidus* and the third resolvable locus for *B. serra* was also polymorphic.

Mulder (1989) resolved only two loci, whilst Engelbrecht & Van der Bank (1994, 1996a, 1996b, 1997) resolved three to four loci, but this is possibly because they used liver tissue as well. Machordom et al. (1995) was also only able to resolve two loci in muscle, with a third left out of their analysis, because of "difficulties in interpreting the pattern". However, Karakousis et al. (1995) was able resolve three esterase loci. Difficulties in interpreting certain esterase loci make it difficult to infer evolutionary patterns, but it seems that a high degree of diploidization occurred in all the polyploid *Barbus* spp. studied to date.

2.3.5 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase has a tetrameric structure (Murphy et al., 1996). Two loci were resolved only for *B. serra*, and no heterozygotes were observed. GAPDH was best resolved on RW buffer, but staining intensity was low.

2.3.6 Glucose dehydrogenase (GDH/GLD)

According to Murphy et al. (1996), the stain used for glucose dehydrogenase can also yield lactate dehydrogenase. It was indeed found to be the case in the present study. The stain used was adapted from Harris & Hopkinson (1976). A tris buffer was used in place of a phosphate buffer. The L-iditol dehydrogenase or sorbitol dehydrogenase (SORDH, SDH or IDDH) stain described by Harris & Hopkinson (1976) also yielded similar enzyme activity. This enzyme was considered to be LDH, because the LDH stain described by Murphy et al. (1996) produced the best resolution.

2.3.7 Glucose-6-phosphate isomerase (GPI/PGI/PHI)

Glucose-6-phosphate isomerase was previously known as phosphohexose isomerase (PHI) or phosphogluco-isomerase (PGI) (Murphy et al., 1996). One interpretable locus was observed for all the cyprinids on the RW buffer system in the present study. However, when the MF buffer system was used in the investigation of *B. serra*, a further five loci were observed with weak activity. The one locus resolved for all the species, called GPI-1, was polymorphic for the Olifants River populations of *P. phlegethon*, but all the other populations investigated were monomorphic for this locus. A dimeric structure was observed in heterozygotes, as described by Murphy et al. (1996). The GPI-1 locus resolved for all the species were observed close to the origin of the gel on the annodal side. Of the five other loci resolved on MF buffer for *B. serra*, three were expressed across the gel on the annodal side, whilst two migrated cathodally.

Diploid African and Asian barbs appear to have two glucose-6-phosphate isomerase loci (Agnèse et al., 1990; Berrebi et al., 1990; Engelbrecht & Van der Bank, 1994, 1996a, 1996b, 1997). Tetraploid European and North African *Barbus* spp., and hexaploid West African *Barbus* spp. in different studies done by Agnèse et al. (1990), Berrebi et al. (1990), Berrebi et al. (1995) and Karakousis et al. (1995) expressed three to five loci.

The present investigation of *B. serra*, as well as other studies on other tetraploid barb species (Agnèse et al., 1990; Berrebi et al., 1990; Berrebi et al., 1995; Karakousis et al., 1995), suggests that many loci is expressed in tetraploid *Barbus* spp. The use of different buffer systems to resolve glucose-6-phosphate isomerase is advised.

2.3.8 L-iditol dehydrogenase (IDDH/SORDH/SDH)

L-iditol dehydrogenase is also known as sorbitol dehydrogenase (Harris & Hopkinson, 1976). The stain described by Harris & Hopkinson (1976) resulted in LDH activity (see Section 2.3.9).

2.3.9 Lactate dehydrogenase (LDH)

According to Ward et al. (1992) and Murphy et al. (1996), lactate dehydrogenase has a tetrameric structure. Three loci of lactate dehydrogenase are known to occur in diploid fish (Murphy & Crabtree, 1985). Mulder (1989) observed three loci in the large barbs of South Africa, although only one was interpreted using muscle tissue. In the present study, two loci were interpretable from muscle tissue in all species investigated. Banding patterns were observed on the bottom half of the gel on the annodal side. This enzyme worked well on MF (Markert & Faulhaber, 1965), TC (Whitt, 1970) and RW (Ridgeway et al., 1970) buffer systems. Better intensity was observed on MF, although TC yielded better separation of bands. Engelbrecht & Van der Bank (1994, 1996a, 1996b, 1997) used TC and MF for lactate dehydrogenase analyses, which revealed two loci. Other studies done on *Barbus* spp. of different ploidy levels (Agnèse et al., 1990; Karakousis et al., 1995; Machordom et al., 1995), also revealed only two loci in muscle tissue.

2.3.10 Malate dehydrogenase (MDH)

The subunit structure of malate dehydrogenase is dimeric (Murphy et al., 1996). Two to four loci were interpretable in the present study. *B. anoplus* and *B. aenus* (diploid and hexaploid respectively) expressed only two loci each. The serrated barbs expressed three loci, whilst the

Pseudobarbus spp. expressed four loci. It appears as if the two loci, expressed by the diploid species in the present study, represent the ancestral condition. However, Agnèse et al. (1990) and Machordom et al. (1995) resolved three loci for diploid West African and Asian barbs, respectively. Mulder (1989) also found two loci for hexaploid barbs and three loci for tetraploid barbs. Engelbrecht & Van der Bank (1994, 1996a, 1996b, 1997) also interpreted two loci for the South African barbs they studied.

All the studies done by Agnèse et al. (1990), Berrebi et al. (1995) and Machordom et al. (1995), which included West African hexaploid and North African and European tetraploid barbs, revealed three loci. This is consistent with the present study (except for *Pseudobarbus*) and work done by Mulder (1989). It appears as if a high degree of diploidization occurred for this enzyme.

2.3.11 Malate dehydrogenase ($NADP^+$) (MDHP/ME)

$NADP^+$ dependent malate dehydrogenase was formerly known as malic enzyme (ME) and has a tetrameric structure (Murphy et al., 1996). It displayed a monomeric structure in studies done on certain vertebrates (Frydenberg & Simonsen, 1973; Hedgecock & Ayala, 1974). Ward et al. (1992) assume this is due to degradation of the true tetrameric structure, whilst keeping activity in the degraded monomeric state. No heterozygotes were observed in the present study. The enzyme was expressed on the bottom half of the gel on the annodal side.

Two loci were observed for all the species investigated in the present study. Engelbrecht & Van der Bank (1994, 1996a, 1996b, 1997) observed a single locus. Mulder (1986) did resolve two loci in the muscle tissue of hexaploid barbs, but in his later work on the same group (1989), only one of the two loci was resolved. A single locus was interpreted by Karakousis et al. (1995).

2.3.12 Octopine dehydrogenase (OPDH/ODH)

Weak activity of octopine dehydrogenase was observed on the bottom half of the gel from muscle extracts of some of the species investigated in the present study. At times as many as four loci were observed, but only two were consistently interpretable after numerous re-runs. The two loci were monomorphic in all cases in the present investigation. No loci were resolved for *B. serra* and *B. aenus*. This enzyme has a monomeric structure (Murphy et al., 1996).

2.3.13 Peptidases: Leucyl-Tyrosine (Pep-LT) and Leucyl-Glycyl-Glycine (Pep-LGG)

The subunit structure of peptidases is variable (Ward et al., 1992; Murphy et al., 1996). There is difficulty in assignment of homology, because of multiple substrate affinities of this group of enzymes (Murphy et al., 1996). It was thus difficult to assign E.C. numbers. The multiple substrate affinity of peptidases was observed in the present study by using (Pep-LT) and (Pep-LGG) stains. The protocol for staining peptidases described by (Murphy et al., 1996) was followed.

In total, six loci were observed using the Pep-LT stain, of which the Pep-LGG stain also stained for the bottom two. The bottom two loci had better intensity with the Pep-LGG stain, and were thus considered to be Pep-LGG. Only one of the two loci was consistently interpretable. The other four loci were not expressed with the Pep-LGG stain and considered to be the four Pep-LT loci. Pep-LT further yielded in total four loci for the serrated barbs studied here and only two loci in the other species. Pep-LT was invariable in all the species studied, whilst Pep-LT-4 was polymorphic in all the populations from the Olifants River. Because of the variable affinity towards different staining methods, care must be taken in trying to assign homologies with other studies. Mulder (1989), however, also interpreted one Pep-LGG and two Pep-LT loci for the hexaploid barbs, as was the case in the present study.

2.3.14 Phosphogluconate dehydrogenase (PGDH/PGD)

Phosphogluconate dehydrogenase was formerly known as 6-phosphogluconate dehydrogenase (PGD or PGDH) and is a dimer (Murphy & Crabtree, 1985; Murphy et al., 1996). According to Turner (1974), glucose-6-phosphate dehydrogenase sometimes appears on the gel, but this was not observed in the present study.

Although the banding patterns were interpretable in the present study, activity was not very intense, suggesting that other tissues might work better. Mulder (1989) observed phosphogluconate dehydrogenase activity in a wide range of tissues (see also Harris & Hopkinson, 1976).

Two loci were observed in tetraploid barbs on the bottom quarter of the gel from muscle tissue extracts. A single locus was observed in *B. anoplus* and *B. aenus* in the present study. A single locus for phosphogluconate dehydrogenase was observed in small *Barbus* spp. and large *Barbus* spp. (Mulder, 1989; Engelbrecht & Van der Bank, 1994, 1996a, 1996b, 1997). Although liver tissue was used, Machordom et al. (1995) found two loci in all the Iberian Peninsula and North African tetraploid *Barbus* spp. surveyed and a single locus in one diploid species from Saudi Arabia. They also analyzed European tetraploid barbs and found one or two loci.

2.3.15 Phosphoglucomutase (PGM)

Phosphoglucomutase is polymorphic for *B. calidus* and it exhibits a monomeric structure as described by Murphy & Crabtree (1985) and Murphy et al. (1996).

Two loci were observed in the middle towards bottom part of the gel for all the species analyzed, except for *B. anoplus* that has a single locus in muscle tissue. All studies on diploid *Barbus* spp. species to date (Agnèse et al., 1990; Berrebi et al., 1990; Engelbrecht & Van der Bank, 1994, 1996a, 1996b, 1997; Machordom et al., 1995) revealed a single locus whilst tetraploid species expressed the duplicated number of loci (Mulder, 1989; Agnèse et al., 1990; Berrebi et al., 1990; Berrebi et al., 1995; Karakousis et al., 1995; Machordom et al., 1995). The only tetraploid species surveyed to date showing the diploid state is *B. barbus* (an European barb). Agnèse et al. (1990), Berrebi et al. (1990) and Machordom et al. (1995) recorded a single locus for the latter species. Hexaploid barbs investigated by Mulder (1989), Agnèse et al. (1990) and Berrebi et al. (1990) expressed two loci like most tetraploid species.

There appears to be a high consistency in the expression of one locus versus two loci in the diploids and tetraploids respectively for this enzyme. It appears as if much diploidization occurred in hexaploid species or, alternatively, isoloci are present.

2.4 DISCUSSION

This chapter outlines 14 enzymes that can be routinely screened from muscle tissue extracts in members of *Barbus* and *Pseudobarbus*. Of these, only GAPDH was not routinely recorded for *B. calidus*, *B. erubescens* and *P. phlegethon*, leaving 13 enzymes that will be used in the population genetic study of these taxa (Chapter 3).

There is much variation in the number of loci resolved for the different enzyme systems in various allozyme studies in *Barbus*. Different lab conditions can influence the number of loci recorded by different authors and some loci might have been omitted from analysis because they were not interpretable. However, differences in the number of loci observed also seem to be due to the different ploidy levels in *Barbus* spp., because most of the variation in the number of loci occur in the polyploid species.

The polyploid species generally show less loci than the tetraploid or hexaploid number of loci expected just after the polyploid event. This is due to either the occurrence of isoloci, or functional diploidization. The latter process is consistent with karyological studies (Golubtsov & Krysanov, 1993; Naran, 1997) of *Barbus* and *Pseudobarbus* spp. An absence of multivalents in meiotic spreads of chromosomes was observed, and this may indicate that the ploidy event is distant (Golubtsov & Krysanov, 1993). Isoloci were not observed in the present study, but may

be present. Isoloci should be detectable where polymorphism occurs in one of the isoloci, or where different staining intensities occur (Utter et al., 1987; Grant & Robinson, 1989).

Variation in the number of active loci (see Berrebi et al., 1990) was observed amongst the taxa investigated in the present study at AAT, CK, EST, GPI, MDH, PEP-LT, PGDH and PGM. AAT-1 and -2, EST-3 and GPI-2 to -6 were not consistently recorded, but the differences in the number of active loci in MDH, PEP-LT and PGDH are considered to be due to diploidization. *P. phlegethon* and *P. burchelli* expressed four MDH loci, whilst the serrated tetraploid barbs expressed three loci and *B. aenus* and *B. anoplus* expressed only two each. The serrated tetraploid barbs expressed four PEP-LT loci, whilst all the other taxa expressed only two. All the taxa expressed two PGDH loci, except for *B. anoplus* and *B. aenus*. *B. anoplus* expressed only one locus for CK and PGM respectively, whilst all the other taxa expressed two loci each for these two enzymes. Apart from the enzymes that were not resolved for all the taxa, *B. aenus* expressed 6.2 % of the duplicated loci expected after the hexaploid event. The tetraploid serrated *Barbus* and *Pseudobarbus* spp., expressed 37.5 % and 31.2 % of the duplicated loci expected after the tetraploid event, respectively. Thus for the population genetic study (Chapter 3), 27 loci for *B. calidus* and *B. erubescens* and 26 loci for *P. phlegethon* can be routinely screened from muscle tissue extracts.

Fixed allele differences among species of the present study were recorded at AAT-1, AAT-2, AK, CK-1, CK-2, EST-1, EST-2, GPI-1, LDH-1, MDH-1, MDH-3, MDH-4, MDHP-1, MDHP-2, LGG, LT-2, LT-3, LT-4, PGDH-1, PGDH-2, PGM-1 and PGM-2. Fixation of the same allele was recorded at LDH-2, MDH-2, ODH-1, ODH-2 and LT-1 amongst species investigated here.

This suggests that allozyme electrophoresis will have potential in delineating species boundaries of *Barbus* and *Pseudobarbus* of the present study.

Polymorphism of allele frequencies was observed at EST-2 (*P. burchelli*), GPI-1 (*P. phlegethon*, *B. anoplus* and *B. serra*), MDH-1 (*B. serra*), PEP-LT-4 (*P. phlegethon*), PGDH-1 (*P. burchelli* and *B. anoplus*), PGM-1 (*B. calidus*) and PGM-2 (*B. calidus* and *B. anoplus*). No polymorphic loci were observed for *B. erubescens*. Only two polymorphic loci each for *B. calidus* (PGM-1 and -2) and *P. phlegethon* (GPI-1 and PEP-LT-4) were recorded, suggesting that allozyme electrophoresis might be too invariable to study genetic diversity of redfin populations (Chapter 3). Leberg (1992) warned against the use of less than eight polymorphic loci that most studies employ to make inferences about population bottlenecks. The low number of individuals screened in this study may have contributed to the lack of polymorphism detected.

The similar amount of diploidization in the tetraploid serrated *Barbus* and *Pseudobarbus* spp. investigated suggests that the ploidy event in these lineages may be of the same age (Table 2.4). The almost complete diploidization of *B. aenus* suggests that its hexaploid event is more ancient than the possibly shared tetraploid event of *Pseudobarbus* and serrated *Barbus* spp. of South Africa. The number of active loci is not a good indication of the ploidy level of African barbs, and cannot be assumed from allozyme electrophoresis. A distant ploidy event in *B. aenus* is consistent with the possibility of a Pan African lineage of hexaploid barbs (see Oellermann & Skelton, 1990; Golubtsov & Krysanov, 1993; Guégan et al., 1995).

Table 2.4. Summary of the amount of diploidization occurring in polyploid species of *Barbus* and *Pseudobarbus*, if *B. anoplus* is considered as a representative of the typical ancestral diploid state and if no isoloci occur. The loci of 11 enzymes resolvable for all the taxa investigated were considered.

Species	Active loci	Ploidy level	% Diploidization
Barbus anoplus	16	2N	Ancestral state?
Barbus aenus	18	6N	93.8 %
Barbus calidus	22	4N	62.5 %
Barbus erubescens	22	4N	62.5 %
Barbus serra	22	4N	62.5 %
Pseudobarbus phlegethon	21	4N	68.8 %
Pseudobarbus burchelli	21	4N	68.8 %

CHAPTER 3

POPULATION GENETICS OF *BARBUS CALIDUS*, *BARBUS ERUBESCENS* AND *PSEUDOBARBUS PHLEGETHON* (TELEOSTEI: CYPRINIDAE) OF THE OLIFANTS RIVER SYSTEM, WESTERN CAPE PROVINCE.

3.1 INTRODUCTION

Human induced fragmentation of riverine habitat is ever increasing in the Western Cape Province, especially as rivers here are extensively utilized for agricultural purposes. Water extraction, habitat degradation and the introduction of alien organisms have led to the severe decline and fragmentation of native fish populations in this province (Impson & Hamman, in prep.). As pressures on the scarce water resources of the province increase, the situation is unlikely to improve in the near future. In light of the above, Cape Nature Conservation (CNC) initiated a program not only to monitor and survey existing populations, but also to describe the genetic structure of indigenous fishes in the Western Cape. The program was launched to assist in CNC's management of threatened fishes and to prioritize conservation effort, as resources within CNC are limited.

As part of this initiative, the population genetic structure of three cyprinid minnows of the Olifants River System is described in the present study. The fiery redfin (*Pseudobarbus phlegethon*) and Clanwilliam redfin (*Barbus calidus*) occur mostly sympatrically in tributaries of both the Olifants and Doring Rivers draining the Cedarberg Mountains of western South Africa. Both are endemic to the Olifants River System (Skelton, 1987). The third minnow, the Twee River redfin (*Barbus erubescens*), is endemic to the Twee River, a tributary of the Doring River

(Skelton, 1974). *B. calidus* and *P. phlegethon* persist in a few fragmented populations in mountain streams of the Cedarberg Mountains, and is believed to reflect only a fraction of their historic distribution in the Olifants River System (Skelton, 1987). South African Museum records, for instance, indicate that *B. calidus* previously existed in the main stream Olifants River (SAM 215/216, SAM 2012-2015). Harrison (1938) furthermore noted great numbers of small indigenous *Barbus* spp. in Keerom pool, which is in the main stream Olifants River south of Citrusdal. According to Barnard (1943), these "small indigenous *Barbus* spp." included both *B. calidus* and *P. phlegethon*.

The main cause of fragmentation of redfin populations was the introduction of alien bass by man. Largemouth bass (*Micropterus salmoides*) was first introduced in the Olifants River System in 1933 (Harrison, 1938), followed by Smallmouth bass (*M. dolomieu*) and spotted bass (*M. punctulatus*) in 1943 and 1945 (Harrison, 1948; Harrison, 1952; Harrison, 1953). Bass spread quickly and a reduction in distribution and numbers of indigenous fish was soon apparent (Harrison, 1963; Jubb, 1965). By the 1960's, redfins were apparently already extinct from the main stream Olifants River (Van Rensburg, 1966). *M. dolomieu* and *M. punctulatus* are particularly well adapted to move into mountain streams where remaining redfins occur. There is no record of sympatry between bass and redfins in the Olifants River System (Van Rensburg, 1966; Gaigher, 1973b; Bruton, 1997; Bills, 1999), suggesting that bass displace these minnows through predation rather than competition. Bluegill sunfish (*Lepomis macrochirus*), another alien species, was recently introduced above the major natural barrier in the Middeldeur stream (headwaters of the Twee River) and now has the potential to dominate all the areas where *B. erubescens* is found (Bills, pers. comm.). Rainbow trout is present in the Krom River (another tributary of the Doring River), where it may have caused the extinction of redfins.

Another threat to the survival of redfin populations is water extraction, often associated with bulldozing of riverbeds and subsequent destruction of habitat (Bills, 1999). Bulldozing is done to secure orchards, mostly of citrus trees. Agro-chemicals and sewage entering the system near human settlements and farms are also a threat (Mariott, 1998; Bills, 1999). Mariott (1998) mentioned sedimentation due to agricultural activities as a threat to the survival of *B. erubescens*. Pollution, sedimentation and the bulldozing of rivers contribute to the fragmentation of redfin populations and increase the possibility of local extinction. Because of these threats and the restricted and declining distribution of the redfins, *B. calidus* and *P. phlegethon* are listed as endangered, and *B. erubescens* is listed as critically endangered in the IUCN red list (Baillie & Groombridge, 1996).

Relatively little is known about the ecology and life history of these three redfin species. *B. calidus* and *P. phlegethon* seem to occupy different ecological niches in the Cedarberg mountain streams (Bills, 1999). *B. calidus* has a short gut (Skelton, 1988), feeds mainly on drifting food in the open and surface waters of these streams (Skelton 1980; Bills, personal comm.) and is carnivorous (Nthimo, 1997). *B. erubescens* was also found to be a carnivorous open water feeder (Mariott, 1998). *P. phlegethon* has a long gut and probably feeds mainly on algae (Skelton, 1988). Adults are found in small groups or singly (Skelton, 1996), usually near the bottom substrate. Skelton (1987, 1988) observed territoriality in breeding males. Juvenile *P. phlegethon* was found to occur in big groups, often in association with juvenile *B. calidus*, sawfin (*B. serra*), Clanwilliam yellowfish (*B. capensis*) and Cape galaxias (*Galaxias zebratus*). Juvenile *B. erubescens* were found to school during surveys in 1998. Marriott (1998) made similar observations. *Galaxias zebratus* is the only other indigenous fish occurring with *B. erubescens* in the Twee River. *Barbus calidus* and *P. phlegethon* are usually abundant in pristine tributary

streams in the absence of alien predatory fish, and often occur with *B. serra*, *B. capensis*, *Austroglanis gilli* (Clanwilliam rock catfish), *A. barnardi* (spotted rock catfish) and *G. zebratus*. Bills (1999) found *B. calidus* to be more common in both lower and headwater sections of the tributaries, whilst *P. phlegethon* concentrate in the mid-to-lower sections of the tributary streams. *B. calidus* also seems to prefer larger pools and deeper water, whilst *P. phlegethon* prefers shallow sand, cobble or rock based pools and rocky riffle habitat (Skelton, 1996; Bills, 1999). The differential feeding, niche preference, behaviour and body form between *B. calidus* and *P. phlegethon* (Skelton, 1980, 1988; Bills, 1999) may have played an important role in gene flow, dispersability and consequently their genetic structure before the introduction of bass.

Together with all other *Pseudobarbus* spp., the named species are popularly referred to as "redfins" due to the bright red pigmentation on their fins, which is particularly evident during the breeding season. When Skelton (1988) defined *Pseudobarbus*, he did not include *B. calidus* and *B. erubescens*, mainly because they have a bony unbranched dorsal ray that is serrated. All *Pseudobarbus* spp. have soft flexible dorsal rays (Skelton, 1988). *Barbus erubescens* can be distinguished from *B. calidus* mainly in having weak or no serration in the last unbranched dorsal ray. It also has no prominent markings on the dorsal surface and males develop an overall reddish hue in the breeding season. *Barbus calidus*, in contrast, has a strongly serrated dorsal ray, black markings on the dorsal surface, and no red hue during the breeding season (Skelton, 1974). *Barbus erubescens* has been shown to be the sister species of *B. calidus* on the basis of cytogenetic, morphological, osteological and meristic data (Skelton, 1974, 1988; Naran, 1997). The taxonomic position of *Pseudobarbus* and "serrated redfins" in relation to other barbs is generally uncertain (Skelton 1986, 1994; Naran, 1997; see Chapter 1).

The present study offers the opportunity to test conflicting hypotheses on the biogeography of redfins. It is generally believed that *Barbus* originated in Asia (Darlington, 1957; Bowmaker et al., 1978) and found their way into Africa via the north-eastern contact between Africa and Asia around the Miocene (Lowe-McConnell, 1975; Van Couvering, 1977). Cyprinids of the Cape ichthyofauna are considered to have originated, mostly in a dispersalist manner, from relatively recent (Pliocene to Pleistocene) invasions from the north (Barnard, 1943; Jubb, 1964; Jubb & Farquharson, 1965; Gaigher & Pot, 1973; Mulder, 1989). Skelton (1980, 1986, 1994), however, suggested that redfins are remnants of the early Tertiary, and that they speciated largely through vicariance with dispersal playing a less important role. Naran (1997) found all redfins and some South African serrated *Barbus* spp. to be tetraploid. She proposed on the basis of a phylogenetic analysis of morphological, osteological and meristic characters, that the tetraploid event might be shared between *Pseudobarbus* spp. and some serrated barbs.

Although some systematic work has been done on redfins (e.g. Barnard, 1943; Skelton, 1974, 1976, 1980, 1988; Naran, 1997), almost nothing is known about their genetic structure. No previous work has been done on the genetic structure of fish in the Olifants River System, which makes conservation management difficult for this particular system (Impson, pers. comm.). Previous recommendations on the conservation of fish in the Olifants River System centered on captive breeding and restocking into suitable habitats (Gaigher, 1973b; Skelton, 1977, 1987; Scott, 1982; Mariott, 1998). Captive breeding of fish, however, invariably leads to loss of genetic diversity (e.g. Quattro & Vrijenhoek, 1989; Briscoe et al., 1992; Leary et al., 1993) and artificial selection in a hatchery environment can eliminate adaptive gene complexes (Garcia de Leániz et al., 1989; Waples & Teel, 1990). It may be important to conserve genetic diversity, as

it has been shown to play a role in the fitness of populations (Quattro & Vrijenhoek, 1989) and resistance to parasites (Leberg &Vrijenhoek, 1994).

Restocking of natural habitats without prior knowledge of the genetic structure of the taxa concerned can also lead to the loss of unique evolutionary lineages of that taxon (Dowling & Childs, 1992; Leary et al., 1993; Quattro et al., 1996; Avise, et al., 1997). This may be especially true for the Cape ichthyofauna in the light of genetic investigations on *P. burgi* (Bloomer & Impson, in press) and *G. zebratus* (Waters & Cambray, 1997). Much genetic differentiation was found within the latter two species. Rehabilitation of streams and restocking might well be a necessary future strategy for conserving redfins in the Olifants River System. If future restocking of indigenous species in the Olifants River System is envisaged to improve the conservation status of threatened fish species, it is important to consider the genetic structure of taxa and the genetic diversity of populations.

The purpose of the present chapter is to describe the genetic structure of *B. calidus* and *P. phlegethon*, and the genetic divergence between *B. erubescens* and populations of *B. calidus*, using allozyme electrophoresis. It will also be investigated whether differential feeding, behaviour and body form between *B. calidus* and *P. phlegethon* (Bills, 1999; Skelton, 1980, 1988) can explain differences in their genetic structure. It will be done by comparing gene flow patterns within these two sympatric species. Their genetic structure will also be related to their current distribution and niche preference. An attempt will also be made to assess the impact of population fragmentation caused by bass on genetic structure and diversity of the redfin populations. A phylogenetic analysis of allozyme electrophoretic characters will be done to investigate the uncertain taxonomic position of redfins in relation to other barbs. Time since

divergence of these taxa will be inferred from genetic distances to test the conflicting models previously proposed for the biogeography of the redfins. It is envisaged that the present investigation will encourage the establishment of management plans for the conservation of genetic diversity in redfins of the Olifants River System (see Chapter 4).

3.2 MATERIALS AND METHODS

3.2.1 Study Area

The study area is shown in Fig. 3.1. Redfins were collected in tributary streams of the Olifants River System draining the Cedarberg Mountains (Fig. 3.2, 3.3 & 3.4). The Olifants River System is comprised of two main basins, drained by the Olifants and Doring Rivers. Redfins are absent from the more saline tributaries of the Doring River draining the Karoo in the east and the small western tributaries of the Olifants River (Fig. 3.2). The mountain streams characteristically have a low pH and low mineral content (Skelton, 1980). The Doring River has a much larger drainage area than the Olifants River, but is situated in a much drier area on the rain shadow side of the Cedarberg Mountains and carry much less water than the Olifants River (Skelton, 1980). The Cedarberg Mountains forms part of the Cape Fold Mountains and are composed mainly of Table Mountain sandstone with Bokkeveld marine sediments in the valleys (Rust, 1967; Theron, 1972) (Fig. 3.2). The vegetation type in the Cedarberg Mountains is typically Mountain Fynbos (Acocks, 1988). Karoo vegetation elements penetrate deep valleys of the tributaries of the Doring River and a karriod zone extends southward from Clanwilliam into the valleys of the Jan Dissels and Rondegat Rivers (Taylor, 1996).

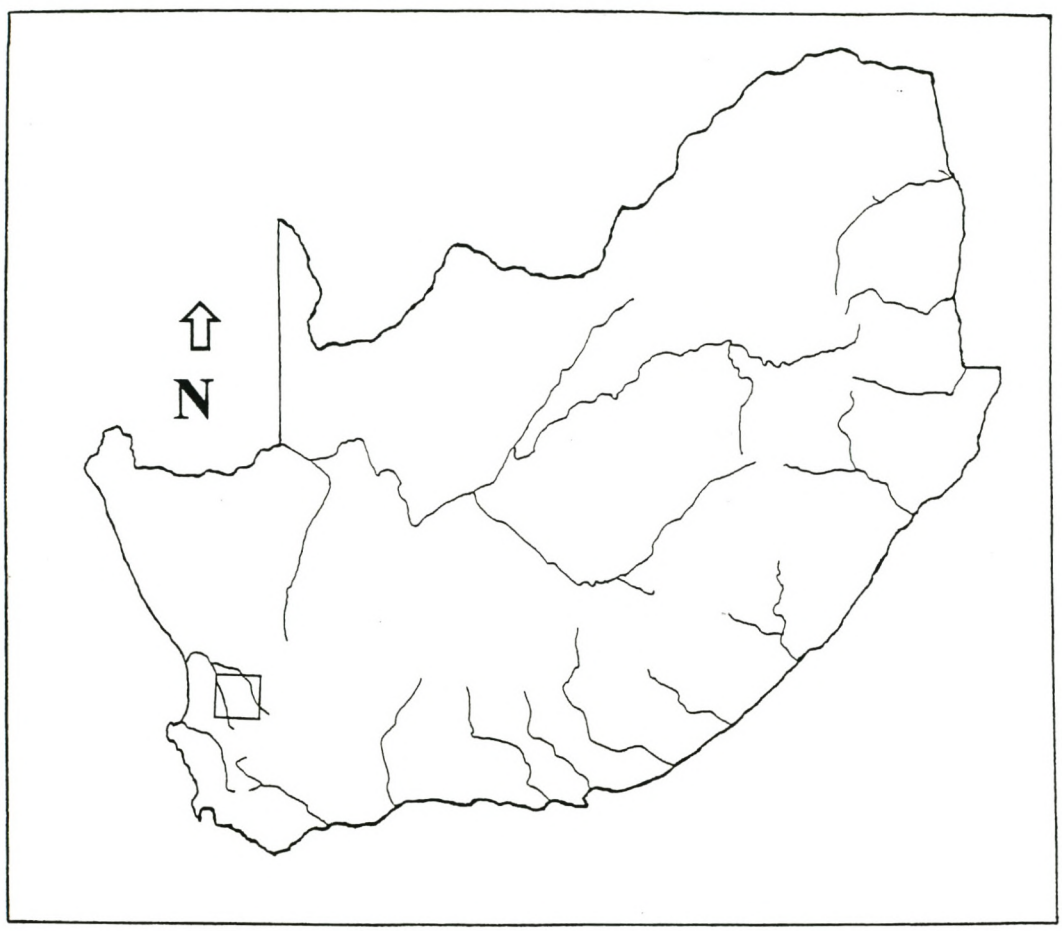

Fig. 3.1 Map of South Africa showing the upper reaches of the Olifants River System where the redfin samples were collected for present study (indicated by an open square).

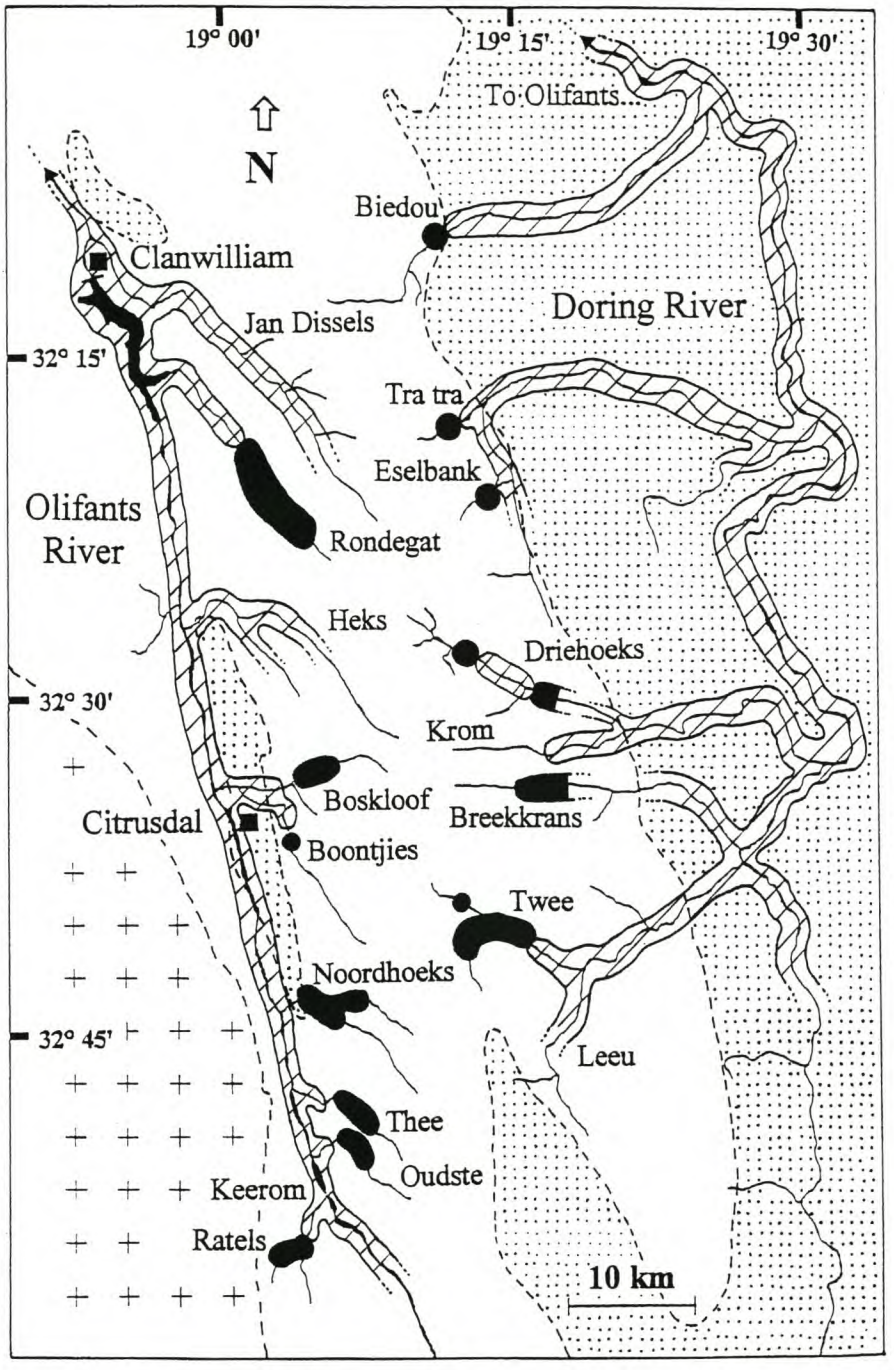

Fig. 3.2 Distribution of redfins in the Olifants River System in relation to the geology of the area, and the distribution of bass (*Micropterus salmoides*, *M. dolomieu* and *M. punctulatus*). The distribution of redfins is indicated by the solid black areas, whilst the distribution of bass is indicated with left slanting lines. Where distributions are uncertain, these were left "open". The Bokkeveld marine sediments and Karoo Supergroup are given as dotted areas. The Malmesbury group in the nearby Berg River catchment is shown with crosses.

3.2.2 Sample collection

Specimens were collected from 19 localities in the Olifants River System during February and March 1998 through electrofishing, seine netting, and snorkeling with a hand net. More specimens were collected in November 1998 and January 1999 from two localities in the same system (Table 3.1; Fig. 3.3 & 3.4). Fish were placed in plastic bags and stored in liquid nitrogen in the field, and transferred into an -80 °C Ultra Deep Freeze upon returning to the lab. Only muscle tissue was dissected from the fish. Some muscle tissue from each individual was stored in dimethyl sulphoxide saturated with NaCl and 70 % alcohol for future genetic work. Additional voucher specimens were collected from each locality, fixed in formalin, and deposited in the National Fish Collection of the J.L.B. Smith Institute in Grahamstown (Table 3.2).

3.2.3 Electrophoretic analysis

After initial electrophoretic screening (see Chapter 2), 13 enzymes were selected for routine analyses in this study. Only a few loci proved to be polymorphic. Individuals (N=375) from 13 tributaries and 19 localities were analyzed. In total, four populations of *P. phlegethon*, five populations of *B. calidus* and one population of *B. erubescens* were analyzed for all the enzymes selected (Table 3.1). To infer minnow gene flow patterns between all the known redfin populations in the Olifants River System, a further three populations of *P. phlegethon* and six populations of *B. calidus* were analyzed for polymorphic loci only (Table 3.1).

Table 3.1. List of *Pseudobarbus phlegethon, Barbus calidus* and *Barbus erubescens* populations included in the allozyme electrophoretic analysis. Unmarked populations were analyzed for all 27 interpretable allozyme loci. The populations marked with an asterisk were analyzed only for polymorphic loci found in the analyses of unmarked populations. The double asterisk indicates populations included as outgroups (see Chapter 2).

Sample number, tributary & species	N	Collection date	Collectors
OLIFANTS RIVER (OLIFANTS RIVER SYSTEM)			
9) Ratels *Barbus calidus*	20	24/03/1998	ES
22) Olifants gorge *Barbus serra* **	24	11/03/1998	ES, RB, DN, DI
11) Oudste *Barbus calidus* *	20	24/03/1998	ES
12) Oudste *Pseudobarbus phlegethon* *	17	24/03/1998	ES
1) Thee *Barbus calidus*	20	24/03/1998	ES
2) Thee *Pseudobarbus phlegethon*	20	24/03/1998	ES
13) Noordhoeks *Barbus calidus* *	20	18/02/1998	ES, RB, DN
14) Noordhoeks *Pseudobarbus phlegethon* *	20	18/02/1998	ES, RB, DN
15) Boskloof *Barbus calidus* *	20	23/02/1998	ES, RB, DN
16) Boskloof *Pseudobarbus phlegethon* *	20	23/02/1998	ES, RB, DN
4) Rondegat *Barbus calidus*	20	17/02/1998	ES, RB, DN
5) Rondegat *Pseudobarbus phlegethon*	20	17/02/1998	ES, RB, DN

Table 3.1 continued

DORING RIVER (OLIFANTS RIVER SYSTEM)			
17) Biedouw *Barbus calidus* *	20	30/03/1998	ES, AF
3) Tra-tra *Barbus calidus*	20	31/03/1998	ES, AF
18) Eselbank *Barbus calidus* *	20	15/11/1998	ES, RB
10) Driehoeks *Pseudobarbus phlegethon*	28	26-27/03/1998	ES
19) Driehoeks *Barbus calidus* *	20	20/11/98	ES, RB
6) Breekkrans *Barbus calidus*	20	25/03/1998	ES
7) Breekkrans *Pseudobarbus phlegethon*	11	25/03/1998	ES
8) Twee *Barbus erubescens*	19	29/03/1998	ES
BREEDE RIVER SYSTEM			
20) Witte *Pseudobarbus burchelli* **	5	21/03/1998	ES, RB, DN
ORANGE RIVER SYSTEM			
21) Sout *Barbus anoplus* **	5	23/03/1997	ES
23) Kraai *Barbus aenus* **	5	22-24/02/1999	RB

ES = E.R. Swartz; RB = I.R. Bills; DN = D. Naran; DI = N.D. Impson; AF = A.F. Flemming

Table 3.2. List of additional voucher specimens deposited into the National Fish Collection at the J.L.B. Smith Institute (Grahamstown) for future identification of redfin populations analyzed in the present electrophoretic survey. Not enough specimens were encountered to collect additional voucher specimens of *Barbus erubescens* from the Twee River.

Tributary	Species	N	RUSI number	Date	Collectors
OLIFANTS RIVER (OLIFANTS RIVER SYSTEM)					
Ratels	*Barbus calidus*	18	58907	24/03/1998	ES
Oudste	*Pseudobarbus phlegethon*	24	58913	24/03/1998	ES
	Barbus calidus	3	59572	01/11/1998	RB, SM
Thee	*Barbus calidus*	12	58909	24/03/1998	ES
		8	58912	24/03/1998	ES
	Pseudobarbus phlegethon	15	58908	24/03/1998	ES
		4	58911	24/03/1998	ES
	Barbus calidus & *Pseudobarbus phlegethon*	13	58910	24/03/1998	ES
Noordhoeks	*Barbus calidus*	6	58930	26/05/1998	DI, ES, FJ
		4	58942	05/08/1998	RB, DI, ES

Table 3.2 continued

Noordhoeks	*Pseudobarbus phlegethon*	1	58926	01/04/1998	ES
		34	58929	26/05/1998	DI, ES, FJ
		25	58941	05/08/1998	RB, DI, ES
Boskloof	*Barbus calidus*	12	58360	23/02/1998	ES, RB, DN
	Pseudobarbus phlegethon	12	58361	23/02/1998	ES, RB, DN
		18	58413	25/02/1998	ES, RB, DN
Rondegat	*Barbus calidus*	2	58935	27/05/1998	ES
		3	58937	06/08/1998	RB, DI, ES
		1	58945	06/08/1998	RB, DI, ES, PS
	Pseudobarbus phlegethon	2	58927	15/05/1998	ES
		1	58934	27/05/1998	ES
		1	58938	06/08/1998	RB, DI, ES
Olifants Gorge	*Barbus serra*	21	58389	11/03/1998	RB, DN, DI, ES
Olifants Gorge	*Barbus serra*	5	58392	10/03/1998	RB, DN, DI, ES
DORING RIVER (OLIFANTS RIVER SYSTEM)					
Biedouw	*Barbus calidus*	7	59605	16/11/1998	RB, ES
Tra-tra	*Barbus calidus*	19	58922	31/03/1998	ES
		29	59596	14/11/1998	RB, ES

Table 3.2 continued

Eselbank	*Barbus calidus*	22	59599	15/11/1998	RB, ES
	Barbus calidus	14	59603	15/11/1998	ES
Driehoeks	*Pseudobarbus phlegethon*	3	58916	27/03/1998	ES
		5	58917	27/03/1998	ES
		9	59612	18/11/1998	RB, ES
		3	59621	22/11/1998	ES
	Barbus calidus	31	59615	20/11/1998	RB, ES
Breekkrans	*Barbus calidus*	10	58914	25/03/1998	ES
		1	59614	19/11/1998	RB, ES
	Pseudobarbus phlegethon	4	58411	15/02/1998	RB, DN
Rietkloof	*Barbus anoplus*	9	59617	22/11/1998	RB, ES
BREEDE RIVER SYSTEM					
Witte	*Pseudobarbus burchelli*	9	58390	21/3/1998	RB, DN
ORANGE RIVER SYSTEM					
Sout	*Barbus anoplus*	1	55109	23/3/1997	ES
Kraai	*Barbus aenus*	87	59936	22-24/2/1999	RB

DI = Dean Impson DN = Daksha Naran ES = Ernst Swartz FJ = Francois Jooste PS = Peter Hill

RB = Roger Bills SM = Stuart Mangold.

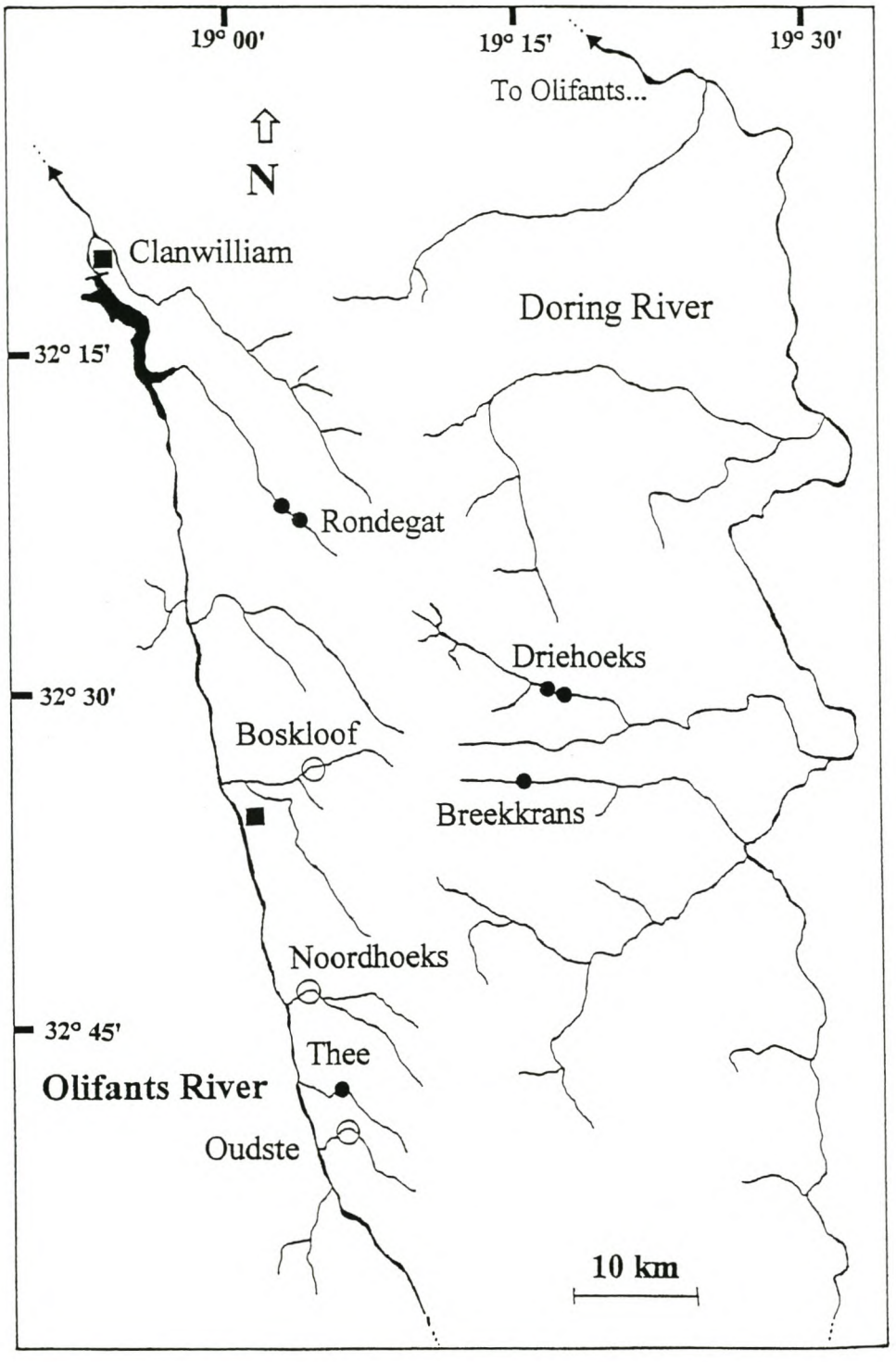

Fig. 3.3 Map of the Olifants River System indicating where *Pseudobarbus phlegethon* were collected. Solid circles refer to populations of *Pseudobarbus phlegethon* analyzed for 14 enzymes, whilst open circles refer to *Pseudobarbus phlegethon* populations analyzed for polymorphic loci only.

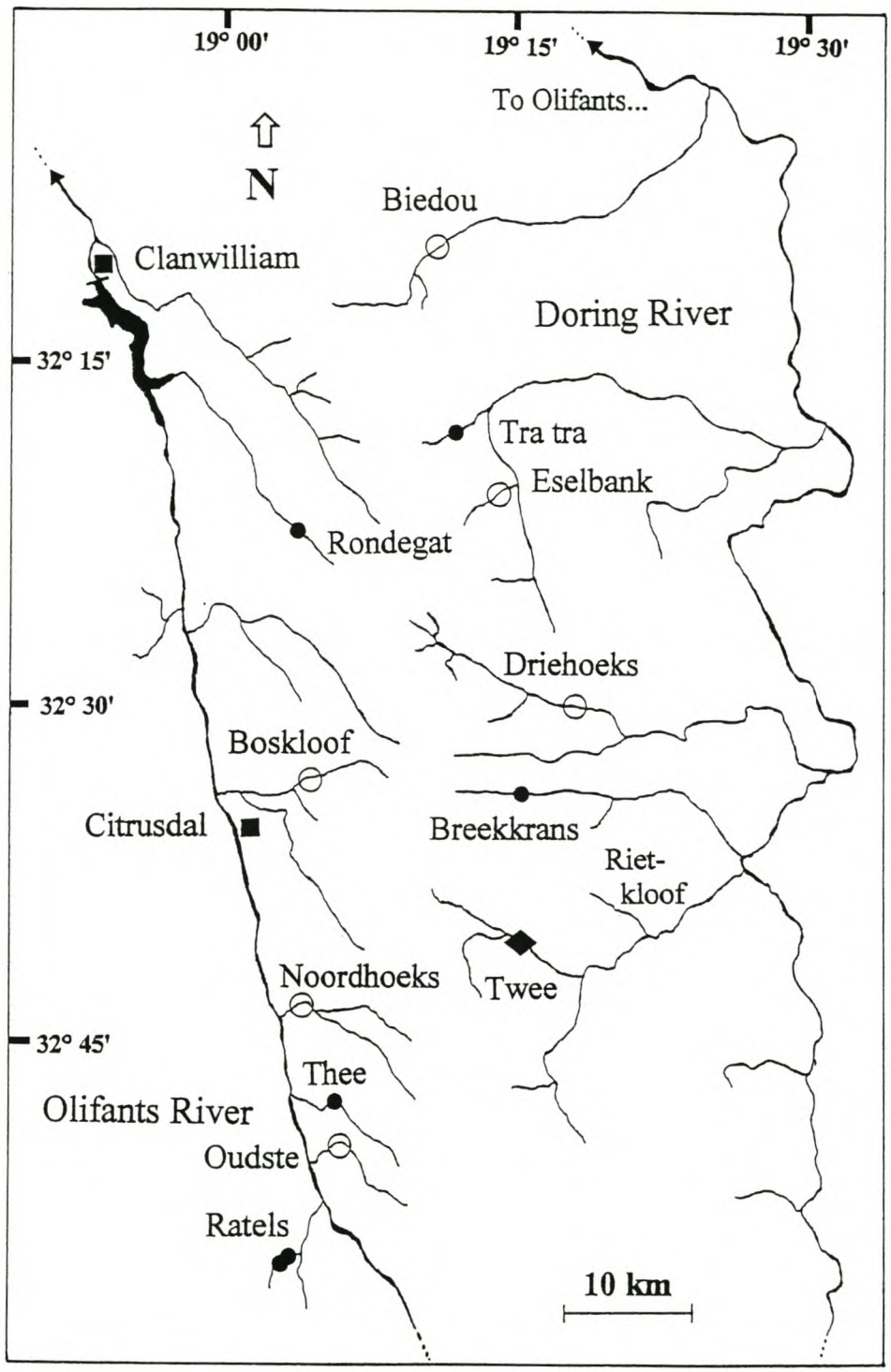

Fig. 3.4 Map of the Olifants River System showing where *Barbus calidus* and *Barbus erubescens* were collected. Solid circles refer to populations of *Barbus calidus* analyzed for 14 enzymes, whilst open circles refer to *Barbus calidus* populations analyzed for polymorphic loci only. The diamond symbol shows where the sample of *Barbus erubescens* was collected.

Muscle samples were homogenized in 0.01 M tris buffer (pH 8) in plastic Eppendorf tubes, using a glass rod attached to a variable speed motor. Samples were stored at -80°C. Allozyme allelic variation was examined on horizontal starch gel (13% hydrolyzed potato starch, Sigma Chemicals). Three electrophoretic buffer systems were used: i) a discontinuous tris-citrate-borate-lithium hydroxide buffer system with the gel buffer at pH 8.7 and the electrode buffer at pH 8.0 (Ridgeway et al., 1970); ii) a continuous tris-borate-EDTA buffer system with gel and electrode buffer at pH 8.6 (Markert & Faulhaber, 1965); iii) a continuous tris-citrate buffer system with the gel and electrode buffer at pH 6.9 (Whitt, 1970).

Procedures followed for electrophoresis are described in detail in paragraph 2.2.3. Staining for enzymatic activity was done according to Shaw & Prasad (1970), Harris & Hopkinson (1976) and Murphy et al. (1996). Sequential numbering of loci started from the cathodal end (Shaklee et al., 1990). The most common allele was assigned a value of 100 and the other alleles were scored relative to this value.

3.2.4 Interpretation of zymograms

The polyploid species investigated in the present study expressed a differential number of active loci (Section 2.4; Table 2.4) due to functional diploidization (Ohno, 1970a, 1970b; Ferris & Whitt, 1977; Buth, 1983, 1984a; Allendorf & Thorgaard, 1984). Berrebi et al. (1990) proposed "maximizing" and "minimizing" interpretation methods to avoid the mathematical difficulty in comparing taxa with different numbers of loci. In both methods Berrebi et al. (1990) considered the silent locus to have a null allele in the homozygote form that they coded as a "fixed

difference" from the expressed loci. The assumption with the minimizing method is then that these silent loci share the same null allele in each taxa, whilst it is assumed in the maximizing method that different evolutionary events led to the functional diploidization of duplicated genes in the different taxa (Machordom et al., 1995). In the latter method, it is assumed that the silent locus of each taxon has a distinct null allele and is then coded as "fixed differences" (Berrebi et al., 1990). In the present study the maximizing method was only performed in comparisons where a differential number of active loci was found.

3.2.5 Genetic analyses

Statistical analyses were preformed using the BIOSYS-1 computer program (Swofford & Selander, 1981). Allelic and genotype frequencies were calculated. Chi-square analyses were performed on genotype frequencies for each polymorphic locus to test whether or not they deviate from Hardy-Weinberg equilibrium. Levene's (1949) correction for small sample size was employed in the Chi-square analyses. Deviation from Hardy-Weinberg equilibrium was also tested using exact significance probabilities (Haldane, 1954; Vithayasai, 1973; Elston & Forthofer, 1977). This was done because of Nei & Chesser's (1983) observation that Levene's (1949) correction is based on the assumption of random mating. When more than two alleles were present, deviation from Hardy-Weinberg equilibrium was also tested by pooling genotype frequencies into three categories: homozygotes for the most common allele; common and rare heterozygotes; rare homozygotes and all other heterozygotes. The mean number of alleles and percentage of polymorphic loci in each population was determined. A locus was considered to be polymorphic when the frequency of the most common allele did not exceed 0.95. Where it did exceeded 0.95, remaining alleles were considered to be rare. Average heterozygosity was

calculated according to Nei et al. (1975). Mean expected heterozygosity was calculated for each population using Nei's (1978) unbiased estimates. In order to avoid a deceiving heterozygosity estimate, weighting factors were calculated for each enzyme according to the average heterozygosity indices which Ward et al. (1992, 1994) compiled for fish. This was done because enzymes differ in their levels of variability and average heterozygosity estimates can be strongly influenced by the combination of enzymes that are selected (Ward et al., 1992).

F-tests were performed according to the method of Wright (1978) to determine the degree of genetic substructuring, differentiation and inbreeding in the different species. The fixation index measures the reduction in heterozygosity expected with random mating at a particular level of a population hierarchy, relative to a more inclusive level of hierarchy (Hartl & Clark, 1997). Fixation indices calculated included F_{SR}, F_{RT} and F_{ST} values. Symbols were defined as follow: F is the fixation index; S (subpopulation) was defined as the currently isolated populations in the tributaries (in the case of *B. erubescens* this would constitute the whole species); R (region) was defined as the combination of populations of the two main rivers (Olifants and Doring Rivers); T (total population) as the total population in the Olifants River System, which can be defined as the whole species in the case of *P. phlegethon* and *B. calidus*. Measures of inbreeding related to hierarchy that included F_{IS}, F_{IR} and F_{IT} were calculated, where I is the inbreeding coefficient and S, R and T are as stated above.

Chi-square analyses were performed to test for heterogeneity of allele frequencies. Allele frequencies were also used to calculate genetic distance (D) and genetic identity (I) among populations (Nei, 1978). Average unbiased genetic distances (Nei, 1978) were analyzed with the unweighted pair group method (UPGMA) of cluster analysis (Sneath & Sokal, 1973) and was

used to construct a dendrogram of genetic distance. One population each of *B. serra*, *B. aenus* and *B. anoplus* (Table 2.1, Table 2.3 & Table 3.1) were included as outgroups. Cladograms were constructed with the PAUP (Swofford, 1985) computer program, using the branch-and-bound search option. Two methods were used to code the 23 loci that were interpretable for all taxa. In the first the presence or absence of alleles was coded as characters (Mickevich & Johnson, 1976), and in the second the loci were used as characters and the alleles as character states (Mickevich & Mitter, 1981). Both methods yielded only unordered characters. Tree Length (TL), Consistency Index (CI), Homoplasy Index (HI), Retention Index (RI) and Rescaled Consistency Index (RCI) were calculated as tree description data using PAUP (Swofford, 1985). Branch lengths, bootstrap (Felsenstein, 1985) and Bremer support values (Bremer, 1994) were calculated using the same computer program. Bootstrap proportions > 90 % were considered to be highly significant, those of 70-89 % as marginally significant and those < 70 % as little evidence of monophyly (Shaffer et al., 1997). Bremer support is the extra length needed to lose a branch in the consensus of near-most-parsimonious trees (Bremer, 1994). The same outgroups as in the above-mentioned dendrogram were included in the PAUP analysis.

Nei's (1978) average unbiased genetic distances were used to calculate time since divergence between taxa. A calibration of 1 Nei distance unit equaling about 15-20 million years of divergence (Sarich, 1977), was used. It was also assumed that the rate of evolution was equal in all taxa and that the rate of evolution in the taxa of the present study is comparable to other vertebrates.

3.3 RESULTS

Enzymes stained for; their abbreviations, presumptive loci, enzyme commission numbers, and buffer systems used are listed in Table 3.3. Relative mobility and frequency of alleles are given in Table 3.4 and Table 3.5. Silenced loci are indicated as "NULL" in these tables. A more detailed discussion on the enzymes, the electrophoretic and staining conditions under which they worked and comparison to other studies can be found in Sections 2.2.3, 2.3 and 2.4.

The 13 enzymes stained for yielded a variable number of loci. Of the 27 loci found, *P. phlegethon* expressed 25 loci and *B. calidus* and *B. erubescens* expressed 26 loci. The difference in the number of loci occurred when staining for Pep-LT and MDH. The serrated barbs expressed four loci (Pep-LT-1 to 4), whilst *P. phlegethon* only expressed two loci (Pep-LT-1 and Pep-LT-4). The serrated barbs had only three MDH loci, whilst *P. phlegethon* expressed four loci (see Table 2.4).

Of the 18 cases of polymorphism (no allele frequency criterion), 94 % were in Hardy-Weinberg equilibrium for allele frequencies (Table 3.6), using Levene's (1949) correction for small sample size. The same conclusions on the deviation from Hardy-Weinberg equilibrium was drawn from the exact significance probabilities test (Haldane, 1954; Vithayasai, 1973; Elston & Forthofer, 1977). This suggests that most of the populations investigated in the present study conform to the assumption of random mating. The only exceptions were GPI for *P. phlegethon* from the Rondegat River where a deficiency of heterozygotes was found ($\chi^2 = 5.505$; df = 1; $p < 0.05$) and Pep-LT-4 from the Noordhoeks River, also for *P. phlegethon* ($\chi^2 = 9.000$; df = 3; $p < 0.05$). The

latter case, however, was in Hardy-Weinberg equilibrium with pooling of genotype frequencies ($\chi^2 = 2.714$; df = 1; $p > 0.05$).

Percentages of polymorphic loci ranged from 3.8 to 7.7 in *B. calidus* and was 8 in Olifants River populations of *P. phlegethon*. No polymorphic loci were detected in *B. erubescens* and Doring River populations of *P. phlegethon*. The polymorphic loci were GPI and PEP-LT-4 in the Olifants River populations of *P. phlegethon* and PGM-1 and PGM-2 in *B. calidus* (0.95 criterion). The observation that GPI and Pep-LT-4 were polymorphic in *P. phlegethon* populations from the Olifants River, and fixed for the 60 % and 100 % alleles respectively in the *P. phlegethon* populations from the Doring River, is an indication of absence of gene flow between these two systems for this species. CK-1 and LGG each expressed a single rare allele in two *P. phlegethon* individuals from the Driehoeks and the Rondegat Rivers, respectively. Rare alleles (frequency less than 0.01) were encountered in LDH-2 and MDH-1 for *B. calidus*. The mean number of alleles per locus in all the populations ranged from 1 to 1.2 (SE 0 - 0.1). Only two loci yielded more than two alleles in any population. Of these, four alleles were found for Pep-LT-4 in *P. phlegethon* from the Oudste River, whilst PGM-1 yielded three alleles in *B. calidus* from the Boskloof River. The mean number of alleles per locus and percentage of polymorphic loci are summarized in Table 3.7.

When weighting factors were calculated to compensate for the differential variability of the enzymes selected (Ward et al., 1992), average observed heterozygosity ranged from 0 (*P. phlegethon* from the Breekkrans River and *B. erubescens*) to 0.032 (*P. phlegethon* from the Olifants River populations). The highest heterozygosity was 0.040 in *P. phlegethon* from the

Rondegat River. The adjusted average observed heterozygosity in *B. calidus* was lowest in the Tra tra River (0.003) and highest in the Thee River (0.016). The enzymes selected were slightly more variable (0.054) than that selected for fish normally (0.051) (Ward, 1992). Observed, expected and adjusted heterozygosity values are summarized in Table 3.7.

Out of the total 27 loci screened, 23 loci were monomorphic (0.95 criterion) in all the populations investigated. Of these, AK, MDH-2, ODH-1, ODH-2, PEP-LT-1 and PGD-2 were fixed for the same allele in all populations. Between *B. erubescens* and *B. calidus*, only one fixed allele difference occurred at MDHP-1 (D = 0.063). Only GPI, MDH, MDHP-1, PGD-1 and PGM-1 could distinguish *B. serra* from *B. calidus* (D = 0.238) and *B. erubescens* (D = 0.244). Surprisingly, AAT-1, LDH-1, MDH-1, MDH-3, MDH-4, PGM-1 and PGM-2 showed fixed allele differences between *P. phlegethon* populations from the Olifants and Doring Rivers (D = 0.355). *B. calidus* did not show this divergence between the Olifants and Doring Rivers (D = 0.005) and was generally homogenous over its distribution (D = 0-0.009). CK-1, EST-1, LDH-1, MDH-1, -3, -4, MDHP-1, LGG, PGM-1 and PGM-2 distinguished *P. burchelli* from *P. phlegethon* from the Olifants River (D = 0.737), but only EST-1, LDH-1, MDHP-1, LGG, LT-4 and PGM-1 were diagnostic between the former and *P. phlegethon* from the Doring River (D = 0.420). The genetic distance between *P. phlegethon* and "serrated barbs" (*B. calidus*, *B. erubescens* and *B. serra*) was 0.9 and 0.858 under the minimizing and maximizing methods, respectively. Nei's (1978) unbiased genetic distance and unbiased genetic identity values under the minimizing method (Berrebi et al., 1990) among populations are summarized in Table 3.8. Nei's (1978) unbiased genetic distance under the minimizing and maximizing methods (Berrebi et al., 1990) for distinct lineages found here is given in Table 3.9.

Table 3.3. Summary of enzyme names, enzyme commission numbers, locus abbreviations and buffer systems used in the genetic analysis of redfin populations. The asterisk mark cases where it is difficult to assign enzyme commission numbers, because of multiple substrate affinities (Murphy et al., 1996).

Enzyme	Enzyme commission number	Locus	Buffer system
Aspartate aminotransferase	2.6.1.1	AAT-1	TC
		AAT-1	TC
Adenylate kinase	2.7.4.3	AK	TC, RW, MF
Creatine kinase	2.7.3.2	CK-1	RW
		CK-2	RW
Esterase	3.1.1.1	EST-1	MF
		EST-2	MF
Glucose-6-phosphate isomerase	5.3.1.9	GPI-1	RW
Lactate dehydrogenase	1.1.1.27	LDH-1	TC, RW, MF
		LDH-2	TC, RW, MF
Malate dehydrogenase	1.1.1.37	MDH-1	MF
		MDH-2	MF
		MDH-3	MF
		MDH-4	MF

Table 3.3 continued

Malate dehydrogenase (NADP+)	1.1.1.40	MDHP-1	MF
		MDHP-2	MF
Octopine dehydrogenase	1.5.1.11	OPDH-1	TC
		OPDH-2	TC
Peptidases:			
Leucyl-glycyl-glycyl tripeptidase	*	LGG	MF
Leucyl-tyrosine dipeptidase	*	LT-1	MF
		LT-2	MF
		LT-3	MF
		LT-4	MF
Phosphogluconate	1.1.1.44	PGDH-1	MF
dehydrogenase		PGDH-2	MF
Phosphoglucomutase	5.4.2.2	PGM-1	MF
		PGM-2	MF

RW = Discontinuous tris-citrate-borate-lithium hydroxide buffer system; gel buffer pH = 8.7; electrode buffer pH = 8.0 (Ridgeway *et al*, 1970).

MF = Continuous tris-borate-EDTA buffer system; pH = 8.6 (Markert & Faulhaber, 1965).

TC = Continuous tris-citrate buffer system; pH = 6.9 (Whitt, 1970).

Table 3.4. Summary of relative mobilities and allelic frequencies for the redfin populations investigated for all 13 interpretable enzymes in the present study. Silenced loci are indicated as "NULL". Sample numbers are explained at the bottom of the table. Locus abbreviations are explained in Table 3.3. Sample sizes are indicated next to the locus abbreviation.

Locus abbreviation		Populations (Sample 1 - 10)									
& relative mobility		1	2	3	4	5	6	7	8	9	10
AAT-1	N =	20	20	20	20	20	20	11	19	18	28
100		-	1.000	-	-	1.000	-	-	-	-	-
97		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
125		-	-	-	-	-	-	1.000	-	-	1.000
AAT-2	N =	20	20	20	20	20	20	11	19	20	28
100		-	1.000	-	-	1.000	-	1.000	-	-	1.000
75		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
AK	N =	20	20	20	20	20	18	7	16	20	28
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
CK-1	N =	20	20	20	20	20	20	11	19	20	28
100		-	1.000	-	-	1.000	-	1.000	-	-	0.982
80		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
82		-	-	-	-	-	-	-	-	-	0.018

Table 3.4 continued

		1	2	3	4	5	6	7	8	9	10
CK-2	N =	20	20	20	20	20	20	11	19	20	28
100		-	1.000	-	-	1.000	-	1.000	-	-	1.000
78		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
Est-1	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
97		-	1.000	-	-	1.000	-	1.000	-	-	1.000
Est-2	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GPI	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	0.250	1.000	1.000	0.525	1.000	-	1.000	1.000	-
60		-	0.750	-	-	0.475	-	1.000	-	-	1.000
LDH-1	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
83		-	-	-	-	-	-	1.000	-	-	1.000
78		-	1.000	-	-	1.000	-	-	-	-	-
LDH-2	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.975	1.000
55		-	-	-	-	-	-	-	-	0.025	-

Table 3.4 continued

		1	2	3	4	5	6	7	8	9	10
MDH-1	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	1.000	1.000	1.000	1.000	0.925	-	1.000	1.000	-
115		-	-	-	-	-	0.075	-	-	-	-
88		-	-	-	-	-	-	1.000	-	-	1.000
MDH-2	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-3	N =	20	20	20	20	20	20	11	19	20	28
100		-	-	-	-	-	-	1.000	-	-	1.000
88		1.000	1.000	1.000	1.000	1.000	1.000	-	1.000	1.000	-
MDH-4	N =	20	20	20	20	20	20	11	19	20	28
100		-	1.000	-	-	1.000	-	-	-	-	-
65		-	-	-	-	-	-	1.000	-	-	1.000
Null		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
ME-1	N =	20	20	20	20	20	20	11	19	20	28
100		-	1.000	-	-	1.000	-	1.000	1.000	-	1.000
50		1.000	-	1.000	1.000	-	1.000	-	-	1.000	-

Table 3.4 continued

		1	2	3	4	5	6	7	8	9	10
ME-2	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
85		-	1.000	-	-	1.000	-	1.000	-	-	1.000
ODH-1	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ODH-2	N =	20	20	20	20	20	20	11	19	20	20
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
LGG	N =	20	20	20	19	20	20	11	19	20	28
100		-	1.000	-	-	0.975	-	1.000	-	-	1.000
50		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
45		-	-	-	-	0.025	-	-	-	-	-
LT-1	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
LT-2	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
Null		-	1.000	-	-	1.000	-	1.000	-	-	1.000

Table 3.4 continued

		1	2	3	4	5	6	7	8	9	10
LT-3	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
Null		-	1.000	-	-	1.000	-	1.000	-	-	1.000
LT-4	N =	20	20	20	20	20	20	11	19	20	28
100		-	0.625	-	-	0.475	-	1.000	-	-	1.000
93		1.000	0.100	1.000	1.000	0.100	1.000	-	1.000	1.000	-
110		-	0.275	-	-	0.425	-	-	-	-	-
PGD-1	N =	20	20	20	20	20	20	11	19	20	28
100		-	1.000	-	-	1.000	-	1.000	-	-	1.000
85		1.000	-	1.000	1.000	-	1.000	-	1.000	1.000	-
PGD-2	N =	20	20	20	20	20	20	11	19	20	28
100		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PGM-1	N =	20	20	20	20	20	19	11	17	20	28
100		0.400	1.000	0.050	0.300	1.000	0.079	-	1.000	0.425	-
128		0.600	-	0.950	0.700	-	0.921	-	-	0.575	-
104		-	-	-	-	-	-	1.000	-	-	1.000

Table 3.4 continued

		1	2	3	4	5	6	7	8	9	10
PGM-2	N =	20	20	20	20	20	19	11	15	20	28
100		0.600	-	0.975	0.900	-	0.974	1.000	1.000	0.925	1.000
86		0.350	1.000	0.025	0.100	1.000	0.026	-	-	0.075	-
70		0.050	-	-	-	-	-	-	-	-	-

1 = Thee *Barbus calidus*; 2 = Thee *Pseudobarbus phlegethon*; 3 = Tra tra *Barbus calidus*; 4 = Rondegat *Barbus calidus*; 5 = Rondegat *Pseudobarbus phlegethon*; 6 = Breekkrans *Barbus calidus*; 7 = Breekkrans *Pseudobarbus phlegethon*; 8 = *Barbus erubescens*; 9 = Ratels *Barbus calidus*; 10 = Driehoeks *Pseudobarbus phlegethon*.

Table 3.5. Summary of relative mobilities and allelic frequencies of additional *Pseudobarbus phlegethon* and *Barbus calidus* populations analyzed for polymorphic loci (0.95 criterion) and loci found to be fixed for alternative alleles within the same species, in the analyses of samples 1 - 10 (Table 3.4). Sample numbers are explained at the bottom of the table. Locus abbreviations are explained in Table 3.3. Sample sizes are indicated next to the locus abbreviation.

Locus abbreviation		Populations (Sample 11 - 19)								
& relative mobility		16	14	12	11	13	15	17	18	19
AAT-1	N =	20	20	17						
100		1.000	1.000	1.000						
GPI	N =	20	20	15						
100		0.050	0.025	0.233						
60		0.950	0.975	0.767						
LDH-1	N =	20	20	17						
78		1.000	1.000	1.000						
MDH-1	N =	20	20	17						
100		1.000	1.000	1.000						
MDH-3	N =	20	20	17						
88		1.000	1.000	1.000						
MDH-4	N =	20	20	17						
100		1.000	1.000	1.000						

Table 3.5 continued

		16	14	12	11	13	15	17	18	19
LT-4	N =	20	20	17						
100		0.625	0.650	0.735						
93		0.300	0.150	0.206						
110		0.075	0.200	-						
70		-	-	0.059						
PGM-1	N =	20	20	17	18	20	20	17	20	20
100		1.000	1.000	1.000	0.278	0.300	0.150	0.559	0.400	-
128		-	-	-	0.722	0.700	0.475	0.441	0.600	1.000
136		-	-	-	-	-	0.375	-	-	-
PGM-2	N =	20	20	17	18	20	20	19	20	20
100		-	-	-	0.639	0.900	0.975	0.868	0.025	0.975
86		1.000	1.000	1.000	0.361	0.100	0.025	0.132	0.975	0.025

11 = Oudste *Barbus calidus*; 12 = Oudste *Pseudobarbus phlegethon*; 13 = Noordhoeks *Barbus calidus*; 14 = Noordhoeks *Pseudobarbus phlegethon*; 15 = Boskloof *Barbus calidus*; 16 = Boskloof *Pseudobarbus phlegethon*; 17 = Biedouw *Barbus calidus*; 18 = Eselbank *Barbus calidus*; 19 = Driehoeks *Barbus calidus*.

Table 3.6. Results of Chi-square tests for deviation from Hardy-Weinberg equilibrium of redfin populations of the Olifants River System. Levene's (1949) correction for small sample size was employed in Chi-square analyses. The exact significance probabilities (Haldane, 1954; Vithayasai, 1973; Elston & Forthofer, 1977) yielded the same conclusions. No allele frequency criteria were set for polymorphism (all polymorphisms are reported). Significant deviation from Hardy-Weinberg equilibrium is indicated with an asterisk. Allele frequencies that deviated from Hardy-Weinberg equilibrium, but were in Hardy-Weinberg equilibrium with pooling of genotype frequencies are indicated by a double asterisk. Locus abbreviations are explained in Table 3.3.

Sample number	Population	Polymorphic loci	Chi-square	df	p
1)	Thee *Barbus calidus*	PGM-1	0.01	1	0.94
		PGM-2	5.05	3	0.17
2)	Thee *Pseudobarbus phlegethon*	GPI	1.06	1	0.3
		LT-4	3.58	3	0.31
3)	Tra tra *Barbus calidus*	PGM-1	0.03	1	0.87
		PGM-2	0	1	1
4)	Rondegat *Barbus calidus*	PGM-1	0.11	1	0.74
		PGM-2	0.18	1	0.67

Table 3.6 continued

5)	Rondegat *Pseudobarbus phlegethon*	GPI	5.51	1	0.02 *
		LGG	0	1	1
		LT-4	2.55	3	0.47
6)	Breekkrans *Barbus calidus*	MDH-1	0.09	1	0.77
		PGM-1	0.09	1	0.76
		PGM-2	0	1	1
7)	Breekkrans *Pseudobarbus phlegethon*	No polymorphic loci			
8)	Twee *Barbus erubescens*	No polymorphic loci			
9)	Ratels *Barbus calidus*	LDH-2	0	1	1
		PGM-1	1.92	1	0.17
		PGM-2	0.09	1	0.77
10)	Driehoeks *Pseudobarbus phlegethon*	CK-1	0	1	1
11)	Oudste *Barbus calidus*	PGM-1	2.35	1	0.13
		PGM-2	1.59	1	0.21
12)	Oudste *Pseudobarbus phlegethon*	GPI	0.17	1	0.68
		LT-4	1.92	3	0.59
13)	Noordhoeks *Barbus calidus*	PGM-1	0.11	1	0.74
		PGM-2	0.18	1	0.67
14)	Noordhoeks *Pseudobarbus phlegethon*	GPI	0	1	1
		LT-4	9	3	0.03 **

Table 3.6 continued

15)	Boskloof *Barbus calidus*	PGM-1	1.45	3	0.69
		PGM-2	0	1	1
16)	Boskloof *Pseudobarbus phlegethon*	GPI	0.03	1	0.87
		LT-4	3.57	3	0.31
17)	Biedou *Barbus calidus*	PGM-1	0.03	1	0.86
		PGM-2	0.34	1	0.56
18)	Eselbank *Barbus calidus*	PGM-1	1.01	1	0.31
		PGM-2	0	1	1
19)	Driehoeks *Barbus calidus*	PGM-2	0	1	1

Table 3.7. Index of mean number of alleles per locus, percentage of polymorphic loci and mean heterozygosity estimates as measures of genetic variability of redfin populations, derived from the present electrophoretic analyses. Standard errors of genetic variability measures are given in parentheses. All samples were analyzed for all 27 interpretable loci, except for samples 12, 14 and 16 of *P. phlegethon* (analyzed only for the nine variable loci found) and samples 11, 13, 15 and 17-19 of *B. calidus* (analyzed only for the two polymorphic loci found). A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95. The unbiased estimate of Nei (1978) was used to calculate the mean heterozygosity expected under Hardy-Weinberg equilibrium. Mean observed heterozygosity values were adjusted by calculating weighting factors according to Ward (1992) (only for samples analyzed for all 27 interpretable loci). This was done to compensate for the differential variability of the enzymes selected for the present study. Sample numbers are explained at the bottom of the table.

Population sample number	Mean sample size per locus	Mean number of alleles per locus	% Poly-morphic loci	Mean heterozygosity		
				Direct count	Hardy-Weinberg expected	Adjusted according to Ward et al. (1995)
1)	20 (0)	1.1 (0.1)	7.4	0.03 (0.02)	0.04 (0.03)	0.016 (0.011)
2)	20 (0)	1.1 (0.1)	7.4	0.03 (0.02)	0.03 (0.02)	0.025 (0.019)

Table 3.7 continued

3)	20 (0)	1.1 (0.1)	3.7	0.01 (0)	0.01 (0)	0 (0)
4)	20 (0)	1.1 (0.1)	7.4	0.02 (0.02)	0.02 (0.02)	0.01 (0.01)
5)	20 (0)	1.1 (0.1)	7.4	0.04 (0.03)	0.04 (0.03)	0.04 (0.03)
6)	19.9 (0.1)	1.1 (0.1)	7.4	0 (0)	0.01 (0.01)	0.01 (0.01)
7)	10.9 (0.1)	1 (0)	0	0 (0)	0 (0)	0 (0)
8)	18.7 (0.2)	1 (0)	0	0 (0)	0 (0)	0 (0)
9)	19.9 (0.1)	1.1 (0.1)	7.4	0.02 (0.01)	0.03 (0.02)	0.01 (0.01)
10)	27.7 (0.3)	1 (0)	0	0 (0)	0 (0)	0 (0)
11)	18 (0)	2 (0)	100	0.58 (0.03)	0.44 (0.03)	-
12)	16.8 (0.2)	1.3 (0.2)	22.2	0.1 (0.07)	0.09 (0.06)	-
13)	20 (0)	2 (0)	100	0.3 (0.1)	0.31 (0.12)	-
14)	20 (0)	1.3 (0.2)	11.1	0.06 (0.06)	0.06 (0.06)	-
15)	20 (0)	2.5 (0.5)	50	0.30 (0.25)	0.34 (0.29)	-

Table 3.7 continued

16)	20 (0)	1.3 (0.2)	22.2	0.09 (0.08)	0.07 (0.06)	-
17)	18 (1.0)	2 (0)	100	0.4 (0.13)	0.37 (0.14)	-
18)	20 (0)	2 (0)	50	0.33 (0.28)	0.27 (0.22)	-
19)	20 (0)	1.5 (0.5)	0	0.03 (0.03)	0.03 (0.03)	-

1 = Thee *Barbus calidus*; 2 = Thee *Pseudobarbus phlegethon*; 3 = Tra tra *Barbus calidus*; 4 = Rondegat *Barbus calidus*; 5 = Rondegat *Pseudobarbus phlegethon*; 6 = Breekkrans *Barbus calidus*; 7 = Breekkrans *Pseudobarbus phlegethon*; 8 = *Barbus erubescens*; 9 = Ratels *Barbus calidus*; 10 = Driehoeks *Pseudobarbus phlegethon*; 11 = Oudste *Barbus calidus*; 12 = Oudste *Pseudobarbus phlegethon*; 13 = Noordhoeks *Barbus calidus*; 14 = Noordhoeks *Pseudobarbus phlegethon*; 15 = Boskloof *Barbus calidus*; 16 = Boskloof *Pseudobarbus phlegethon*; 17 = Biedouw *Barbus calidus*; 18 = Eselbank *Barbus calidus*; 19 = Driehoeks *Barbus calidus*.

Table 3.8. Matrix of genetic distance and identity coefficients under the minimising method (Berrebi, 1990) between redfin populations analyzed for all 27 interpretable loci. Values reported above the diagonal are Nei's (1978) unbiased genetic distance and those below the diagonal are Nei's (1978) unbiased genetic identity. Sample numbers are explained at the bottom of the table.

	Populations (Sample 1-10)									
Population	1	2	3	4	5	6	7	8	9	10
1)		0.852	0.009	0.003	0.823	0.008	1.125	0.058	0.003	1.124
2)	0.426		0.932	0.892	0.003	0.932	0.314	0.765	0.884	0.315
3)	0.991	0.394		0.002	0.901	0.000	1.099	0.073	0.005	1.098
4)	0.997	0.410	0.998		0.862	0.002	1.098	0.057	0.000	1.098
5)	0.439	0.997	0.406	0.422		0.901	0.333	0.738	0.854	0.334
6)	0.992	0.394	1.000	0.998	0.406		1.095	0.071	0.004	1.094
7)	0.325	0.730	0.333	0.333	0.716	0.334		0.993	1.097	0.000
8)	0.944	0.465	0.929	0.944	0.478	0.931	0.370		0.051	0.993
9)	0.997	0.413	0.995	1.000	0.426	0.996	0.334	0.950		1.096
10)	0.325	0.730	0.334	0.334	0.716	0.335	1.000	0.371	0.334	

1 = Thee *Barbus calidus*; 2 = Thee *Pseudobarbus phlegethon*; 3 = Tra tra *Barbus calidus*; 4 = Rondegat *Barbus calidus*; 5 = Rondegat *Pseudobarbus phlegethon*; 6 = Breekkrans *Barbus calidus*; 7 = Breekkrans *Pseudobarbus phlegethon*; 8 = *Barbus erubescens*; 9 = Ratels *Barbus calidus*; 10 = Driehoeks *Pseudobarbus phlegethon*.

Table 3.9. Matrix of genetic distance coefficients under the minimising and maximising methods (Berrebi, 1990) between *Barbus* and *Pseudobarbus* lineages, analyzed for 23 interpretable loci. Values reported above the diagonal are Nei's (1978) unbiased genetic distance under the minimising method and below the diagonal is Nei's (1978) unbiased genetic distance under the maximising method. "*Pseudobarbus* Olifants" and "*Pseudobarbus* Doring" refer to the divergent *Pseudobarbus phlegethon* populations of the tributaries of the Olifants and Doring Rivers respectively. See Table 3.1 and 2.1 for localities of the samples.

		Taxa (a-h)							
Taxa	N	a	b	c	d	e	f	g	h
a) *Barbus calidus*	100		0.072	0.918	1.194	0.851	1.135	0.238	1.329
b) *Barbus erubescens*	19	0.120		0.761	1.055	0.851	1.159	0.244	1.344
c) "*Pseudobarbus* Olifants"	40	0.918	0.761		0.330	0.737	0.968	1.151	1.223
d) "*Pseudobarbus* Doring"	39	1.194	1.055	0.462		0.420	1.132	1.184	1.055
e) *Pseudobarbus burchelli*	5	0.851	0.851	0.946	0.564		1.444	0.944	1.163
f) *Barbus anoplus*	5	1.288	1.313	1.245	1.458	1.930		1.013	0.495
g) *Barbus serra*	24	0.296	0.302	1.151	1.184	0.944	1.145		1.052
h) *Barbus aenus*	5	1.511	1.526	1.583	1.343	1.494	0.843	1.185	

F-statistics for redfin population are reported in Table 3.10. In *P. phlegethon* 88 % of the variation is ascribable to differentiation among populations, and only 12 % of the total variation is found within populations. Most of this substructuring (87 %) can be explained by differentiation between the Olifants and Doring River populations of *P. phlegethon*, whilst only 8 % can be explained by variation among populations within these drainages. Much less substructuring was found within *B. calidus*, where 87 % of the total genetic variation was found within populations. Similar F_{RT} (0.064) and F_{SR} (0.058) values reflect the lack of substructuring between the Olifants and Doring Rivers for *B. calidus*. When *B. erubescens* and *B. calidus* are considered together, only 47 % of the total genetic variation can be found within populations. Differentiation between *B. calidus* and *B. erubescens* accounts for 42 % of the genetic structuring, whilst 17 % can be explained for by variation within these two species.

Significant heterogeneity of allele frequencies occurred at 90 % of the 10 cases of between population comparisons of *P. phlegethon* from the Olifants River for both or either GPI and Pep-LT-4 (Table 3.11). Significant frequency differences of allele frequencies were also observed for *B. calidus* at 85.5 % of the 55 between population comparisons across their distribution at both or either PGM-1 and PGM-2. Less cases of heterogeneity of allele frequencies were found within the Olifants (73 %) and Doring Rivers (60 %), than between these two basins (98 %) in the latter species (Table 3.12). Significant frequency differences of alleles were observed between populations as close to each other along the river as the Thee and Oudste Rivers (*P. phlegethon*) (± 12 km) and the Tra tra and Eselbank Rivers (*B. calidus*) (± 11 km). *B. calidus* populations as far apart along the river as the Biedou and Ratels Rivers (± 250 km) did not show heterogeneity of allele frequency.

Table 3.10. Summary of F statistics across all 27 allozyme loci for *Barbus calidus*, *Pseudobarbus phlegethon* and "serrated" barbs. "Serrated" refers to F statistics calculated when *B. erubescens* is added as a population of *B. calidus*. "F" is the fixation index; "S" (subpopulation) was defined as the currently isolated populations in the tributaries; R (region) was defined as the combination of populations of the two main rivers (Olifants and Doring Rivers); T (total population) as the total population in the Olifants River System.

Taxa	F statistics				
	F(SR)	F(RT)	F(IS)	F(IT)	F(ST)
P. phlegethon	0.067	0.865	0.159	0.897	0.877
B. calidus	0.058	0.064	0.069	0.196	0.135
"Serrated"	0.171	0.415	0.069	0.559	0.527

Table 3.11. Matrix of chi-square statistics testing heterogeneity of allele frequencies between *Pseudobarbus phlegethon* populations. Only two loci were polymorphic (0.95 criterium). Chi-square values for between population comparisons are reported for GPI above the diagonal and for Pep-LT-4 below the diagonal. Degrees of freedom are given in parentheses. Sample numbers are explained at the bottom of the table.

Sample number	Population (Sample numbers)						
& population	2	5	7	10	12	14	16
2) Thee		6.373* (1)	6.558* (1)	15.628** (1)	0.026 (1)	8.538* (1)	6.275* (1)
5) Rondegat	2.104 (2)		17.466** (1)	37.632** (1)	6.076* (1)	25.078** (1)	22.029** (1)
7) Breekkrans	10.883* (2)	17.466** (2)			5.932* (1)	0.559 (1)	1.137 (1)
10) Driehoeks	24.889** (2)	37.632** (2)			14.224** (1)	1.415 (1)	2.860 (1)
12) Oudste	13.420* (3)	20.283** (3)	6.939* (2)	16.471** (2)		7.350* (1)	5.143* (1)
14) Noordhoeks	0.893 (2)	4.729 (2)	9.946* (2)	22.946** (2)	9.674* (3)		0.346 (1)
16) Boskloof	8.571* (2)	14.618** (2)	10.883* (2)	24.889** (2)	5.868 (3)	4.292 (2)	

* $p < 0.05$; ** $p < 0.001$

Table 3.12. Matrix of chi-square statistics testing heterogeneiety of allele frequencies between *Barbus calidus* and *Barbus erubescens* populations. Only two loci were polymorphic (0.95 criterium). Chi square values for between population comparisons are reported for PGM-1 above the diagonal and for PGM-2 below the diagonal. Degrees of freedom are given in parentheses.

	Populations (Sample numbers)					
Sample number & population	1	3	4	6	8	9
1) Thee *Barbus calidus*		14.050** (1)	0.879 (1)	10.901** (1)	30.192** (1)	0.052 (1)
3) Tra tra *Barbus calidus*	16.838** (2)		8.658* (1)	0.272 (1)	66.394** (1)	15.531** (1)
4) Rondegat *Barbus calidus*	9.956* (2)	1.920 (1)		6.131* (1)	38.287** (1)	1.352 (1)
6) Breekkrans *Barbus calidus*	15.996** (2)	0.001 (1)	1.764 (1)		60.939** (1)	12.240** (1)
8) Twee *Barbus erubescens*	15.556** (2)	0.761 (1)	3.182 (1)	0.801 (1)		28.367** (1)
9) Ratels *Barbus calidus*	11.888* (2)	1.053 (1)	0.157 (1)	0.949 (1)	2.351 (1)	
11) Oudste *Barbus calidus*	1.853 (2)	14.244** (1)	7.439* (1)	13.508** (1)	13.491** (1)	9.332* (1)
13) Noordhoeks *Barbus calidus*	9.956* (2)	1.920 (1)	0.000 (1)	1.764 (1)	3.182 (1)	0.157 (1)
15) Boskloof *Barbus calidus*	16.838** (2)	0.000 (1)	1.920 (1)	0.001 (1)	0.761 (1)	1.053 (1)

Table 3.12 continued

	1	3	4	6	8	9
17) Biedou *Barbus calidus*	7.638* (2)	3.117 (1)	0.190 (1)	2.895 (1)	4.261* (1)	0.678 (1)
18) Eselbank *Barbus calidus*	34.952** (2)	72.200** (1)	61.596** (1)	70.200** (1)	66.048** (1)	64.962** (1)
19) Driehoeks *Barbus calidus*	16.838** (2)	0.000 (1)	1.920 (1)	0.001 (1)	0.761 (1)	1.053 (1)

Table 3.12 continued

	Populations (Sample numbers)					
Sample number & population	11	13	15	17	18	19
1) Thee *Barbus calidus*	1.258 (1)	0.879 (1)	20.127** (2)	1.860 (1)	0.000 (1)	20.000** (1)
3) Tra tra *Barbus calidus*	7.393* (1)	8.658* (1)	23.333** (2)	23.410** (1)	14.050** (1)	2.051 (1)
4) Rondegat *Barbus calidus*	0.045 (1)	0.000 (1)	18.723** (2)	5.058* (1)	0.879 (1)	14.118** (1)
6) Breekkrans *Barbus calidus*	5.047* (1)	6.131* (1)	20.703** (2)	19.474** (1)	10.901** (1)	3.284 (1)
8) Twee *Barbus erubescens*	39.066** (1)	38.287** (1)	53.465** (2)	19.245** (1)	30.192** (1)	74.000** (1)
9) Ratels *Barbus calidus*	1.793 (1)	1.352 (1)	20.642** (2)	1.317 (1)	0.052 (1)	21.587** (1)
11) Oudste *Barbus calidus*		0.045 (1)	16.925** (2)	5.692* (1)	1.258 (1)	12.795** (1)
13) Noordhoeks *Barbus calidus*	7.439* (1)		18.723** (2)	5.058* (1)	0.879 (1)	14.118** (1)
15) Boskloof *Barbus calidus*	14.244** (1)	1.920 (1)		21.888** (2)	20.127** (2)	28.475** (2)
17) Biedou *Barbus calidus*	5.291* (1)	0.190 (1)	3.117 (1)		1.860 (1)	30.075** (1)

Table 3.12 continued

	11	13	15	17	18	19
18) Eselbank *Barbus calidus*	33.048** (1)	61.596** (1)	72.200** (1)	56.376** (1)		20.000** (1)
19) Driehoeks *Barbus calidus*	14.244** (1)	1.920 (1)	0.000 (1)	3.117 (1)	72.200** (1)	

* $p < 0.05$

** $p < 0.001$

The dendrogram of Nei's (1978) unbiased genetic distances, under the minimizing method (Berrebi et al., 1990), shows two distinct groups within *P. phlegethon* and the close genetic similarity between *B. calidus* and *B. erubescens* (Fig. 3.5). The cladograms confirm this organization. Coding the alleles as characters (Fig. 3.7) allowed for more resolving power than using loci as characters (Fig. 3.6). Buth (1984b) warned against the latter method, because the absence of characters is coded in outgroups that may never have been present in the first place. Coding loci as characters, however, presented a similar problem, because of differential diploidization of the taxa investigated. Berrebi's (1990) minimizing method were used, which led to coding the absence of silenced alleles in outgroups. In both the cladograms and the dendrogram of Nei (1978) genetic distance, the redfins form a monophyletic group with *B. serra*. Monophyly of the tetraploid barbs investigated in the present study is supported by highly significant bootstrap proportions (97-99 %) and high Bremer support (2-8) (Fig. 3.6 & 3.7). There was little support for monophyly of *P. phlegethon* populations from the Olifants and Doring Rivers (bootstrap < 72 %; Bremer support < 2), because of the inclusion of *P. burchelli* as an outgroup. The close relationship between *B. calidus* and *B. erubescens* is reflected in the lack of support for monophyly of *B. calidus* populations (bootstrap < 64 %; Bremer support < 1). Monophyly of the serrated tetraploid barbs (*B. calidus*, *B. erubescens* and *B. serra*) is supported by marginally to highly significant bootstrap proportions (82-100 %) and low to high Bremer support (1-8). Monophyly of *Pseudobarbus* spp. received much support where alleles were coded as characters (bootstrap 96 %; Bremer support = 5). Character matrixes for phylogenetic analysis are summarized in Table 3.13 and 3.14 for cladograms in Fig. 3.6 and Fig. 3.7 respectively. Estimated time since divergence between lineages found here is summarized in Table 3.15.

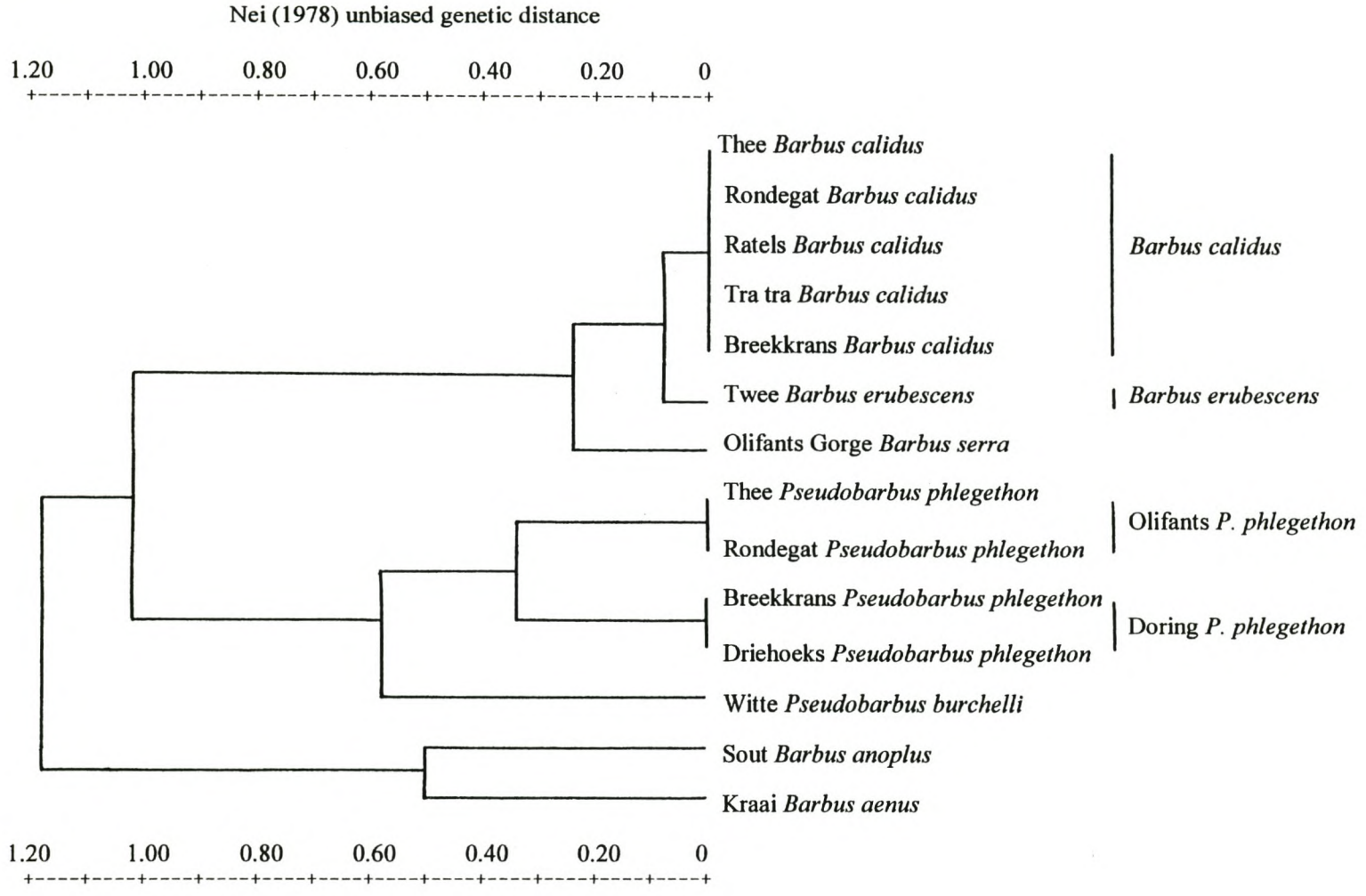

Fig. 3.5. Dendrogram of Nei's (1978) unbiased genetic distance of *Barbus* and *Pseudobarbus* populations, constructed through cluster analysis using the Unweighted Pair Group Method (Sneath and Sokal, 1973).

Table 3.13. Character matrix for phylogenetic analysis (Fig. 3.6) of the five populations of *Barbus calidus*, one population of *Barbus erubescens* and four populations of *Pseudobarbus phlegethon* that was analyzed for all 27 interpretable loci. Allozyme alleles were used as characters (Mickevich & Johnson, 1976). Question marks indicate characters not recorded. Dots indicate characters that were found to be the same as the outgroup. Character names are given at the bottom of the table. *Barbus anoplus*, *Barbus aenus*, *Barbus serra* and *Pseudobarbus burchelli* were included as outgroups.

Taxa	Characters																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Barbus anoplus (outgroup)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Barbus aenus (outgroup)	?	?	.	?	?	?	?	.	1	1	1	.	.	1	.	.	1	1	.	.	.	.	.	.	.
Barbus serra (outgroup)	?	?	.	?	?	?	?	1	1	.	1	1	.	.	.	1	.	1	1	.	1	1	.	1	.
Pseudobarbus burchelli (outgroup)	1	.	.	1	1	.	1	1	1	.	1	.	1	.	1	.	.	1	1	.	1	1	1	1	.
Thee *Barbus calidus*	.	1	.	1	.	1	1	1	1	.	1	1	.	.	.	1	.	1	1	.	1	1	.	1	1
Thee *Pseudobarbus phlegethon*	1	.	.	1	1	.	1	1	1	.	.	.	.	.	1	.	.	1	.	1	1	1	.	1	1

Table 3.13 continued

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Tra tra *Barbus calidus*	.	1	.	1	.	1	1	1	1	.	1	1	.	.	.	1	.	1	1	.	1	1	.	1	1
Rondegat *Barbus calidus*	.	1	.	1	.	1	1	1	1	.	1	1	.	.	.	1	.	1	1	.	1	1	.	1	1
Rondegat *Pseudobarbus phlegethon*	1	.	.	1	1	.	1	1	1	.	.	.	.	.	1	.	.	1	.	1	1	1	.	1	1
Breekkrans *Barbus calidus*	.	1	.	1	.	1	1	1	1	.	1	1	.	.	.	1	.	1	1	.	1	1	.	1	1
Breekkrans *Pseudobarbus phlegethon*	.	.	1	1	1	.	1	1	1	.	.	.	.	.	1	.	.	1	.	1	1	1	.	1	.
Twee *Barbus erubescens*	.	1	.	1	.	1	1	1	1	.	1	1	.	.	.	1	.	1	1	.	1	1	.	1	1
Ratels *Barbus calidus*	.	1	.	1	.	1	1	1	1	.	1	1	.	.	.	1	.	1	1	.	1	1	.	1	1
Driehoeks *Pseudobarbus phlegethon*	.	.	1	1	1	.	1	1	1	.	.	.	.	.	1	.	.	1	.	1	1	1	.	1	.

Table 3.13 continued

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Barbus anoplus (outgroup)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Barbus aenus (outgroup)	.	1	.	.	.	.	.	1	.	1	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.
Barbus serra (outgroup)	1	.	.	.	.	.	1	1	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	1
Pseudobarbus burchelli (outgroup)	.	1	1	.	.	1	.	1	.	1	1	1	.	.	.	1	1	.	.	1	1	1	1	.	.
Thee *Barbus calidus*	1	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	1
Thee *Pseudobarbus phlegethon*	.	1	1	.	1	.	1	1	.	.	.	.	.	.	1	.	1	1	.	1	.	1	1	1	.
Tra tra *Barbus calidus*	1	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	1
Rondegat *Barbus calidus*	1	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	1
Rondegat *Pseudobarbus phlegethon*	.	1	1	.	1	.	1	1	.	.	.	.	.	.	1	.	1	1	.	1	.	1	1	1	.
Breekkrans *Barbus calidus*	1	1	.	.	.	.	1	1	1	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	1
Breekkrans *Pseudobarbus phlegethon*	.	1	1	1	.	.	.	1	.	1	1	1	.	.	.	1	1	1	.	1	.	1	1	1	.

Table 3.13 continued

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Twee *Barbus erubescens*	1	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	1	.	1	.	.	.	.	1
Ratels *Barbus calidus*	1	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	1
Driehoeks *Pseudobarbus phlegethon*	.	1	1	1	.	.	.	1	.	1	1	1	.	.	.	1	1	1	.	1	.	1	1	1	.

Table 3.13 continued

	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Barbus anoplus (outgroup)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Barbus aenus (outgroup)	.	.	.	.	.	.	1	.	.	1	1	.	1	1	.	.	.	.	.	.	1	1	.	1	.
Barbus serra (outgroup)	1	.	1	1	1	1	1	1	.	.	.	.	.	1	1	1	.	.	.	.	1	1	1	1	1
Pseudobarbus burchelli (outgroup)	1	1	.	.	.	.	1	1	.	.	1	1	1	1	1	1	.	.	.	1	.	1	1	1	1
Thee *Barbus calidus*	1	.	1	1	1	1	1	1	.	.	.	1	1	1	1	1	1	1	.	.	.	1	1	.	.
Thee *Pseudobarbus phlegethon*	1	.	.	.	.	.	.	1	1	.	1	.	1	1	1	1	1	.	.	.	.	1	.	.	1
Tra tra *Barbus calidus*	1	.	1	1	1	1	1	1	.	.	.	1	1	1	1	1	1	1	.	.	.	1	1	1	1
Rondegat *Barbus calidus*	1	.	1	1	1	1	1	1	.	.	.	1	1	1	1	1	1	1	.	.	.	1	1	.	1
Rondegat *Pseudobarbus phlegethon*	1	.	.	.	.	.	.	1	1	.	1	.	1	1	1	1	1	.	.	.	.	1	.	.	1
Breekkrans *Barbus calidus*	1	.	1	1	1	1	1	1	.	.	.	1	1	1	1	1	1	1	.	.	.	1	1	1	1
Breekkrans *Pseudobarbus phlegethon*	1	.	.	.	.	.	.	.	.	.	1	.	1	1	1	1	.	.	1	.	.	1	1	1	1

Table 3.13 continued

	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Twee *Barbus erubescens*	1	.	1	1	1	1	1	1	.	.	.	1	1	1	1	1	1	1	.	.	.	1	1	1	1
Ratels *Barbus calidus*	1	.	1	1	1	1	1	1	.	.	.	1	1	1	1	1	1	1	.	.	.	1	1	.	1
Driehoeks *Pseudobarbus phlegethon*	1	.	.	.	.	.	.	.	.	.	1	.	1	1	1	1	.	.	1	.	.	1	1	1	1

Table 3.13 continued

Characters: (1) AAT-1^{100}, (2) AAT-1^{97}, (3) AAT-1^{125}, (4) AAT-1^{64}, (5) AAT-2^{100}, (6) AAT-2^{75}, (7) AAT-2^{Null}, (8) AK^{100}, (9) AK^{75}, (10) AK^{130}, (11) CK-1^{100}, (12) CK-1^{80}, (13) CK-1^{82}, (14) CK-1^{75}, (15) CK-2^{100}, (16) CK-2^{78}, (17) CK-2^{70}, (18) CK-2^{Null}, (19) Est-1^{100}, (20) Est-1^{97}, (21) Est-1^{90}, (22) Est-2^{100}, (23) Est-2^{90}, (24) Est-2^{93}, (25) GPI^{100}, (26) GPI^{60}, (27) GPI^{50}, (28) LDH-1^{100}, (29) LDH-1^{83}, (30) LDH-1^{78}, (31) LDH-1^{94}, (32) MDH-1^{100}, (33) MDH-1^{Null}, (34) MDH-1^{115}, (35) MDH-1^{88}, (36) MDH-3^{100}, (37) MDH-3^{88}, (38) MDH-3^{105}, (39) MDH-3^{Null}, (40) MDH-4^{100}, (41) MDH-4^{65}, (42) MDH-4^{Null}, (43) ME-1^{100}, (44) ME-1^{50}, (45) ME-1^{120}, (46) ME-1^{140}, (47) ME-2^{100}, (48) ME-2^{85}, (49) LGG^{100}, (50) LGG^{50}, (51) LGG^{70}, (52) LGG^{90}, (53) LT-2^{100}, (54) LT-2^{Null}, (55) LT-3^{100}, (56) LT-3^{Null}, (57) LT-4^{100}, (58) LT-4^{93}, (59) LT-4^{110}, (60) LT-4^{106}, (61) PGD-1^{100}, (62) PGD-1^{85}, (63) PGD-1^{77}, (64) PGD-1^{52}, (65) PGD-2^{100}, (66) PGD-2^{Null}, (67) PGM-1^{100}, (68) PGM-1^{128}, (69) PGM-1^{104}, (70) PGM-1^{112}, (71) PGM-1^{76}, (72) PGM-1^{Null}, (73) PGM-2^{100}, (74) PGM-2^{86}, (75) PGM-2^{70}

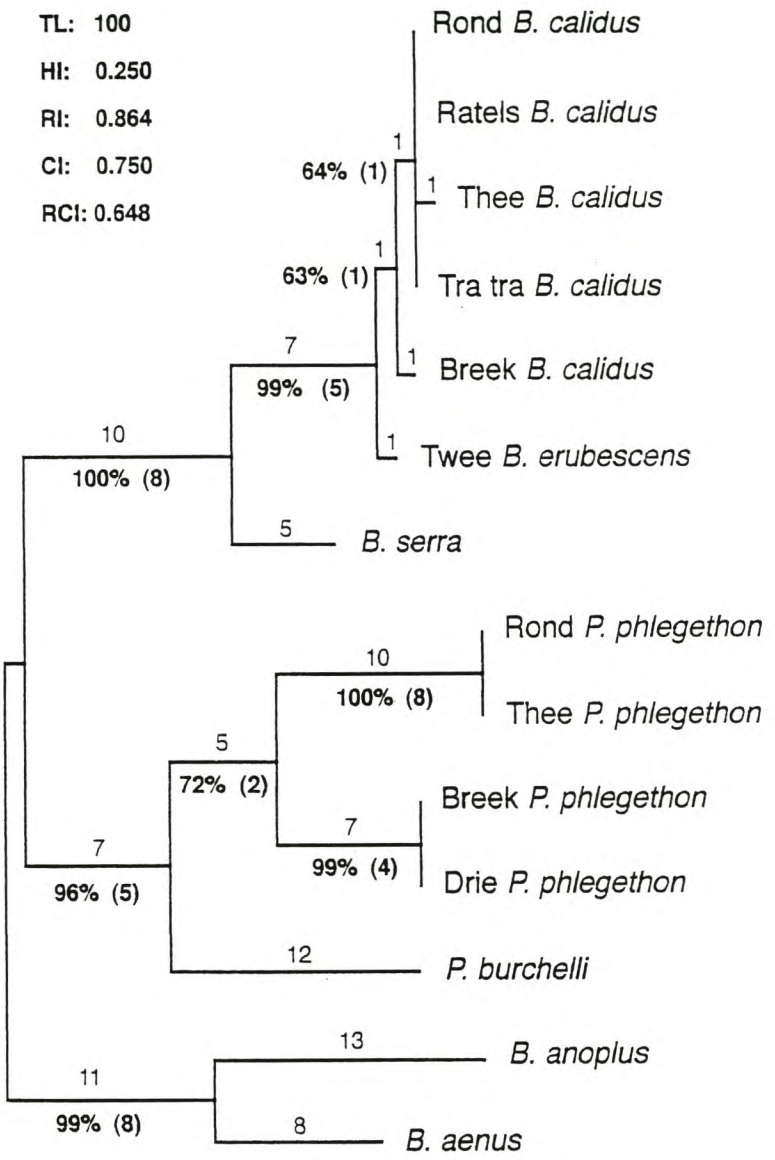

Fig. 3.6. The single most-parsimonious tree showing the relationships among *Barbus* and *Pseudobarbus* populations, constructed with the PAUP computer program by coding allozyme alleles as characters. Bold numbers below the branches are bootstrap proportions for groups recovered in at least 50% of the bootstrap replicates, based on 100 bootstrap replicates. Bremer support values (Bremer, 1994) are given in brackets. Numbers reported above the branches are branch lengths. Tree Length (TL), Consistency Index (CI), Homoplasy Index (HI), Retention Index (RI) and Rescaled Consistency Index (RCI) are given at the top of the page as tree description data.

Table 3.14. Character matrix for phylogenetic analysis (Fig. 3.7) of the five populations of *Barbus calidus*, one population of *Barbus erubescens* and four populations of *Pseudobarbus phlegethon* that was analyzed for all 27 interpretable loci. Allozyme loci were used as characters and alleles as character states (Mickevich & Mitter, 1981). Question marks indicate characters not recorded. Dots indicate characters that were found to be the same as the outgroup. Character names are given at the bottom of the table. *Barbus anoplus*, *Barbus aenus*, *Barbus serra* and *Pseudobarbus burchelli* were included as outgroups.

Taxa	Characters																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Barbus anoplus (outgroup)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Barbus aenus (outgroup)	?	?	1	1	1	.	.	2	.	1	1	.	.	.	.	.	.	1	1	.	1	1	.	.	.
Barbus serra (outgroup)	?	?	2	2	2	1	1	1	.	2	2	.	.	.	1	1	1	2	2	1	1	2	.	.	.
Pseudobarbus burchelli (outgroup)	1	1	2	3	3	1	2	2	1	1	3	1	1	1	2	.	.	2	3	1	2	2	.	.	.
Thee *Barbus calidus*	2	2	2	2	2	1	1	4	.	2	.	.	2	.	1	1	1	2	4	1	4	3	.	.	.
Thee *Pseudobarbus phlegethon*	1	1	2	.	3	2	1	3	2	2	.	2	3	1	3	.	.	3	1	1	3	4	.	.	.

Table 3.14 continued

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Tra tra *Barbus calidus*	2	2	2	2	2	1	1	4	.	2	.	.	2	.	1	1	1	2	4	1	4	2	.	.	.
Rondegat *Barbus calidus*	2	2	2	2	2	1	1	4	.	2	.	.	2	.	1	1	1	2	4	1	4	3	.	.	.
Rondegat *Pseudobarbus phlegethon*	1	1	2	.	3	2	1	3	2	2	.	2	3	1	3	.	.	3	1	1	3	4	.	.	.
Breekkrans *Barbus calidus*	2	2	2	2	2	1	1	4	.	3	.	.	2	.	1	1	1	2	4	1	4	2	.	.	.
Breekkrans *Pseudobarbus phlegethon*	3	1	2	.	3	2	1	2	3	1	3	1	3	1	3	.	.	.	1	1	5	2	.	.	.
Twee *Barbus erubescens*	2	2	2	2	2	1	1	4	.	2	.	.	3	.	1	1	1	2	4	1	3	2	.	.	.
Ratels *Barbus calidus*	2	2	2	2	2	1	1	4	.	2	.	.	2	.	1	1	1	2	4	1	4	3	.	.	.
Driehoeks *Pseudobarbus phlegethon*	3	1	2	.	3	2	1	2	3	1	3	1	3	1	3	.	.	.	1	1	5	2	.	.	.

Table 3.14 continued

Characters: (1) AAT-1, (2) AAT-2, (3) AK, (4) CK-1, (5) CK-2, (6) EST-1, (7) EST-2, (8) GPI, (9) LDH-1, (10) MDH-1, (11) MDH-3, (12) MDH-4, (13) MDHP-1, (14) MDHP-2, (15) PEP-LGG, (16) PEP-LT-2, (17) PEP-LT-3, (18) PEP-LT-4, (19) PGD-1, (20) PGD-2, (21) PGM-1, (22) PGM-2, (23) MDH-2, (24) LDH-2, (25) PEP-LT-1

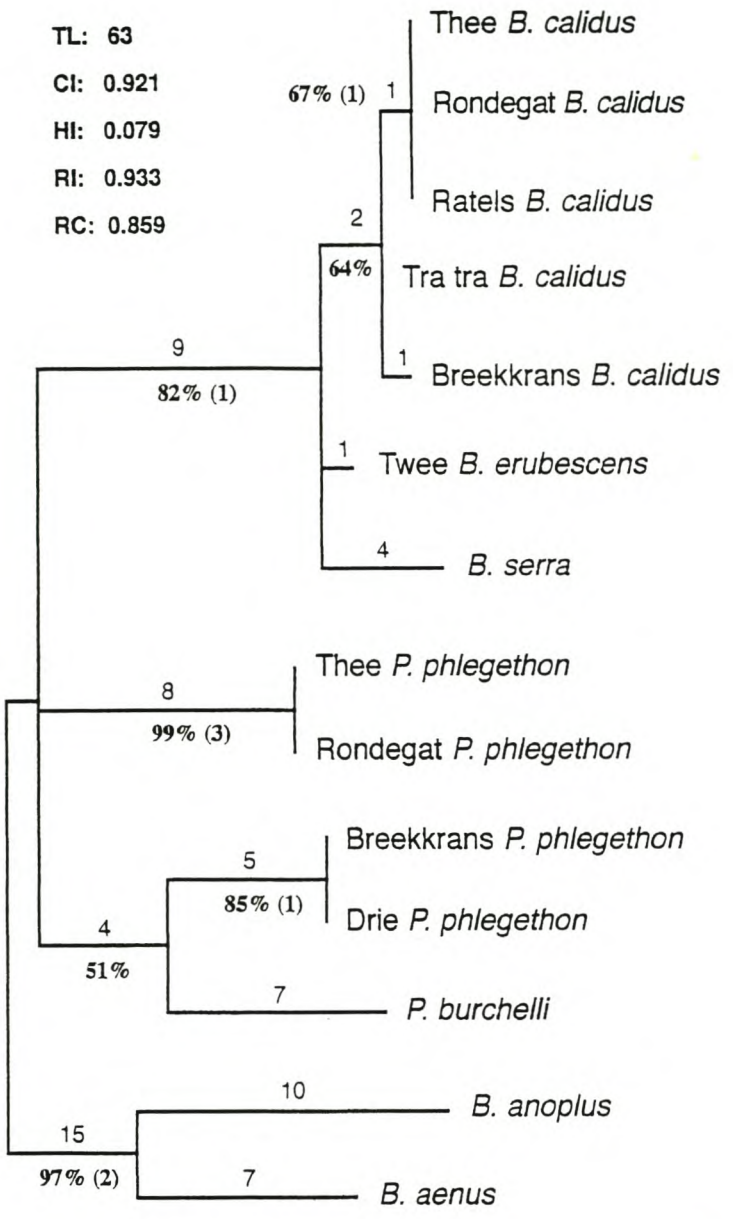

Fig. 3.7. Adams consensus tree of 14 most-parsimonious trees showing the relationships among *Barbus* and *Pseudobarbus* populations, constructed with the PAUP computer program by considering the allozyme loci as characters and alleles as character states. Bold numbers below the branches are bootstrap proportions for groups recovered in at least 50% of the bootstrap replicates, based on 100 bootstrap replicates. Bremer support values (Bremer, 1994) are given in brackets. Numbers reported above the branches are branch lengths. Tree Length (TL), Consistency Index (CI), Homoplasy Index (HI), Retention Index (RI) and Rescaled Consistency Index (RCI) are given at the top of the page as tree description data.

Table 3.15. Estimated time since divergence (sensu Sarich, 1977) between *Barbus* and *Pseudobarbus* taxonomic groupings as estimated from unbiased genetic distances in the present study. Estimated times of divergence have been adjusted for standard errors.

Taxonomic comparison	Minimum time	Maximum time	Epoch
"Tetraploid serrated" & *Pseudobarbus* spp.	12.5	18.5	Early to mid-Miocene
Barbus calidus & *Barbus erubescens*	0.9	1.3	Pleistocene
Barbus serra & *Barbus calidus - Barbus erubescens*	3.4	4.6	Pliocene
Olifants *Pseudobarbus phlegethon* & *Pseudobarbus burchelli*	10.8	15	Mid to late-Miocene
Doring *Pseudobarbus phlegethon* & *Pseudobarbus burchelli*	6.3	8.4	Late-Miocene
Olifants & Doring *Pseudobarbus phlegethon*	5.2	7.2	Late-Miocene
Pseudobarbus spp. & *Barbus anoplus*	13.3	27.4	Late-Oligocene to mid-Miocene
4N serrated & *Barbus anoplus*	15.9	24.8	Late-Oligocene to mid-Miocene
Barbus anoplus & *Barbus aenus*	7.4	16.8	Mid to late-Miocene
Pseudobarbus spp. & *Barbus aenus*	15.1	27.7	Late-Oligocene to middle-Miocene

Table 3.15 continued

4N serrated & *Barbus aenus*	18	28.9	Middle-Oligocene to late-Miocene

* Both the maximizing and minimizing methods had to be used to estimate time since divergence, because of differential diploidization of loci, hence a wider time range was estimated.

3.4 DISCUSSION

There is a clear divergence between the five populations of *P. phlegethon* from the Olifants River on the one hand, and the two populations of *P. phlegethon* from the Doring River on the other. *P. phlegethon* specimens from the Doring River tributaries were consistently distinguishable from those from the Olifants River tributaries at seven loci out of a possible 25 loci investigated. The latter were also distinguishable from *P. burchelli* specimens of the Breede River System at 10 loci, but *P. phlegethon* specimens of the Doring River tributaries were only distinguishable from *P. burchelli* at six out of a possible 25 loci. The Nei (1978) similarity index of 70.1 % between Olifants and Doring River populations of *P. phlegethon* suggests that these two groups should be considered different species. Genetic similarity lower than 85 % is generally considered characteristic for between species differences in fishes (Avise, 1975). The genetic distance (Nei, 1978) of 0.355 between Olifants and Doring River populations of *P. phlegethon* is also characteristic of typical species or subspecies differences often reported in the literature for *Barbus* (Karakousis et al., 1995; Machordom et al., 1995; Engelbrecht & Van der Bank 1996a, 1996b, 1997). The range of genetic distance estimates between taxonomic categories, however, overlap considerably in this genus. For instance, the Unbiased genetic distance (Nei, 1978) overlapped between populations (0.228 - 0.342) and species (0.245 - 0.797) of South African diploid barbs (Engelbrecht & Van der Bank 1996a, 1996b, 1997), and the genetic distance (Nei, 1972) overlapped between populations (0.005 - 0.269) and species (0.192 - 1.017) of European barbs (Karakousis et al., 1995). A decision on the taxonomic status of *P. phlegethon* from the Doring River is further complicated by their allopatric distribution in relation to *P. phlegethon* from the Olifants River. There is, however, presently no evidence for a genetic cline between these two groups. All the known populations of *P. phlegethon* were sampled and analyzed. They

all fall within either of these two clusters, with most of the genetic variation occurring between the Olifants and Doring River populations (87 %). The maximum genetic distance (Nei, 1978) within these two lineages is 0.003 and the minimum distance between them is 0.314.

Skelton (1980) did find variation in certain meristic characters between *P. phlegethon* specimens from the Driehoeks (the only population known from the Doring River Drainage at that stage) and those from the Olifants River. These included differences in the mean number of fin rays in dorsal and pectoral fins and in the mean number of lateral line scales. He also noted that his small sample from the Driehoeks River included larger than average specimens and "a male with the best developed tubercles yet seen". It was suggested that some of these differences might be environmentally determined. The high altitude of the Driehoeks River and sandy, marshy habitat, as compared to the rocky riffle habitat of most of the Olifants River tributaries, were named as ecological differences. The Breekkrans population of *P. phlegethon* that is very closely related to *P. phlegethon* from the Driehoeks (D = 0), however, occurs at a lower altitude. Where *P. phlegethon* was collected in the Breekkrans River, there are no marshy plateaus comparable to those in the Driehoeks River. The Breekkrans River descends in a steep gradient like most of the Olifants River tributaries. All the *P. phlegethon* populations that were sampled occur in streams draining Table Mountain sandstone. Although there might be water chemistry, stream structure and other ecological differences among Olifants and Doring tributary streams, it is proposed that the high genetic differentiation found between *P. phlegethon* populations from the latter two areas is due to a long period of isolation rather than just environmental selection. In the light of their genetic differentiation, the total absence of gene flow, and clear genetic structuring between the Olifants and Doring River populations of *P. phlegethon*, it is suggested that the taxonomic status of *P. phlegethon* from the Doring River should be further investigated.

The close relationship between *B. calidus* and *B. erubescens* evident from morphometric (Skelton, 1974; 1988) and cytogenetic (Naran, 1997) studies, is supported by the present study. Divergence below the species level is generally characterized by genetic similarity greater than 85 % (Avise, 1975). The genetic similarity of 93.9 % between *B. calidus* and *B. erubescens* falls within this category. This implies that the genetic distance (Nei, 1978) estimated between these two taxa (D = 0.063) is more characteristic of conspecific populations than congeneric species. The area downstream of the Twee River, where possible historical hybridization or a genetic cline could have existed between *B. calidus* and *B. erubescens*, is now dominated by bass. Minnows were probably eliminated from this area if they indeed occurred there previously (Skelton, 1974). The nearest downstream population of *B. calidus* occurs in the Breekkrans River, and shows typical morphometric characters for that species (Skelton, 1974). Specimens from this population are fixed for the diagnostic *B. calidus* 50 % ME-1 allele found in the present investigation. No redfins and only *B. anoplus* were found in a recent survey of the Rietkloof River, a tributary situated between the Breekkrans and Twee Rivers. Most of the genetic structuring in the serrated minnows of the Olifants River System is between these two taxa, providing no evidence for a genetic cline between *B. calidus* and *B. erubescens*. It does suggest that the divergence occurred relatively recently. Because very little genetic structuring was found within *B. calidus*, and because of the low genetic variability found in both *B. calidus* and *B. erubescens*, it is not possible to determine the origin of *B. erubescens* in relation to *B. calidus* populations (Fig. 3.5 – 3.7). The apparent closer relationship between Doring River *B. calidus* and *B. erubescens*, rather than to Olifants River *B. calidus* populations in Fig. 3.7 is not due to the occurrence of private alleles. The frequency of the rare (86 %) allele for PGM-2 in the Olifants River populations of *B. calidus* had a frequency higher than 0.05. This allele was thus coded as present. It had a frequency of less than 0.05 in the Doring River populations and had to

be coded as absent. This allele was not observed in *B. serra* and *B. erubescens*. As in *P. phlegethon*, decisions on the taxonomic status should be made on the basis of geographic patterns of variation and not the magnitude of variation (Buth & Mayden, 1981; Buth et al., 1991). In the absence of a genetic cline or a hybrid zone, and in the light of morphometric results of Skelton (1974), it is suggested that *B. erubescens* should retain some taxonomic recognition.

When Wright's (1978) qualitative guidelines for interpreting F-statistics were followed, it was evident that both *B. calidus* and *P. phlegethon* populations had at least moderate genetic substructuring, mostly explainable by low gene flow among populations. The pattern of genetic structuring, however, differed between *P. phlegethon* and *B. calidus*. There was less overall genetic structuring in *B. calidus* and most of the variation was within populations (87 %). In contrast, there was a great divergence between the Olifants and Doring River populations of *P. phlegethon*, with 87 % of the genetic structuring explained by variation between these two drainages.

Estimated gene flow between populations was found to be low. It is difficult, however, to distinguish between historical gene flow and recent fragmentation (e.g. Cunningham & Moritz, 1998). Small populations like the Tra tra, Eselbank and Biedou populations of *B. calidus* may show frequency differences in alleles because of severe fragmentation caused by bass. Certain populations may have been isolated earlier than others. The effect of sampling relatively small numbers of individuals per population over a geographically restricted area may also lead to allele frequency differences. This may explain the divergence from Hardy-Weinberg equilibrium for GPI of *P. phlegethon* from the Rondegat River. Skelton (1987, 1988) found that *P. phlegethon* males are territorial, and this could possibly contribute to deviation from random

mating. The allele frequency differences between tributaries were encountered more in *P. phlegethon* than in *B. calidus*. This may be further evidence that *P. phlegethon* preferred the tributary streams and that *B. calidus* was better able to disperse through the main stream. There has apparently been more historic gene flow among Olifants River tributaries and among Doring River tributaries, than between tributaries of these two rivers for *B. calidus*, based on allele frequency differences.

The differential feeding, niche preference, behaviour and body form between *B. calidus* and *P. phlegethon* (Skelton, 1980, 1988; Bills, 1999) apparently played an important role in gene flow, dispersability and consequently their genetic structure. Skelton (1980) noted that the morphological adaptations of *B. calidus* are towards active swimming habitats. It is proposed that, with its more slender body, bony serrated dorsal spine, and open-water feeding behaviour (Skelton, 1980, 1988), it is better adapted to disperse to other tributaries through the main stream than *P. phlegethon*. The bony serrated dorsal spine of *B. calidus* could be an adaptation against predation and possibly acts as a keel in strong currents (Skelton, 1988). *P. phlegethon* has a flexible primary dorsal spine (Skelton, 1980, 1988) and may prefer the habitat of the small tributary streams. The observation that *P. phlegethon* prefers shallow sand, cobble or rock based pools and rocky riffle habitat (Skelton, 1996; Bills, 1999) and that it concentrates in the mid to lower reaches of tributary streams where shallow riffle habitat is plentiful (Bills, 1999), does indicate possible adaptation to tributary streams. Further evidence for *P. phlegethon* preferring tributary habitat comes from the Driehoeks River, the only river known where bass does not mark the lower distribution *P. phlegethon*. Here *P. phlegethon* occurs in the upper and middle reaches of the River. *B. serra*, *B. calidus* and *B. capensis* dominate the lower reaches. According to Skelton (1987) the habitat of the Noordhoeks tributary stream may be an optimal habitat for *P.*

phlegethon, because of its high relative abundance in this stream. There are also more historical records of *B. calidus* occurring in the main stream of the Olifants River (Clanwilliam and Keerom) (Harrison, 1938; Barnard, 1943; Skelton, 1988; SAM 215/216; SAM 2012-2015) and it has a wider distribution than *P. phlegethon* (Gaigher, 1973b; Skelton, 1988; Bills, 1999). Fewer populations are known to exist for *P. phlegethon* (Gaigher, 1973b; Skelton, 1988; Bills, 1999) and this species has usually a more restricted distribution where they occur together with *B. calidus*. *P. phlegethon* was also only recorded from the main stream at Keerom (Harrison, 1938; Barnard, 1945). The wider distribution of *B. calidus* could be due to their wider habitat preference, possible ability to survive and compete better in the main stream than *P. phlegethon*, and superior ability to disperse and overcome natural barriers. *B. calidus* is able to occupy upper reaches of mountain streams, whilst bass may have eradicated *P. phlegethon* in the lower reaches of streams like the Ratels, Boontjies, Biedou, Tra tra and Eselbank.

Skelton (1980, 1986, 1988, 1994) mentioned that redfin speciation is closely related to the development of the Cape Fold Mountains. It is suggested that the genetic divergence within *P. phlegethon* is due to allopatric speciation, associated with the availability of suitable habitat. It is proposed that *P. phlegethon* has specialized to middle to lower reaches of clear mountain streams, with low pH and low mineral content, associated with Table Mountain sandstone (Fig. 3.2) (Skelton, 1980). The existence of the proto-Orange River (Skelton, 1986, 1994) could have influenced the divergence in *P. phlegethon*. The current main stream Doring River is very saline and drains a large area of the Karoo Supergroup and Bokkeveld Group, and causes high suspension loads and high salinity (Skelton, 1980). In a proto-Orange River, the main stream habitat would probably have been similar to the current lower Olifants or lower Orange Rivers, with higher suspension loads and higher salinity than the mountain streams where *P. phlegethon*

currently occurs. These physical properties of the main stream, together with competition from large main stream fishes like yellowfish and sawfin, and an apparent preference of *P. phlegethon* for mountain streams, seem to have isolated populations of the Olifants and Doring Rivers for a long time. *B. calidus*, on the other hand, was able to disperse through the main stream. The current distribution of *B. calidus*, therefore, probably reflects only a fraction of its former distribution.

Proposed periods of redfin origin can be tested using the genetic distance calculated. Proposed periods for the origin of redfins are Pliocene and Pleistocene invasions from the north (Jubb, 1964; Jubb & Farquharson, 1965; Gaigher & Pott, 1973; Mulder, 1989) as opposed to speciation in the southern temperate fauna since the early Tertiary (Skelton 1980, 1994, 1986). Nei's (1978) genetic distance between *P. phlegethon* from the Olifants River and *P. phlegethon* from the Doring River in the present study suggests that these two groups may have been isolated from each other for about 5.2 to 7.2 million years. The divergence between Doring River *P. phlegethon* and *P. burchelli* (late Miocene) and between Olifants *P. phlegethon* and *P. burchelli* (mid to late Miocene), is also too distant to be explained by Pliocene invasions from the north. Estimated time since divergence between the tetraploid serrated barbs and *Pseudobarbus*, inferred from the species investigated here, is between 12.5 and 18.5 million years. The estimation of time since divergence from allozyme data must be interpreted with caution, however, because rates of evolution may not be equal for these taxa. This would violate the assumption of a molecular clock. There is currently no geological event associated with speciation in the redfins of the present study that can be confidently used to calibrate enzyme divergence. The range of 15 - 20 million years (Sarich, 1977) was chosen, because most other estimated allozyme evolution rates fall within this range (Kim et al., 1976; Adest, 1977; Carlson

et al., 1978; Vawter et al., 1980; Wyles & Gorman, 1980). Avise (1977) found the rate of evolution to be decelerated in the rapidly speciating cyprinids, suggesting that the rate of allozyme evolution of cyprinids should not be faster than in other taxa. The estimated time since divergence of the species investigated in the present study seems more consistent with the theory of a Tertiary origin in the Cape Fold Mountains (Skelton, 1980, 1986, 1994) than a relatively recent Pliocene to Pleistocene origin (Barnard, 1943; Jubb, 1964; Jubb & Farquharson, 1965; Gaigher & Pot, 1973; Mulder, 1989).

B. anoplus was suggested as the sister group to the redfins (Skelton, 1980, 1986, 1988, 1994), but this is rejected by the current genetic results. The phylogenetic analysis of Naran (1997) and the present results suggest that the South African tetraploid serrated barbs and *Pseudobarbus* are sister groups and share a common tetraploidy event. The similar amount of diploidization occurring in *Pseudobarbus* (68.8 %) and the serrated barbs (62.5 %) investigated in the present study, is further evidence of a common tetraploid event and ancestry (Table 2.4). The possible common tetraploidy event in South African serrated barbs and *Pseudobarbus* seems to be between 12.5 and 18.5 million years old. This estimate is consistent with Naran (1997), who found an absence of multivalents within the meiotic spreads of the tetraploid *Pseudobarbus* and serrated South African *Barbus* spp. Absence of multivalents could indicate that the ploidy event is distant (Golubtsov & Krysanov, 1993). The almost complete diploidization in the hexaploid *B. aenus* 93.8% (Section 2.4; Table 2.4) also supports the view of Golubtsov & Krysanov (1993) that the ploidy event in the African hexaploid species is distant. The hexaploid *B. aenus* studied here, expressed less loci than the tetraploid species, suggesting that the hexaploidy event in this lineage is more ancient than the tetraploid event in the "tetraploid serrated - *Pseudobarbus*" lineage. The relationship of the "tetraploid serrated - *Pseudobarbus*" lineage with other *Barbus*

spp. remains speculative, and more outgroups will have to be investigated (Naran, 1997). The three serrated tetraploid barbs investigated here seems to be very closely related to each other and a smaller genetic distance was found among them (including the large *B. serra*), than between *P. phlegethon* from the Olifants and Doring Rivers. The differentiation among *P. phlegethon* from the Olifants River, *P. phlegethon* from the Doring River and *P. burchelli*, needs to be related to other *Pseudobarbus* spp. There is also a need to investigate the possibility of a monophyletic lineage of tetraploid serrated barbs in South Africa.

The pattern of genetic diversity differed between *B. calidus* and *P. phlegethon*. *B. calidus* had an overall, but uniform low diversity in the Olifants River System. *P. phlegethon* from the Olifants River has the highest genetic diversity (heterozygosity) measured for redfins in the Olifants River System (0.032), which is more characteristic of the mean of 0.051 - 0.054 expected for fish (Avise & Aquadro, 1982; Ward et al., 1992). In contrast, the *P. phlegethon* lineage from the Doring River showed virtually no variability, probably due to a historic bottleneck occurring in this lineage. The Olifants River lineage of *P. phlegethon* may have remained more stable and possibly larger for a longer time than *P. phlegethon* from the Doring River, hence the significantly higher allelic diversity, percentage of polymorphic loci and heterozygosity. River capture associated with a founder event (either from *P. burchelli* or *P. phlegethon* from the Olifants River) can explain the low heterozygosity in *P. phlegethon* from the Doring River. A historical bottleneck, possibly associated with the divergence from *B. calidus*, could also explain the absence of genetic variability found for *B. erubescens*. Alternatively, the bottleneck in the Doring populations of *P. phlegethon* and *B. erubescens* occurred after the isolation of these lineages. It is interesting to note that the Doring River carries much less water than the Olifants River and drains the rain shadow side of the Cedarberg Mountains, which probably makes it a

more variable system. This variability could have caused fragmentation in the past and may explain the low heterozygosity for *P. phlegethon* and *B. erubescens* in the Doring River drainage. The effect of fragmentation caused by bass is difficult to distinguish from historical events, but the smallest *B. calidus* population (Tra tra River) did show the lowest heterozygosity of the *B. calidus* populations. The Breekkrans population of *B. calidus* has a heterozygosity level comparable to the Olifants populations of *B. calidus*, probably due to a larger population size. The Breekkrans *B. calidus* population has not been restricted to the same extend by bass, as is the case in the Tra tra *B. calidus* population. *B. calidus* from the Doring River does not show the same loss of heterozygosity level as *P. phlegethon* from the same river. This is possible further evidence that *B. calidus* was able to maintain some gene flow in the past between these two drainages, whilst *P. phlegethon* remained isolated. According to Archie (1985), at least 40 loci should be sampled to have confidence in heterozygosity estimates and Leberg (1992) recommended that 16 – 25 polymorphic loci are needed to effectively detect bottlenecks in terms of heterozygosity. Allele diversity and the percentage of polymorphic loci are, however, better measures of bottlenecks (Leberg, 1992), but the present allozyme investigation yielded too few polymorphic loci to have confidence in these diversity patterns. Thus more variable markers will be needed to detect loss of diversity, especially in recently fragmented populations.

The low overall genetic diversity for all the species is also difficult to explain. Values often reported in the literature for the genus *Barbus* were generally higher (Mulder, 1989; Machordom et al., 1990; Engelbrecht & Van der Bank, 1994, 1996a, 1996b, 1997; Karakousis et al., 1995). Mulder (1989) found generally lower heterozygosity in large barbs from the Western Cape (*B. andrewi*, *B. serra* and *B. capensis*) compared to other large barbs in South Africa (e.g. 0.216 in *B. mattozi*, a serrated tropical barb). He explained the low heterozygosity in Western Cape species

as a result of fragmentation and inbreeding. Small South African diploid species of *Barbus* studied by Engelbrecht & Van der Bank (1994, 1996a, 1996b, 1997), showed lower heterozygosity than large South African *Barbus* (Mulder, 1989). The lower level of heterozygosity in small diploid barbs investigated by Engelbrecht & Van der Bank (1996a, 1996b, 1997), was explained as an association with smaller number of active loci due to diploidy compared to polyploidy in the fish investigated by Mulder (1989). Diploid species should, however, not have inherently lower heterozygosity levels compared to polyploid species, because heterozygosity estimates are not dependent on the number of active loci. Engelbrecht & Van der Bank (1994 &1996a) gave other reasons for low heterozygosity in diploid South African barbs as environmental degradation and isolation and small population size. Mulder (1989) explained high heterozygosity in large *Barbus* spp. as an indication of big population sizes associated with extensive migration. Similar to this, higher heterozygosity found by Karakousis et al. (1995) for *Barbus* spp. from Greece was explained by suggesting that glacial extensions did not have a great effect on these populations, allowing bigger and more stable population sizes. Generally lower heterozygosity was found in the present study for redfins compared to diploid small barbs (Engelbrecht & Van der Bank, 1994, 1996a, 1996b, 1997). In the light of these and Mulder's (1989) results, it is proposed that the lower heterozygosity in Western Cape barbs is due to historical events. Apart from recent loss of diversity due to isolation of some small populations, low heterozygosity in the Western Cape barbs may be associated with dispersal and founding events. Alternatively, fluctuations in climate since the Tertiary could have resulted in a historic cycle of fragmentation, followed by the expansion of suitable habitat. This may be especially true if these species are adapted for colder habitats and clear mountain streams. The latter explanation may be more consistent with Skelton's (1980, 1986, 1994) view of speciation through mainly vicariance, and the estimated time of divergence calculated for the species in the

present study. There may be a similar situation in European barbs where Berrebi et al. (1988) found low heterozygosity in French populations of *B. meridionalis* and *B. barbus*. Machordom et al. (1990) found higher heterozygosity for *B. meridionalis* populations in Spain. According to Persat & Berrebi (1990) this difference in heterozygosity might have been due to small populations sizes caused by historical glacial extension.

In conclusion, it seems probable that *P. phlegethon* from the Olifants River tributaries and *P. phlegethon* from the Doring River tributaries have been isolated from each other since the late Miocene. Although frequency differences were observed among most of the *B. calidus* populations, no genetic divergence was found between the Olifants and Doring River populations. It is proposed that *B. calidus* is better adapted to disperse through the main stream and most probably did so before the introduction of bass. *P. phlegethon* on the other hand, seems to prefer middle to lower reaches of tributary streams. The divergence between *B. calidus* and *B. erubescens* is relatively recent and characteristic of conspecific populations. It is estimated that the ancestor of the taxa investigated here may have been present in the Fold Mountains since at least the late Tertiary. The relationship between the tetraploid serrated barbs and *Pseudobarbus* spp. found here supports the results of Naran (1997) that these may be sister groups and may share a common and distant tetraploidy event.

CHAPTER 4

MANAGEMENT IMPLICATIONS OF IDENTIFYING CONSERVATION UNITS OF CEDARBERG REDFINS

4.1 INTRODUCTION

There is an urgent need to assess genetic diversity of native fishes of the Western Cape Province, as nine of the 13 species endemic to river systems of this province, are endangered or critically endangered (Baillie & Groombridge, 1996). This is mainly due to habitat destruction, water extraction and the introduction of alien fishes (Skelton, 1987; Skelton et al., 1991; Impson & Hamman, in prep.). The Cape Ichthyofauna is generally associated with the Cape Fold Mountains as well as the Cape Floristic Region, the smallest of the seven plant kingdoms of the world, most of which is incorporated within the boundaries of the Western Cape Province of South Africa. In the Cape Floristic Region, low productivity, as well as ancient and vicariant history of the Cape Fold Mountain rivers have contributed to the evolution of depauperate fish assemblages, with high levels of endemicity (Skelton, 1987; 1988) and largely uncertain biogeographic associations with the rest of Africa (Skelton, 1986, 1994). Rivers of the Cape Floristic Region are generally clear, perennial, acidic, and oligotrophic (Impson & Hamman, in prep.). These ecosystems have a low resilience to disturbance (Skelton, 1987). In the absence of exclusively piscivorous fishes, a group popularly referred to as redfin minnows, has in particular, evolved relatively inflexible life-history strategies (Skelton, 1987). Six of the eight redfins endemic to the Cape Floristic Region are endangered or critically endangered (Baillie & Groombridge, 1996).

The name "redfin" refers to a group of barbine minnows with bright red pigmentation on their fins, which is particularly evident during the breeding season. The present study will focus on three of these species, namely the Clanwilliam redfin (*Barbus calidus*), Twee River redfin (*Barbus erubescens*) and fiery redfin (*Pseudobarbus phlegethon*). When Skelton (1988) defined *Pseudobarbus*, *B. calidus* and *B. erubescens* were not included, despite the presence of red fins, mainly because they have a bony unbranched dorsal ray that is serrated. All *Pseudobarbus* have soft flexible dorsal rays (Skelton, 1988).

Pseudobarbus phlegethon, *B. calidus* and *B. erubescens* are endemic to the Olifants River System of the West Coast of South Africa (Jubb, 1965; Skelton, 1974, 1976, 1987, 1988). The Olifants River System has the highest endemicity of freshwater fish south of the Zambezi River System (Gaigher, 1973a), but because of human intervention, all eight of its endemic fishes are now threatened with extinction (Baillie & Groombridge, 1996). The Olifants River System is comprised of two main basins, drained by the Olifants and Doring Rivers. Central to these basins are the Cedarberg Mountains that form part of the Cape Fold Mountains and are composed mainly of Table Mountain sandstone with Bokkeveld marine sediments in the valleys (Rust, 1967; Theron, 1972). The vegetation type in the Cedarberg Mountains is typically Mountain Fynbos with Karoo vegetation elements penetrating some of the deep valleys (Acocks, 1988).

Populations of *P. phlegethon* and *B. calidus* are isolated in a few fragmented populations in tributary streams of the Olifants and Doring Rivers draining the Cedarberg Mountains (Fig. 3.2, 3.3 & 3.4). This reflects a fraction of their former distribution, as records exist of both *B. calidus* and *P. phlegethon* occurring in the main stream Olifants River (Harrison, 1938; Barnard, 1943; Section 3.1). The main cause of fragmentation of redfin populations in the

Olifants River System is predation by alien bass (*Micropterus salmoides*, *M. dolomieu* and *M. punctulatus*). Because of bass, both *P. phlegethon* and *B. calidus* are listed as endangered (Baillie & Groombridge, 1996). Effective conservation management of *P. phlegethon* and *B. calidus* populations will benefit all the other threatened Cedarberg fishes occurring with them or breed in these mountain tributaries. *Barbus erubescens* is critically endangered (Baillie & Groombridge, 1996) because of its restricted distribution, the introduction of alien fishes and agricultural activities in the Twee River catchment (a tributary of the Doring River) to which it is endemic (Mariott, 1998). *Barbus erubescens* was included in the present genetic study to investigate their apparently close relationship to *B. calidus* (Skelton, 1974; 1988) and to re-evaluate previous recommendations on their conservation (Mariott, 1998; Skelton, 1987).

Cape Nature Conservation (CNC), the statutory provincial conservation agency for the Western Cape Province, changed its policies on freshwater fish conservation during the 1970's - 1980's by removing protective measures for alien fish species in exchange for indigenous ones (Gaigher et. al., 1980; Scott, 1982). The initial focus of the conservation effort was based on captive breeding programs and restocking into suitable habitat (Gaigher, 1973b; Scott, 1982; Hey, 1995). The Clanwilliam hatchery on the Olifants River was built especially for captive breeding and restocking of endemic Olifants River fishes. Mainly the Clanwilliam yellowfish (*Barbus capensis*) was successfully bred here and re-introduced across much of the Olifants River System (Scott, 1982). This was done without prior knowledge of their genetic structure. None of the redfin minnows were bred and restocked.

Concern within CNC over the loss of genetic diversity, often associated with hatchery based introduction programs (Leary et al., 1993) and human induced fragmentation of natural populations (e.g. Quattro & Vrijenhoek, 1989), led them to support conservation genetic

programs on Western Cape fishes (Impson, pers. comm.). The main focus of these programs, including the present study, is to describe genetic diversity, mainly through the identification of conservation units. It is hoped that protection of these conservation units would ensure evolutionary potential (e.g. Leberg & Vrijenhoek, 1994) of Western Cape fishes, which is in line with CNC's policy of preserving biodiversity. Studies by Bloomer & Impson (in press) and Waters & Cambray (1997) indicated that formal taxonomy does not adequately reflect genetic diversity of Western Cape fishes in need of protection. The identification of conservation units would allow conservation agencies concerned with the Olifants River System to prioritize their efforts (see Moritz, 1994), as resources are limited (Impson & Hamman, in prep.).

Surveys were conducted during 1998 and 1999 in collaboration with CNC and Roger Bills (J. L. B. Smith Institute of Ichthyology) as part of his WWF project on *Austroglanis* (Bills, 1999) in the Olifants River System, Western Cape. The main objectives of these surveys were to: (1) identify all the existing populations of *P. phlegethon* and *B. calidus*, (2) collect specimens of *P. phlegethon*, *B. calidus* and *B. erubescens* for a genetic investigation (Chapter 3) and (3) assess threats to Cedarberg redfins. Threats were assessed to identify the underlying reason for declining redfin populations (see Caughley, 1994). Fish were surveyed by snorkeling, as the mountain streams in which they occur are clear and shallow. Fish were mainly collected using small seine nets. Methods followed to resolve allozyme loci have been described in Sections 2.2.3 and 3.2.3. Voucher specimens from each locality were collected, fixed in formalin, and deposited in the National Fish Collection of the J. L. B. Smith Institute in Grahamstown (Table 3.2). During surveys several new distribution localities were recorded. Important for the conservation of redfins, was the discovery of two new populations of *B. calidus* in the Driehoeks (RUSI 59615) and Eselbank Rivers (RUSI 59599, 59603) (Fig. 3.4),

and one new population of *P. phlegethon* in the Breekkrans River (RUSI 58411) (Fig. 3.3) (Bills, 1999). New distribution records were also documented for alien species during these surveys (Bills, 1999). All of the known redfin populations in the Olifants River System were included in the allozyme electrophoretic investigation (Table 3.1). Reference will be made to the population genetic results of Section 3.3.

The purpose of the present chapter is to: (1) Identify Evolutionarily Significant Units (ESU's) and Management Units (MU's) of Cedarberg redfins, (2) identify threats to these conservation units, and (3) make recommendations to assist effective conservation management in future.

4.2 IDENTIFYING UNITS OF CONSERVATION MANAGEMENT

4.2.1 Genetic analysis

Genetic results of Section 3.3 were used to identify units of conservation management. This was specifically based on the electrophoretic analysis of the following enzymes (enzyme commission number in brackets): Aspartate aminotransferase (2.6.1.1); Adenylate kinase (2.7.4.3); Creatine kinase (2.7.3.2); Esterase (3.1.1.1); Glucose-6-phosphate isomerase; (5.3.1.9); Lactate dehydrogenase (1.1.1.27); Malate dehydrogenase (1.1.1.37); Malate dehydrogenase (NADP+) (1.1.1.40); Octopine dehydrogenase (1.5.1.11); Leucyl-glycyl-glycyl tripeptidase; Leucyl-tyrosine dipeptidase; Phosphogluconate dehydrogenase (1.1.1.44); Phosphoglucomutase (5.4.2.2). These enzymes yielded 27 loci for *B. calidus* and *B. erubescens* and 26 loci for *P. phlegethon*. Only two loci each for *B. calidus* (Phosphoglucomutase-1 and -2) and *P. phlegethon* (Glucose-6-phosphate isomerase and

Leucyl-tyrosine dipeptidase-4) proved to be polymorphic (0.95 criterion). No polymorphic loci were found for *B. erubescens*.

Moritz (1994) suggested that Evolutionarily Significant Units (ESU's) should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci. ESU's should thus be lineages that have been historically isolated and is likely to have a unique genetic potential (Moritz, 1994). According to Moritz (1994), Management Units (MU's) would then be defined as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci and form the logical basis of population monitoring and demographic studies. MU's thus represents sub-populations that are currently demographically independent with low levels of gene flow among them.

ESU's were identified in the present study by assessing the genetic divergence among redfin lineages. This was based on Nei's (1978) unbiased genetic distance (Table 3.8; Fig. 3.5), the occurrence of fixed allele differences (Table 3.4 & 3.5), the genetic structure of populations (Table 3.10) and evaluating morphological results of Skelton (1974, 1988). Identification of MU's was based on frequency differences of polymorphic allozyme loci. Heterogeneity of allele frequencies of allozyme markers gives an indication of low gene flow and should give an indication of functionally independent populations that Moritz (1994) defined as MU's. Chi-square analyses of allele frequency data from Table 3.11 and 3.12, based on the above-mentioned polymorphic loci, were used to construct maps of hypothesized gene flow among *P. phlegethon* (Fig. 4.1) and *B. calidus* (Fig. 4.2) populations in the Olifants River System. Reference will be made to the genetic diversity of Cedarberg redfins (Table 3.7), the dendrogram in Fig. 3.5, and the cladograms in Fig. 3.6 and 3.7.

4.2.2 *Pseudobarbus* Evolutionarily Significant Units

The divergence between the five populations of *P. phlegethon* from the Olifants River and the two populations of *P. phlegethon* from the Doring River is illustrated in Fig. 3.5 – 3.7. Enzymes AAT-1, LDH-1, MDH-1, MDH-3, MDH-4, PGM-1 and PGM-2 showed fixed differences between *P. phlegethon* populations from the Olifants and Doring Rivers. Nei's (1978) distance of 0.355 between Olifants River populations of *P. phlegethon* and Doring River populations of *P. phlegethon* suggests a relatively long time of isolation and unique evolutionary history between these two groups, possibly since the late-Miocene (Table 3.15). Most of the genetic substructuring (87 %) in *P. phlegethon* can be explained by variation between Olifants and Doring River populations, whilst only 8 % can be explained by variation among populations within these drainages (Table 3.10), leaving no evidence of a genetic cline between these two taxa. Skelton (1980) noted variation in certain meristic characters between *P. phlegethon* specimens from the Driehoeks River and those from Olifants River catchment, but too few samples were available to make broad taxonomic inferences.

In the light of the allozyme divergence found, it is proposed that *P. phlegethon* of the Doring River catchment and *P. phlegethon* of the Olifants River catchment should be recognized as separate ESU's. Olifants River *P. phlegethon* ESU is known to occur in the Oudste, Thee, Noordhoeks, Boskloof and Rondegat Rivers whilst the Doring River *P. phlegethon* ESU only occur in the Driehoeks and Breekkrans Rivers, based on recent surveys (Fig. 3.2 & 3.3).

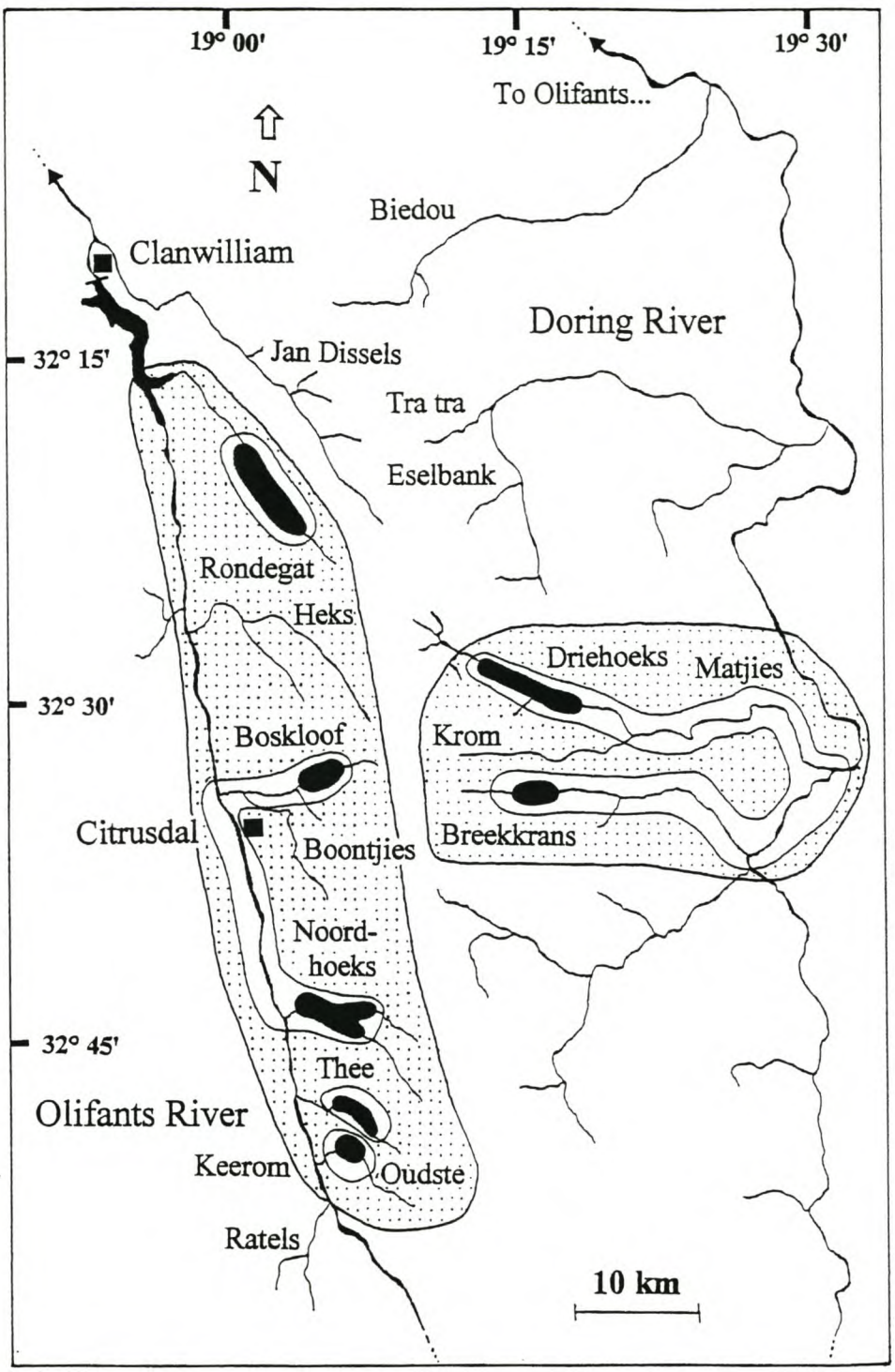

Fig. 4.1 Map of the Olifants River System showing hypothesized gene flow among populations of *Pseudobarbus phlegethon*. Current Management Units (CMU's) are given as solid areas, Historical Management Units (HMU's) are unshaded and Evolutionarily Significant Units (ESU's) are given as dotted areas.

4.2.3 Serrated redfin Evolutionarily Significant Units

The genetic distance (Nei, 1978) of 0.063 between *B. calidus* and *B. erubescens* is characteristic of below species level divergence (see Avise, 1975). MDHP-1, however, is a diagnostic locus between *B. erubescens* and *B. calidus*, suggesting that these two taxa could be recognized as separate ESU's. The two taxa also differ from each other morphologically. *Barbus erubescens* can be distinguished from *B. calidus* mainly in having weak or no serration in the last unbranched dorsal ray, having no prominent markings on the dorsal surface, and males developing an overall reddish hue in the breeding season (not observed in *B. calidus*) (Skelton, 1974). Redfins are extinct in the area between the Twee and Breekkrans Rivers (Mariott, 1998; Skelton, 1974). Alien bass now dominates this area where possible historical hybridization could have occurred between *B. calidus* and *B. erubescens* (Skelton, 1974), leaving no evidence of a genetic cline. Differentiation between *B. calidus* and *B. erubescens* accounts for 42 % of the genetic structuring, whilst 17 % can be explained for by variation within these two species. Allozyme results seem to warrant separate ESU status for these two species.

Based on recent surveys, *B. calidus* populations are known to occur in the Ratels, Oudste, Thee, Noordhoeks, Boskloof, Rondegat, Biedou, Tra tra, Eselbank, Driehoeks and Breekkrans Rivers. A few individuals were also found in the lower Boontjies. *Barbus erubescens* is confined to the Twee River catchment (Mariott, 1998). There is a need to establish more populations of this ESU's because of threats outlined in Section 4.3.

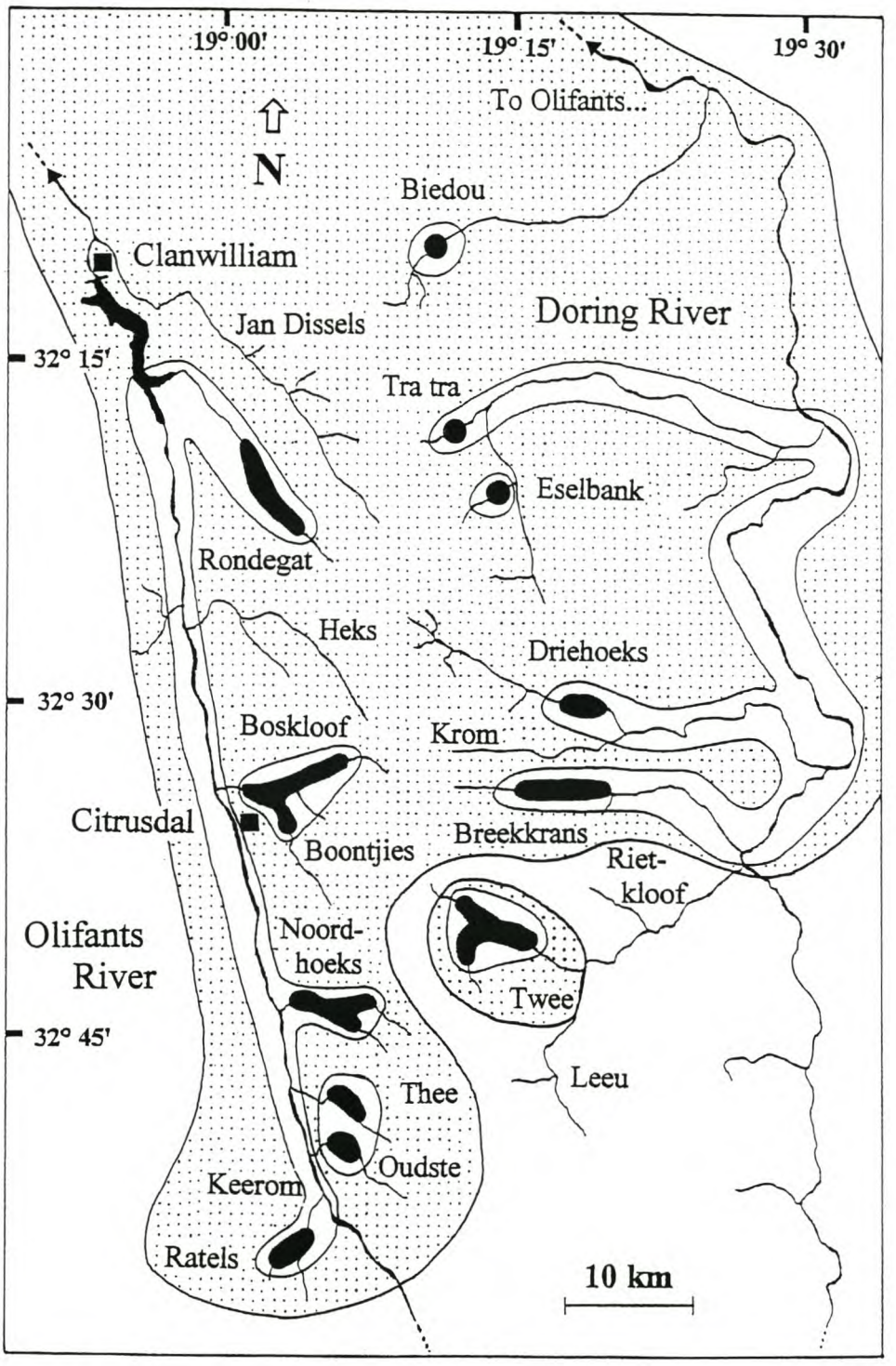

Fig. 4.2 Map of the Olifants River System showing hypothesized gene flow among populations of "serrated redfins" (*Barbus erubescens* and *Barbus calidus*). Current Management Units (CMU's) are given as solid areas, Historical Management Units (HMU's) are unshaded and Evolutionarily Significant Units (ESU's) are given as dotted areas.

4.2.4 Management Units

Gene flow among redfin populations in the Olifants River System has been influenced by "historical" aspects like their habitat preference, ability to disperse throughout the main stream and the occurrence of natural barriers to migration like waterfalls. "Recent" aspects like the building of artificial barriers (dams and weirs) and the introduction of alien fishes have prevented gene flow between previously connected populations of redfins. To interpret the management units, these aspects must be kept in mind. Two types of management units are defined here to prevent confusion between recent and historical events. Current management units (CMU's) would be those populations that are currently isolated because of recent events like the building of dams and weirs and the introduction of alien fishes by man. Qualification of populations as CMU's depends on whether these populations have a large enough effective population size to prevent inbreeding depression, without having to be saved from metapopulations, much like the ecological concept of minimum viable populations (MVP's) (Caughley, 1994). These would typically be larger than 50 breeding individuals. CMU's may still be in danger of general inbreeding associated with the erosion of genetic diversity because of small population size (Caughley, 1994). Measures may therefore be necessary to increase the size of CMU's. CMU's should be the basis for conservation management in terms of monitoring, demographic studies, increasing population size and building weirs to secure populations (Section 4.4).

Historical management units (HMU's) are defined as those units that had enough gene flow among them to prevent heterogeneity of allele frequencies, before recent fragmentation occurred. HMU's would typically be larger units than CMU's and more ideal for the prevention of loss of genetic diversity. HMU's should be the basis on which decisions are

made to establish new populations, as these units represent historical gene flow patterns. When a choice has to be made between HMU's for establishment of new populations, it should be judged on the following criteria:

(1) How many CMU's of the HMU exists?

(2) Which is the most threatened HMU?

(3) Does this HMU have unique alleles?

(4) Which HMU is geographically the closest to the site?

(5) Which HMU has the highest genetic diversity?

(6) Will a combination of HMU's increase genetic diversity and is such a combination necessary?

As many HMU's as possible should be represented in the establishment of new populations, as more genetic diversity may be secured this way than promoting a single HMU. CMU's should not be mixed where they constitute artificially and recently fragmented HMU's, unless the latter became too small for a long period of time and suffer from inbreeding depression (see Caughley, 1994).

Captive breeding programs are seen as one of the most powerful tools for rescuing a species that has declined to very low numbers (Caughley, 1994). Loss of genetic diversity (e.g. Leary et al., 1993; Quattro & Vrijenhoek, 1989; Briscoe et al., 1992) and artificial selection in a hatchery environment that eliminates adaptive gene complexes (Waples & Teel, 1990; Garcia de Leániz, et al., 1989), however, have been associated with captive breeding of fishes. Provided CMU's remain large enough, taking stock from these MU's to establish new populations, would avoid many of the pitfalls of captive breeding. Restocking from natural populations would also be a cheaper option. Enough individuals must be translocated in

rehabilitation efforts to avoid a bottleneck effect (i.e. > 50). Additional seasonal introductions should be considered to establish these refuge populations, especially where the founder stock is small, where the source population is sensitive and if mainly juveniles are available.

The purpose of CMU's and HMU's is to allow more freedom in describing genetic diversity in fragmented populations and to make the MU concept more applicable to the current isolated and fragmented status of redfin populations in the Olifants River System.

4.2.4.1 Doring River *Pseudobarbus* Management Units

The Doring River *P. phlegethon* ESU is also a HMU. Although there is no detectable allozyme divergence between them, the Breekkrans and Driehoeks populations of *P. phlegethon* form two separate CMU's, because they are currently isolated from each other.

The Breekkrans and Driehoeks Rivers are also very important refugia for Doring River populations of *B. capensis*, *B. anoplus*, *B. calidus*, *B. serra*, *A. gilli* and *G. zebratus*. Apart from *B. erubescens* (see Mariott, 1998) and possibly *L. seeberi* (see Skelton, 1998; Bills, 1999), Doring River *P. phlegethon* is the most threatened ESU in the Olifants River System.

4.2.4.2 Olifants River *Pseudobarbus* Management Units

Four management units were found within the Olifants River *P. phlegethon* ESU (Fig. 4.1). The *P. phlegethon* population from the Rondegat River forms a MU with a waterfall at the lower end of their distribution, separating them from the other MU's of this ESU. This waterfall has stopped the intrusion of bass and could have restricted historical gene flow. The

Rondegat MU is thus a CMU and possibly also a HMU. The Boskloof and Noordhoeks Rivers each forms separate CMU's and together a single HMU, separate from the Thee and Oudste Rivers. There was also heterogeneity of allozyme alleles between the Oudste and Thee Rivers, suggesting that these two are separate CMU's and HMU's. *Pseudobarbus phlegethon* has, however, been recorded from Keerom (main stream Olifants River) before the introduction of *M. dolomieu* in 1945 (SAM 215/216, SAM 2012-2015; see Harrison, 1938; see also Barnard, 1943), making it difficult to interpret these "Upper Olifants MU's". The heterogeneity of allele frequencies that is now found, may as a result of genetic drift due to fragmentation caused by bass. Sampling of relatively few individuals may also have played a role.

The Boskloof, Noordhoeks, Thee and Oudste Rivers have a very high diversity of indigenous fish, making this one of the most important areas in the Olifants River System for conservation of biodiversity. *P. phlegethon* occurs with *B. calidus*, *B. capensis*, *A. gilli*, *A. barnardi* and *G. zebratus* in these streams.

4.2.4.3 Serrated redfin Management Units

Gene flow appears to have occurred among populations as far apart as the Rondegat and Ratels Rivers in the Olifants River catchment and the Tra tra and Breekkrans in the Doring River catchment. The apparent gene flow between the Biedou and Ratels may be incidental. It is not known whether the isolation of the Boskloof and Thee-Oudste MU's from the main MU of the Olifants River is due to sampling, fragmentation caused by bass, or low historical gene flow. The same can be said about the Eselbank. Its apparently small population size suggests genetic drift. The Breekkrans, Driehoeks, Tra tra and Eselbank Rivers each forms a

CMU and together probably form a HMU. The Biedou, Boskloof and Thee-Oudste should probably be recognized as three separate CMU's and HMU's. The Ratels, Noordhoeks and Rondegat Rivers each forms a separate CMU and together a single HMU.

Barbus erubescens samples were only collected from the lower Twee River, thus no inferences on MU's can be made for this species.

4.3 THREATS TO REDFIN CONSERVATION UNITS

Threats to the survival of redfins were identified during the surveys, from the available literature and evaluation of database records at the South African Museum (SAM), Albany Museum (AMG) and J. L. B. Smith Institute of Ichthyology (RUSI).

4.3.1 Aliens

Largemouth bass (*Micropterus salmoides*) was first introduced in the Olifants River System 1933 (Harrison, 1938), followed by Smallmouth bass (*M. dolomieu*) and spotted bass (*M. punctulatus*) in 1943 and 1945 (Harrison, 1948, 1952, 1953), to improve fishing. There is no previous record of sympatry between bass and redfin populations in the Olifants River System (Bills, 1999; Van Rensburg, 1966, Gaigher, 1973b). Repeated snorkeling surveys of contact zones between bass and redfin populations in the Olifants River System, also failed to record them in sympatry (Bills, 1999). The redfins are often isolated from the intrusion of bass by small natural waterfalls. Field observations thus suggest an entirely parapatric distribution between bass and redfins and suggest that bass displace the redfins through predation of juvenile and adult individuals.

Micropterus salmoides occurs within the distribution of *P. phlegethon* in the Driehoeks River, placing this population at serious risk of extinction. It is not known why *M. salmoides* has not moved downstream in the Driehoeks River. *Micropterus salmoides* also occurs in the middle reaches of the Krom River, where it probably contributed to the extinction of some of the indigenous fishes in this stream.

From surveys and historical records, *M. dolomieu* apparently caused the extinction of *B. calidus* and Olifants River *P. phlegethon* ESU in the main stream Olifants River (SAM 215/216, SAM 2012-2015; see Harrison, 1938; see also Barnard, 1943) and tributaries of the Olifants River, namely the Jan Dissels (AMG 1850, 9991 & 11603) and Heks Rivers. No historical record exists for *B. calidus* and *P. phlegethon* in the Heks River, but the occurrence of *Austroglanis gilli* and *A. barnardi*, does suggest that they occurred here. *Pseudobarbus phlegethon* seems to prefer middle to lower reaches of mountain streams (Bills, 1999; Chapter 3). For this reason, *M. dolomieu* may have caused their extinction (and not *B. calidus*) in the Ratels (AMG 7672) and Boontjies Rivers (SAM 22484 - composite sample of SAM 19003 and SAM 18767). This may also have occurred in the Eselbank, Tra tra and Biedou Rivers, but no historical records exist to suggest that *P. phlegethon* occurred in the latter three streams. *B. calidus* is able to occupy headwater reaches of mountain streams. Differential niche preference and dispersability between *P. phlegethon* and *B. calidus* may explain why less populations of the former than the latter survived in the Olifants River System (Bills, 1999; Chapter 3). On the Doring River side, Smallmouth bass may have caused the extinction of main stream *B. calidus* and *P. phlegethon*. The occurrence of *M. dolomieu* marks the downstream limit of distribution of *B. calidus* in the Ratels, Rondegat, Biedou, Tra tra, Eselbank and Driehoeks Rivers, and possibly the Breekkrans River. The occurrence of *M. dolomieu* marks the downstream limit of distribution of *B. erubescens* in the Twee River, as

well as *P. phlegethon* in the Rondegat and possibly the Breekkrans Rivers. Recently *M. punctulatus* has also been recorded in mountain tributaries (Bills, 1999). The destruction caused by *M. dolomieu* and *M. punctulatus* is due to their preference for flowing water and their ability to enter small mountain tributaries.

Bluegill sunfish (*Lepomis macrochirus*), introduced as fodder for bass (Harrison, 1954), was well established in the main stream of the Olifants and Doring Rivers by 1963 to 1964 (Van Rensburg, 1966). *Lepomis macrochirus* was recently introduced above the major natural barrier in the Middeldeur stream (Twee River System) and now has the potential to dominate all the areas where *B. erubescens* is found (Bills pers. comm.). *Lepomis macrochirus* was also recently recorded from farm dams in the Krom River valley (Bills, 1999), making it more difficult to rehabilitate this river (see Section 4.4.2.3 & 4.4.5).

The spreading of centrarchids (Bass and Bluegill) in the Olifants River System is also associated with biocontrol as they are effective bio-agents against aquatic insects and frogs that block the filters of water pumps in farm dams used for irrigation.

In the Krom River, redfins are extinct possibly because centrarchids dominate the lower reaches, whilst Rainbow trout (*Oncorhynchus mykiss*) dominates the colder headwater zones of this stream. The occurrence of *Galaxias zebratus* and *Austroglanis gilli* in this stream, does suggest that redfins occurred here, as there are no streams in the Olifants River System where these species occur without redfins, in the absence of alien predatory fishes. *O. mykiss* occurs in low numbers in the middle reaches of the Twee River, but according to Mariott (1998) its impact is possibly not severe.

Banded tilapia (*Tilapia sparrmanii*), also introduced as fodder for bass (Brand, 1954), was established in the Olifants River System by the 1960's (Jubb, 1961; 1965). Gaigher (1981) suggested that *Tilapia sparrmanii* has contributed to the decline of indigenous fish in the Olifants River System, through predation on juveniles and competition for food resources. *Tilapia sparrmanii* has recently been recorded from the Noordhoeks River (Bills, 1999). Clanwilliam yellowfish (*B. capensis*), although native to the Olifants River System, was introduced in the Twee River above their natural distribution as part of the breeding program of the Clanwilliam hatchery. The Cape Kurper (*Sandelia capensis*) was also introduced into the Twee River (Hamman et al., 1984) and together with *Barbus capensis* may compete for food with *B. erubescens* and predate on juvenile *B. erubescens* (Mariott, 1998).

The spread of alien trees (especially *Acacia* spp.) is a problem in riparian zones where they destabilize river banks, change nutrient profiles in rivers and utilize large volumes of water and has probably had an impact in the lower Rondegat on *B. calidus* and *P. phlegethon* numbers.

4.3.2 Habitat destruction and fragmentation

The Olifants River System supports an extensive irrigation scheme with many dams, weirs and channels (Nieuwoudt, 1962; Fourie, 1976). Orchards of mainly citrus are still expanding (Nieuwoudt, 1962; Mariott, 1998; Impson & Hamman, in prep.). Apart from preventing the free movement of indigenous fishes (Gaigher, 1973b; Gaigher et al., 1980; Scott, 1982), dams and weirs are refugia for alien fishes. In some cases, however, weirs may have stopped the intrusion of bass into tributary streams. Pipelines, and especially channels, provide a means for alien species to bypass natural barriers, possibly explaining the intrusion of bass into the

lower Eselbank (Bills, 1999), *Tilapia sparrmanii* in the Noordhoeks River and *Lepomis macrochirus* in the Twee River (Bills, pers. comm.). Bulldozing of rivers for flood control to protect orchards planted within floodlines is destroying habitat and increases erosion and sedimentation. Excessive or total water extraction associated with bulldozing of riverbeds and subsequent destruction of habitat occurs in the lower reaches of the Oudste, Thee, Noordhoeks, and Boskloof Rivers. Excessive water extraction may have an impact on redfins in the Twee (Mariott, 1998), Eselbank and Tra tra Rivers.

The extensive use of fertilizers increases nutrient levels and productivity, changing the normally oligotrophic streams into a more eutrophic state in all the streams where agricultural activities are prevalent. This may assist the establishment of alien species, as more food is available to both omnivores (e.g. *Tilapia sparrmanii*) and carnivores (e.g. Black Bass). The use of agricultural pesticides can harm non-target organisms like indigenous fish, and has been suggested as an important reason of the decline of *B. erubescens* (Mariott, 1998). It is difficult to assess the direct impact of chemical pollution and sedimentation on the redfins, as they are usually part of multiple impacts along middle to lower reaches of tributary streams. Pollution (agro-chemicals and sewage) is a threat near human settlements and farms for redfin populations in the Ratels, Boontjies, Boskloof, Rondegat, Biedou, Driehoeks and Twee Rivers. Mariott (1998) identified the extensive use of pesticides in the Twee River valley as a major threat to *B. erubescens*. Bills (1999) suggested that copper based compounds and insecticides pose a serious threat to indigenous fish in the Olifants River System and recommended that a specialized study should be done on this issue. Sedimentation has been noted as a threat to *B. erubescens* (Mariott, 1998) and certainly has a negative impact on the distribution of *Austroglanis* spp. in the Olifants River System (Bills, 1999).

4.3.4 Small population size

Mariott (1998) estimated that 4100 mature *B. erubescens* occur in the Twee River. Some of the other redfin populations of the Olifants River System, like the Driehoeks and Oudste *P. phlegethon* and the Eselbank, Tra tra and Biedou, Boontjies and Oudste *B. calidus* populations, may have much smaller numbers of mature individuals, evident from their restricted distribution found in surveys during 1998-1999. The size of the Breekkrans population of *P. phlegethon* is also unknown. The density of *P. phlegethon* from the Driehoeks and Breekkrans Rivers observed in the present study was low. Very few *B. calidus* individuals were also observed in the lower Boontjies River.

Much controversy exists on the effective size of a population that must be maintained to prevent loss of genetic diversity (Lynch & Lande, 1988; Billington, 1991; Frankham & Franklin, 1998; Franklin & Frankham, 1998). From the number of mature individuals estimated by Mariott (1998) and tentative observations of other small redfin populations, it seems as if redfin populations in the Olifants River System are not in immediate danger of inbreeding depression. The mentioned small redfin populations are, however, possibly in danger of general inbreeding that leads to erosion of genetic variability due to reduction in population size and the persistence of low numbers as described Caughley (1994). These populations are possibly also in danger of extinction because of demographic instability (Caughley, 1994), due to their restricted and in some cases low density distribution.

4.4. RECOMMENDATIONS

It is believed that Cedarberg redfins will only be safe from extinction if: (1) Conservation agencies of the Olifants River System recognize the different conservation units of redfins before recovery programs are devised; (2) the main reason for their decline, namely the intrusion of centrarchids, is addressed; (3) new populations of critically endangered lineages are established within the appropriate region (ESU); (4) the size of many of the populations is increased; (5) existing populations are secured; and (6) more research, dedicated monitoring, sampling and surveys are done.

Specific recommendations will focus on these six aspects and will be listed in order of highest to lowest priority.

4.4.1 Conservation units

4.4.1.1 Four ESU's of Cedarberg redfins should be recognized, namely *B. calidus*, *B. erubescens*, Olifants River populations of *P. phlegethon* and Doring River populations of *P. phlegethon*. Translocation of *P. phlegethon* between the Olifants and Doring River catchments and "serrated redfins" between the Twee River catchment and the rest of the Olifants River System should be prevented. Hybridization between *B. calidus* and *B. erubescens* and between *P. phlegethon* populations of the Olifants and Doring River is highly likely (Chapter 3).

4.4.1.2 Priorities for conservation effort is firstly *B. erubescens* and Doring River populations of *P. phlegethon*, secondly Olifants River populations of *P. phlegethon* and lastly *B. calidus*. There is a need to secure the Doring River *P. phlegethon* ESU populations and to establish new populations of this ESU as well as *B. erubescens*.

4.4.1.3 Two types of management units should be recognized, namely "historical management units" (HMU's) and "current management units" (CMU's). HMU's should form the basis on which decisions are made on the establishment of new populations (Section 4.4.2). CMU's should be the basis for population monitoring for loss of genetic diversity and the effect of demographic and ecological instability (Section 4.4.6). HMU's and CMU's that is recommended are shown in Fig. 4.1 and

4.4.1.4 If populations qualify as CMU's, translocation of individuals to prevent loss of genetic diversity should not be considered, as local adaptations might be lost (see Lesica & Allendorf, 1992). The size of CMU's should rather be increased (Section 4.4.3) as an immediate measure to prevent loss of genetic diversity.

4.4.2 Establish new populations

4.4.2.1 Farm dams in the Twee River catchment should be stocked with *B. erubescens*, as long as the dams do not allow entry into sensitive areas for indigenous aquatic invertebrates. Cover vegetation (preferably indigenous) must be provided.

4.4.2.2 The establishment of additional *B. erubescens* populations, above natural barriers, should be considered. The loss of possible endemic invertebrate fauna must be avoided.

4.4.2.3 The Krom River should be cleared of exotic fish species and stocked with Doring River *P. phlegethon* ESU. It should be considered not to stock this stream with *B.*

calidus, *B. serra* and *B. capensis* as well, as enough populations of these fish occur elsewhere and they might impede the establishment of *P. phlegethon*. Other options to secure Doring River *P. phlegethon* ESU may have to be considered (recommendation 4.4.2.4).

4.4.2.4 If clearing of the lower reaches of the Eselbank, Tra tra and Biedou Rivers of bass and *L. macrochirus* can be achieved, the introduction of Doring River *P. phlegethon* ESU into these areas, below the natural barrier that is stopping bass from moving upstream, should be considered. Alternatively, the Eselbank, Tra tra and Biedou Rivers can be stocked with Doring River *P. phlegethon* ESU within the distribution of the *B. calidus* populations. Because Doring River *P. phlegethon* ESU possibly never occurred in these tributaries, this option should only be considered if other options fail.

4.4.2.5 Irradication of bass, in otherwise suitable environments, should be done in the lower Ratels, Boontjies, Heks and Jan Dissels River and additional populations of Olifants River *P. phlegethon* ESU should be established in these streams. The lower Ratels should be restocked with *P. phlegethon* from the Oudste and/or Thee River, depending or further genetic investigation of more variable molecular techniques than allozymes. The lower Boontjies and Heks should be restocked with *P. phlegethon* from the Boskloof or Noordhoeks Rivers. The Jan Dissels River should be restocked with *P. phlegethon* from the Rondegat River.

4.4.2.6 To establish additional populations of *B. calidus*, irradication of bass in otherwise suitable environments should be done in the Heks and Jan Dissels Rivers. The Heks River should be stocked with *B. calidus* from the Boskloof or Rondegat Rivers (depending on genetic diversity studies with more variable techniques). If the Boontjies *B. calidus* population goes extinct, it should also be stocked with Boskloof

B. calidus. The Jan Dissels should be restocked with *B. calidus* from the Rondegat River. If it is decided that *B. calidus* would not adversely affect *P. phlegethon* in the Krom River (recommendation 4.4.2.3), then this stream should be stocked with the more genetically diverse Breekkrans *B. calidus*.

4.4.3 Increase population size

4.4.3.1 *Micropterus salmoides* in the Driehoeks River should be irradicated to increase the size of the

Doring River *P. phlegethon* ESU population in this stream.

4.4.3.2 Bass must be irradicated in the lower Breekkrans River and the Doring River *P. phlegethon* ESU population in this stream should be secured from re-intrusion by bass by building a weir.

4.4.3.3 The feasibility of poisoning of the lower reaches of the Twee River where bass occur (Mariott, 1998) should be investigated. As many indigenous fish as possible can be recovered before poisoning.

4.4.3.4 Irradication of alien fishes and restoration of the lower reaches of the Oudste, Thee, Noordhoeks, Boskloof and Rondegat Rivers should be done to increase the size of Olifants River *P. phlegethon* ESU populations in these streams.

4.4.3.5 Restoration and irradication of bass in the lower reaches of the Ratels, Boskloof, Boontjies, Rondegat, Biedou, Tra tra, Eselbank, Driehoeks and Breekkrans Rivers should be done to increase the size of *B. calidus* populations in these streams.

4.4.4 Secure existing populations

4.4.4.1 Permanent weirs should be constructed on the lower reaches of the Noordhoeks, Thee, Oudste and possibly the Eselbank and Breekkrans Rivers, to prevent the intrusion of alien fishes.

4.4.4.2 The Maatjies River and Twee River catchments should be declared national heritage sites (see also Mariott, 1998).

4.4.4.3 The following streams should be formally protected for Olifants River *P. phlegethon* ESU: Oudste, Thee, Noordhoeks, Boskloof and Rondegat. Particular attention should be given to the Boskloof, Noordhoeks, Thee and Oudste Rivers, as they host large populations of various threatened fishes.

4.4.4.4 The Ratels, Oudste, Thee, Noordhoeks, Boskloof, Rondegat, Biedou, Tra tra, Eselbank, Driehoeks and Breekkrans Rivers, should receive formal protection for *B. calidus*.

4.4.4.5 CNC should enforce existing laws to prevent unsustainable water extraction (DWAF, 1997), unwanted translocation of fish (illegal without a permit from CNC), and the structural modification of streams or rivers in sensitive areas (South Africa's Environmental Conservation Act No. 73 of 1989).

4.4.4.6 Conservation agencies should also be made aware of the danger of introgression between closely related species in management planning, for example, inter-basin transfer schemes and stocking of indigenous fish for angling.

4.4.4.7 The local community and visitors must be made aware of the natural heritage of endemic fishes of the Olifants River System and the threat to them, especially the translocation of fish.

4.4.4.8 Land management and future development should be better-planned (see Gaigher et al., 1980).

4.4.5 Suggestions to irradicate aliens

4.4.5.1 Stream invaded by bass can be sectioned off with temporary weirs. It is hoped that winter floods will wash bass downstream and that they would not be able to return in spring and summer due to the weirs.

4.4.5.2 Spearfishing and conventional fishing of bass can be effective in small streams (e.g. Skelton, 1993), especially in combination with recommendation 1.

4.4.5.3 Netting with fine mesh nets should be done with the help of divers that can ensure that the net covers all the areas in a pool. Temporary clearing of structures in the pool and vegetation on the banks of the river should be considered to facilitate netting. Temporary weirs should be built to prevent the re-invasion of alien fishes.

4.4.5.4 Integrated research, survey, monitoring and impact assessment program is needed to investigate the feasibility of using fish poisons to irradicate bass where they do not occur with indigenous organisms of conservation concern (see Meffe, 1983).

4.4.5.5 Alien trees should be irradicated in the lower Rondegat River.

4.4.6 Future field-work and research

4.4.6.1 There is a need to assess the sizes of the Driehoeks and Breekkrans *P. phlegethon* populations and to establish how far bass has intruded into the Breekkrans River.

4.4.6.2 Estimates of size of redfin populations should be done, particularly the Doring River *P. phlegethon* ESU populations. This would give an indication of the density of populations and their susceptibility to genetic inbreeding and demographic instability.

4.4.6.3 Monitoring should be done on the establishment of alien fish populations, particularly *Lepomis macrochirus*, *Barbus capensis* and *Sandelia capensis* in the Twee River and *Tilapia sparrmanii* in the Noordhoeks River, as well as bass in the Boontjies, Noordhoeks, Thee and Oudste Rivers, as well as the Maatjies River catchment. Monitoring of the movement of *M. salmoides*, *M. dolomieu*, *M. punctulatus* and *L. macrochirus*, especially during winter floods, would be valuable information to establish if these species can be irradicated (Section 4.4.5). The contact zones between bass and redfin populations should be monitored to assess the security of redfin populations and the effectiveness of natural or artificial barriers.

4.4.6.4 A re-assessment of the conservation status of the two ESU's of *P. phlegethon* should be done. *P. phlegethon* as a whole is currently listed as endangered (Baillie & Groombridge, 1996).

4.4.6.5 Systematic work accompanied by seasonal invertebrate surveys is needed on aquatic invertebrates of Olifants River System.

4.4.6.6 Genetic samples (i.e. small fin clips) should be taken of founder individuals when new populations of redfins are established and genetic monitoring on the establishment of new populations should be done to prevent excessive loss of genetic diversity.

4.4.6.7 To make informed decisions under time and capacity constraints, an e-mail or internet communication network is suggested to assist conservation authorities to keep in contact with researchers in the field.

4.4.6.8 There is still a need for further ecological and life history information of the Olifants River redfins like those done on *B. erubescens* (Mariott, 1998) and *B. calidus* (Nthimo, 1997).

4.4.6.9 Further population genetic studies should be done on other fishes of the Olifants River System.

4.4.6.10 The CMU's identified in the present study, should be evaluated to see if they are minimum viable populations (Caughley, 1994), through demographic and ecological studies and modeling. Loss of genetic diversity should be further investigated in small populations (e.g. the Tra tra and Biedou populations of *B. calidus*, the Driehoeks and Breekkrans populations of *P. phlegethon* and the Oudste populations of *B. calidus* and *P. phlegethon*).

4.4.6.11 The enzymes used in the present study were too invariable to make clear inferences on the possible loss of genetic diversity of redfins as a result of recent events. More variable genetic markers will be needed to detect and monitor genetic diversity.

4.4.6.12 An assessment of the formal taxonomic status of the two *P. phlegethon* ESU's should be done.

4.4.6.13 Further surveys are needed on the Jan Dissels and Heks Rivers to confirm that *P. phlegethon* and *B. calidus* are extinct in these streams.

4.5 CONCLUSIONS

The Olifants and Doring River populations of *P. phlegethon* form two distinct ESU's. No such divergence was detected in *B. calidus*, but this species does form a separate ESU from *B. erubescens*. Two types of MU's should be used to manage populations of redfins. Historical management units should form the basis on which decisions are made to establish new

populations. Current management units should be the focus of population monitoring for loss of genetic diversity and the effect of demographic and ecological instability. The size of current management units should be expanded and secured to prevent loss of genetic diversity. The intrusion of centrarchids is the main threat to the survival of redfin populations. It is recommended that a recovery program of redfins should establish new populations of at least *B. erubescens* and Doring River *P. phlegethon* ESU, the size of many of the populations should be increased, existing populations should be secured and centrarchids should be irradicated.

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