Phylogeography, phylogenetics and evolution of the redfins (Teleostei, Cyprinidae, *Pseudobarbus*) in southern Africa

by

Ernst Roelof Swartz

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Department of Genetics

Faculty of Natural and Agricultural Science

University of Pretoria

Pretoria

Supervisors:

Prof. Paulette Bloomer (Department of Genetics, University of Pretoria)

Prof. Paul H. Skelton (South African Institute for Aquatic Biodiversity)

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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.

Signature: _____ Date: _____

Thesis summary

The present thesis concerns the population history, evolutionary processes and phylogenetic relationships of lineages of the redfin minnow genus *Pseudobarbus*. First, the population history and evolutionary processes within *P. quathlambae* were determined, mainly to decide the conservation value of the Mohale population. The Mohale dam threatens its survival. A divergence in mitochondrial control region and cytochrome *b* sequences and frequency differences in the distribution of major histocompatibility alleles were found between the Mohale population and an "Eastern" lineage. The Mohale population has therefore been historically isolated and was deemed indispensable for the conservation of *P. quathlambae*. Differentiation among populations of the *P. afer* and *P. phlegethon* complex were investigated, in relation to geological and climatic processes. Sea levels were about – 130 m below present levels during the last glacial maximum, about 18 000 years ago. Five historically isolated lineages were identified through analysis of mitochondrial control region sequences. The four *P. afer* lineages showed a strong association with proposed palaeoriver systems. A "Forest" lineage, however, reaches across two proposed palaeoriver systems. Surprisingly, this lineage is closely related to *P. phlegethon*. *Pseudobarbus asper* and *P. tenuis* were analysed together, because of their close phylogenetic relationship and because they occur in sympatry in the Gourits River system. *Pseudobarbus tenuis* showed divergence in mitochondrial control region only between the Keurbooms and Bitou River systems compared to the Gourits River system. Within *P. asper*, divergence was low, suggesting recent inland exchange opportunities between populations of the Gourits and Gamtoos River systems. River capture of south-eastern tributaries of the Gourits River system by the Keurbooms River would have resulted in unidirectional colonization, suggesting that speciation between *P. asper* and *P. tenuis* occurred within the Gourits River system with or

without the Gamtoos River system being involved. Lower sea levels during the last glacial maximum also played an important role in the population history of *P. burchelli*. Differentiation in *P. burchelli* did not occur between two proposed palaeoriver systems, but rather within a western palaeoriver system. Divergence in mitochondrial control region and cytochrome *b* sequences showed that the "Breede" and "Tradou" lineages diverged within the Breede River system, before the "Heuningnes" lineage became isolated in the Heuningnes River system. Fifteen historically isolated *Pseudobarbus* lineages were included in a phylogenetic analysis on which biogeographic hypotheses were based. Phylogenetic relationships based on mitochondrial control region, cytochrome *b* and 16S and a combined dataset of all these were compared to relationships recovered from a previous morphological dataset. Conflicts between the molecular and morphological analyses, suggests that several morphological characters evolved in a complex manner. The molecular phylogenies suggest that the earliest divergence in the *Pseudobarbus* was between *P. quathlambae* in the Orange River system and the other species that occur in the Cape Foristic Region. *Pseudobarbus* lineages with two pairs of barbels and those with a single pair of barbels (excluding *P. quathlambae*) grouped together. In terms of currently described species, only the two lineages of *P. quathlambae* and the three lineages of *P. burchelli* were clearly monophyletic.

This thesis is dedicated to my parents Doempie and Hilda for their unconditional love and support throughout my studies and my brothers Juan-Francois and Ruan for their friendship…

…and to the plight of the redfins and those who dedicate time and effort towards their conservation.

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Chapter 1

Thesis introduction

Ichthyofaunas and study area

There are several primary freshwater minnow or barb-like cyprinids in the south-western parts of South Africa that have red fins. Those species with red fins belonging to the genus *Pseudobarbus* occur in rivers associated with the Cape Floristic Region (CFR) in the Western Cape and Eastern Cape provinces of South Africa, with one species in the highlands of Lesotho (Fig. 1.1). The CFR is the smallest of the plant regions of the world, but is famous for its spectacular fynbos diversity. The fish fauna associated with the CFR, however, is one of the poorest in terms of species and taxonomic diversity in Africa. Skelton (1986; 1994b) classified the freshwater fish of southern Africa into temperate and tropical Ichthyofaunas. Within the temperate ichthyofauna, he distinguished between the Cape and Karoo Ichthyofaunas, on the basis of regional ecological differences. *Pseudobarbus* is the most diverse lineage within the Cape ichthyofauna, and is therefore a prominent element of the river ecology of rivers associated with the CFR. The same can be said of the one species that occurs in Lesotho, *P. quathlambae*, since it is essentially the only indigenous fish species that occurs in the high altitude tributary streams in the eastern and central highlands. It is also Lesotho's only endemic vertebrate species, since the extinction of a population that occurred in the Kwa-Zulu-Natal province in South Africa.

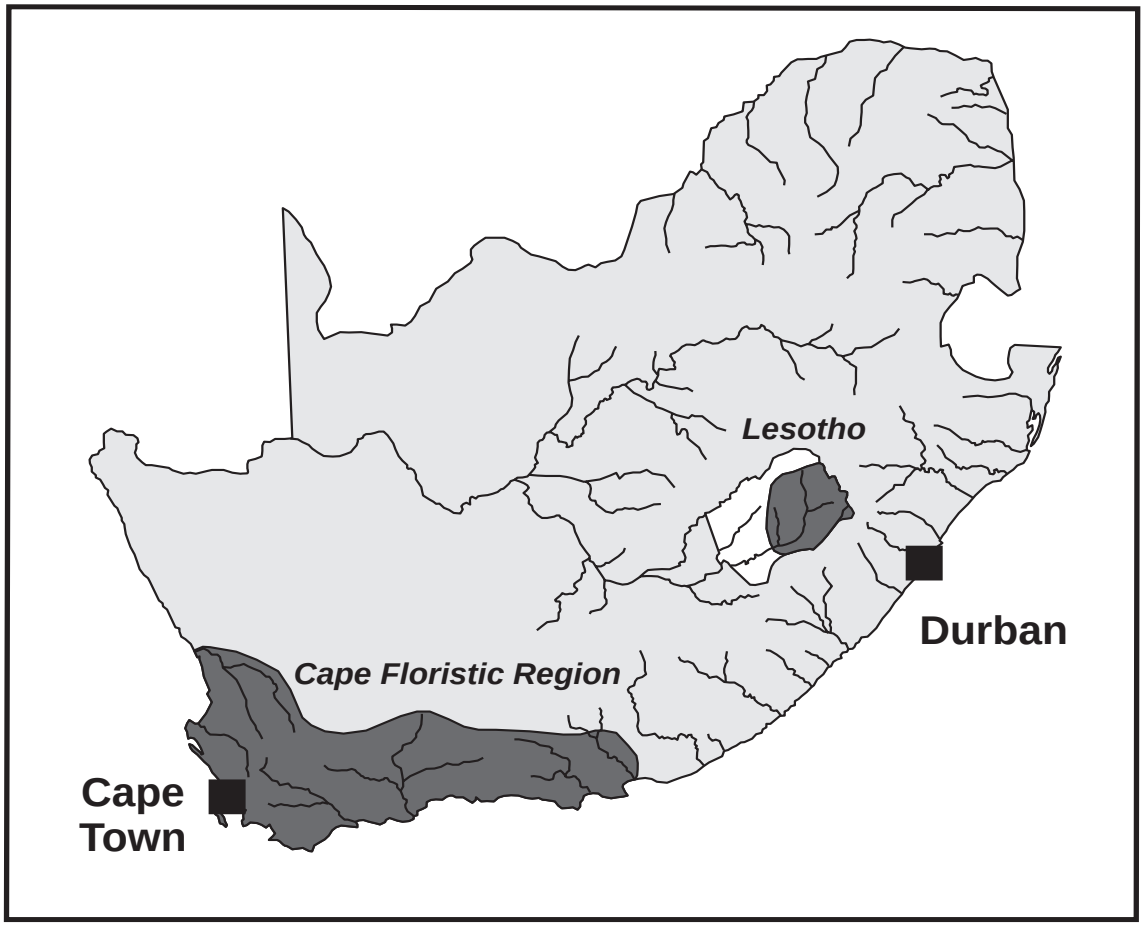


Fig. 1.1. Map of South Africa and Lesotho showing the range of the genus *Pseudobarbus*, which is associated with the Cape Floristic Region and the Lesotho highlands.

The Orange River system has its origins in the highlands of Lesotho and is the largest river system in South Africa. The Olifants, Berg, Breede, Gourits, Gamtoos and Sundays River systems are the larger systems in the Cape Floristic Region and drain from interior regions of the Western Cape and Eastern Cape provinces associated with the semi-arid Karoo, through the CFR. The Keurbooms and Swartkops River systems penetrate coastal mountain ranges, but originate within the CFR. There are also several small coastal river systems that do not penetrate coastal mountain ranges, especially along the southern coastline of the CFR.

Taxonomic history and currently recognised species and genetic lineages

Ludwig Krebs collected the earliest known fish specimens from South Africa in about 1820-1822 (Skelton, 1997). His collection included specimens of the Eastern Cape redfin (*Pseudobarbus afer*). However, Andrew Smith working with collections in the 1830's described the first redfin species, *Barbus (Pseudobarbus) burchelli* in 1841. Smith recorded the type locality as "Various rivers of the Cape Colony" and there is no known type material (Barnard, 1943). The illustration that accompanied the species description, however, clearly shows that the species had red fins and two pairs of barbels. Castelnau described *Gnathendalia vulnerata* in 1861 and Steindachner described *Barbus multimaculatus* in 1861 from the Breede River system. Both descriptions were of species with two pairs of barbels.

Valenciennes in Cuvier and Valenciennes (1842) described *Barbus gobionides* that was placed in synonymy with *Gnathendalia vulnerata* by Günther in 1868. However, Barnard (1943) declared that *Barbus gobionides* was a *nomen dubium*. Boulenger (1911) placed *Barbus multimaculatus* in synonymy with *Gnathendalia vulnerata* in 1905, a decision that was confirmed by Barnard (1943), Jubb (1965) and Skelton (1988). Boulenger (1911) also

described a redfin species with two pairs of barbels from Berg River system and called it *Barbus burgi*. Barnard (1943), working with a better understanding of the distribution of the species, had to decide whether the Breede or Berg species should be placed in synonymy with Smith's *Barbus (Pseudobarbus) burchelli*. He decided to place *Barbus burgi* in synonymy with *Barbus burchelli* and therefore recognised *Barbus vulneratus* for the Breede River system.

Jubb (1965), however, reversed this decision and placed *Gnathendalia vulnerata* in synonymy with Smith's *Barbus (Pseudobarbus) burchelli*. According to Jubb (1965), this was done after P. H. Greenwood examined the skins of Castelnau's types of *Gnathendalia vulnerata*, compared it to Smith's description of *Barbus (Pseudobarbus) burchelli* and concluded that they should be placed in synonymy. However, Jubb (1965) gave no justification why *Barbus burgi* does not also agree with Smith's description and why Barnard's (1943) arrangement was not kept. It may be possible that *Barbus burgi* was in general usage, but that was probably also the case for *Barbus vulnerata*. When Skelton (1988) defined a monophyletic redfin genus, he raised Smith's (1841) subgenus name to a full generic name and accepted Jubb's (1965) nomenclatural changes to maintain taxonomic stability. A specimen from the Tradou catchment was assigned as neotype material for *P. burchelli*.

Peters (1864) was the earliest description of a redfin with a single pair of barbels (*Barbus (Capoeta) afer*), from the Krebs collection. The specimens were most likely taken from the Swartkops River system near Uitenhage (Jubb, 1965). Smith (1936) described *Barbus senticeps* from the Krom River on the south coast, which was later placed in synonymy with *Barbus afer* by Jubb (1965). Boulenger (1911) described *Barbus asper* from the Gamtoos River system at Steytlerville and Barnard (1938b) described *Barbus phlegethon* from the

Boontjies tributary of the Olifants River system and *Barbus tenuis* from the Gourits river system. These all had a single pair of barbels and were recognised as redfins by Barnard (1943) and Jubb (1965). Confusion of these species with *Barbus anoplus*, a species occurring mostly in Karoo-type streams, was largely resolved by Barnard (1943). However, there was still considerable confusion regarding the status of *B. afer* and the distinction between the latter and *B. asper* (Barnard, 1943; Jubb, 1965). When Skelton (1988) defined *Pseudobarbus*, he clearly defined *P. asper*. Redfin populations from coastal river systems that drain the Afromontane forests were earlier placed with *P. asper* (Jubb, 1965). Skelton (1988) placed these with *P. afer*, but also recognised that *P. afer* was a polytypic species.

Earlier Barnard (1938a) described a cyprinid minnow from the Mkhomazana River in KwaZulu-Natal as *Labeo quathlambae*. However, Greenwood & Jubb (1967) placed it in its own genus (*Oreodiamon*), after they failed to establish a clear relationship to other African cyprinids. Skelton (1974b) collected live specimens from a population discovered in Lesotho and confirmed that they had red fins, which suggested a relationship with the redfin species in river systems associated with the Cape Floristic Region. The subsequent confirmation that *Oreodiamon quathlambae* was related to the redfin species with a soft primary dorsal spine, was an important step in defining the genus *Pseudobarbus* and had interesting biogeographic implications (Skelton, 1980; 1988). The present thesis therefore builds on the revision that was done by Skelton (1988), whom recognised the lineages *Pseudobarbus quathlambae*, *P. phlegethon*, *P. burgi*, *P. burchelli*, *P. tenuis*, *P. asper* and *P. afer*.

Apart from *P. burchelli* and *P. burgi*, two other minnow species with red fins and two pairs of barbels were described in the 20th century. Barnard (1938b) described *Barbus calidus* from the Jan Dissels and Skelton (1974a) described its sister species *Barbus erubescens* from the Twee River, both tributaries of the Olifants River system. *Barbus calidus* occur in several tributaries of both the Olifants and Doring Rivers, but *B. erubescens* is restricted to the Twee River catchment. Both species, however, were excluded when Skelton (1988) defined the genus *Pseudobarbus*. This was done on the basis of a complex of characters including one of significance in African *Barbus* classification, having a primary dorsal spine that is serrated, compared to all the *Pseudobarbus* species that have a soft primary dorsal spine.

Bloomer & Impson (2000) investigated the mitochondrial DNA differentiation among populations of *P. burgi* and found a major divergence between the localities of the Berg and Verlorenvlei River systems. Skelton (1980; 1988) reported that Verlorenvlei specimens had a much longer gut than specimens from the Berg River system. Tubercles have also never been noticed in specimens from Verlorenvlei. Swartz *et al.* (2004) recorded seven fixed allozyme allelic differences between populations of *P. phlegethon* from the Olifants and Doring River systems. In addition, there seems to be colour pattern differences between Olifants and Doring populations and Skelton (1988) noticed frequency differences in the number of lateral line scales, pectoral fin rays and dorsal branched fin rays. The only other intraspecific genetic investigations that has been done on a *Pseudobarbus* species, was when Van der Bank *et al.* (2001) found little allozyme differentiation among localities of *P. quathlambae* within the Senquenyani catchment (Mohale population).

Several other intraspecific problems remain. Apart from the above and recognising that *P. afer* is a polypypic species, Skelton (1988) also found intraspecific variation in fin lengths and caudal peduncle proportions between Gourits and Keurbooms River system populations of *P. tenuis*. Gephard (1978) found mainly colour pattern differences between eastern populations of *P. quathlambae*. Only *P. quathlambae* (after the extinction of the Mkhomazana population) and *P. phlegethon* occur in a single river system. There would therefore have been many opportunities for historical isolation to occur between different river systems in the other species, which warrants further taxonomic investigation.

Phylogeography

Very little population genetic or phylogenetic information is available for the genus *Pseudobarbus*. Apart from allozyme electrophoresis studies on *P. phlegethon* (Swartz *et al.*, 2004) and *P. quathlambae* (Van der Bank *et al.*, 2001), which can be classified as population genetic studies, there was a mitochondrial control region study of *P. burgi* (Bloomer & Impson, 2000). The latter study falls more in the field of phylogeography. Phylogeography deals with processes relating to the geographic distribution of genetic lineages within and between related species and may be considered to be a bridge between population genetics and phylogenetics (Avise *et al.*, 1987). Mitochondrial DNA especially has been used in many phylogeographic studies on freshwater fish (Bermingham & Martin, 1998; Durand *et al.*, 1999; Kotlík & Berrebi, 2001; Waters & Wallis, 2000), because of the information that can be gained regarding population history and evolutionary processes.

Based on extensive surveys, a phylogeography study can form a good platform on which population genetics, phylogenetic, taxonomic and biogeographic studies can be based. Geographically representative molecular sequencing provides a basis for taxonomic investigations, since it can be a powerful tool in identifying potential new or cryptic taxa. Depending on the species, its habitat and nature and period of isolation, historically isolated lineages may have undergone speciation. In addition, evolutionary processes are identified that can be tested in greater depth with increased sample sizes in population genetic studies.

Historically isolated lineages identified in phylogeographic studies should be used as the basic units for phylogenetic and biogeographic studies instead of species, since they represent more of the existing genetic diversity and therefore provide more appropriate information. The wide integrated distribution of *Pseudobarbus* species in rivers of the CFR and relatively well studied morphological diversity (Skelton, 1980; 1988), makes it a particularly suitable group with which to study drainage evolution specifically in the CFR and evolutionary processes of riverine cyprinid minnows in general.

Phylogenetics and biogeographic hypotheses

The outgroup relationships of *Pseudobarbus* have been well established. The *Barbus anoplus*-group was earlier suggested as a possible sister group to *Pseudobarbus*, since they share a number of similarities, including a soft primary dorsal spine, and are distributed contiguously (Skelton, 1980). The karyological study by Naran (1997) showed that *B. calidus*, *B. erubescens*, *B. serra*, *B. andrewi*, *B. hospes*, *B. trevelyani* and all the *Pseudobarbus* species were tetraploid. Naran (1997) also suggested that all these species had a common ploidy event, which suggested that *Pseudobarbus* and the serrated tetraploid *Barbus* species were

sister groups. This was confirmed through molecular phylogenies based on mitochondrial DNA (Machordom & Doadrio, 2001b; Tsigenopoulos *et al.*, 2002; E. R. Swartz *et al.*, unpublished). All the species of the *B. anoplus*-group are diploid and form part of a pan-African lineage of diploid *Barbus* species.

Skelton (1980; 1986; 1994b) formulated biogeographic hypotheses regarding the evolution of the genus *Pseudobarbus*, based on a morphologically derived phylogeny. Relationships recovered from the morphology, suggested that *P. quathlambae* from Orange River system in Lesotho is the sister species of *P. tenuis* from the Gourits, Keurbooms and Bitou River systems in the central parts of the Cape Floristic Region. This relationship was explained, by suggesting that ancestral populations were extirpated in central Karoo tributaries of the Orange River system. These populations would have been the ancestor of both *P. quathlambae* and *P. tenuis*. *Pseudobarbus phlegethon* was inferred as their closest relative, which could be justified through the common confluence that the Orange River system had with the Olifants River system (De Wit, 1993; Dingle & Hendey, 1984).

The earliest divergence in the *Pseudobarbus* phylogeny was suggested to be between *P. burgi* and all the other species. Later, there was a divergence between *P. burchelli* and the other *Pseudobarbus* species. However, these two species were also suggested to be closely related to each other. They occur in the neighbouring Berg and Breede River systems. Next, *P. asper* and *P. afer* that were inferred as sister species was hypothesized to have diverged from *P. phlegethon*, *P. tenuis* and *P. quathlambae*. Again, differentiation in neighbouring river systems explained their close relationship. These relationships and biogeographic hypotheses will be tested in the present thesis using mitochondrial DNA.

Conservation concerns

Soon after the introduction of especially smallmouth bass (*Micropterus dolomieu*), Barnard (1943) recognised the threat that some alien fish species posed to indigenous species. He suggested that prompt surveys were needed of the indigenous fish fauna. Even Harrison (1961) whom himself was responsible for several introductions, noted in an editorial response to an article by Jubb (1961), that the impact that North-American black bass species was having on the small indigenous species was regrettable. Since these early concerns, many other authors have expressed concern regarding the conservation status of *Pseudobarbus* species in the Cape Floristic Region (Bills, 1999; Gaigher *et al.*, 1980; Impson *et al.*, 2002; Impson, Hamman, 2000; Skelton, 1996; Skelton, 2002; Swartz, 2000).

However, the circumstances surrounding the discovery and conservation of *P. quathlambae* (Maloti minnow) in Lesotho and KwaZulu-Natal has elevated the conservation profile of this species. It has been at the centre of freshwater fish conservation controversies since its discovery in the 1930's. The name that Greenwood & Jubb (1967) gave to the monotypic genus in which they placed the Maloti minnow, means "spirit of the mountains", as they feared that it was extinct due to predation and competition from trout (Jubb, 1966). It was rediscovered in Lesotho by Pike & Tedder (1973). This brought about renewed controversy, since Crass (1985) argued that the original Maloti minnow type material was collected in westward flowing rivers in Lesotho and not in the Mkhomazana River. However, a letter from one of the original collectors, confirmed that the collection locality as the Mkhomazana River (Jubb, 1966) where they were apparently fairly plentiful in 1938 (Pike & Tedder, 1973).

More recent surveys have failed to record the Maloti minnow in the Mkhomazana River (Pike & Karssing, 1995) and this population is considered to have been extirpated by trout. Further surveys showed that six populations exist within Lesotho (Cambray & Meyer, 1987; Rondorf, 1976; Skelton *et al.*, 2001; Skelton, 2000). The Lesotho Highlands Water Project has brought about renewed threats to *P. quathlambae*. The Mohale dam was built in the Senquenyani catchment in which the largest population of *P. quathlambae* occur. Chapter 2, through an evolutionary and population history perspective, gauges the conservation value of the Mohale population to deal with this problem.

Apart from the Mkhomazana population of *P. quathlambae*, a population of *P. burgi* seems to have been extirpated from the Eerste River system in the south-western part of the Western Cape Province. *Pseudobarbus burchelli* were since introduced into this river system and could have introgressed with any *P. burgi* that remained. During surveys for the present thesis, several other potential extinctions of *Pseudobarbus* populations were noted. These will be discussed in the present thesis and future publications. These observations suggest that important populations that would otherwise have contributed to the overall genetic diversity or even species or subspecies diversity of *Pseudobarbus* may have been extirpated.

The main threat to the continued survival of *Pseudobarbus* species in the Cape Floristic Region and in Lesotho, is alien fish invasion. However, the effects of excessive water extraction have also played a role, especially in the Cape Floristic Region. This problem may be elevated in future, if plans proceed to begin utilising the Table Mountain Sandstone aquifers. These aquifers are responsible for most of the run-off of tributary streams in which most *Pseudobarbus* populations are now isolated in because of the introduction of alien fishes.

As a result of mainly alien fish introductions, most of the *Pseudobarbus* species were included in the first red data lists that were compiled. Skelton (1977; 1987) listed *P. phlegethon* and *P. quathlambae* as Endangered and *P. burchelli* and *P. tenuis* as Rare according to earlier IUCN categories of threat. Skelton (1977) listed *P. burgi* as Rare, which was then changed to Endangered by the same author (1987). Skelton listed all the *Pseudobarbus* species in the evaluation included in Baillie & Groombridge (1996). *Pseudobarbus afer*, *P. asper* and *P. burchelli* were included as Rare, with *P. tenuis* as Vulnerable and *P. burgi*, *P. phlegethon* and *P. quathlambae* as Endangered. Since 1996, *P. afer* has been listed as Lower Risk, *P. asper* as Vulnerable, *P. burchelli*, *P. phlegethon* and *P. tenuis* as Endangered and *P. burgi* and *P. quathlambae* as Critically Endangered. The conservation status of *P. quathlambae* was changed from Endangered to Critically Endangered by Skelton *et al.* (2001) due to the impact of Mohale dam as part of the Lesotho Highlands Water Project. Only the conservation status of *P. afer* and *P. phlegethon* has not declined in terms of IUCN categories. All the other species show a general trend towards being listed in IUCN categories that reflect an increased risk of extinction.

Conservation should seek to maintain evolutionary processes and not merely conserve biological patterns. Historically isolated lineages are important to protect, since they cannot be replaced over a short period of time as can be the case in recently formed phenotypic variants (Moritz, 1999; Moritz *et al.*, 2002). In this respect, molecular markers should be used as the primary tools to define conservation units, since they provide information regarding population history and the current distribution of genetic diversity (Moritz, 1999). There is thus an urgent need to continue to describe the genetic diversity of *Pseudobarbus*, before further decline and extinction of populations occur.

Aims of the thesis

The first aim of the present thesis is to identify historically isolated lineages of *Pseudobarbus* species, based on extensive field surveys and geographically representative sequencing of mainly mitochondrial DNA. Phylogeographic-level inferences regarding population history are made, before broad phylogenetic relationships within *Pseudobarbus* are determined. Biogeographic hypotheses are formulated on these phylogenetic relationships and are augmented by inferences made on more local scales within species complexes. Thus the present thesis deals first with phylogeographic and population history inferences within species complexes (Chapters 2-5) before overall phylogenetic relationships and biogeographic hypotheses are determined (Chapter 6).

Chapter 2

Population history and evolutionary processes in the critically endangered *Pseudobarbus quathlambae* (Teleostei, Cyprinidae), a flagship species in the highlands of Lesotho, southern Africa

Abstract

The future existence of *Pseudobarbus quathlambae* is in doubt, because of the continued impact by alien trout and more recently, because of the building of large dams as part of the Lesotho Highlands Water Project. One of these dams will directly affect the Mohale population in central Lesotho, which is the largest of the six extant *P. quathlambae* populations. Divergence in mitochondrial control region and cytochrome *b* sequences, and the distribution of major histocompatibility alleles attained from restriction site profiles, shows that historical isolation and subsequent divergence has occurred between the Mohale population and the remaining five populations in the eastern parts of Lesotho. Among the eastern populations, the Tsoelikane population appears to have become relatively recently isolated. Both isolation and migration-type processes were inferred among north-eastern populations, but in general historical and recent isolation played a more important role in shaping the genetic patterns than recent migration. The Mohale population is indispensable for the conservation of *P. quathlambae*. It represents a unique evolutionary lineage and conservation efforts should therefore ensure its future survival. Efforts should also be made to secure the remaining populations against further invasion of trout and the size of some of the smaller *P. quathlambae* populations should be increased by eradicating trout from certain rivers.

Introduction

Pseudobarbus quathlambae (Maloti minnow or Maloti redfin) has become a flagship species for conservation in Lesotho as this country's only endemic vertebrate (Skelton, 2000). It was first discovered in the Mkhomazana River at the foot of the Drakensberg Mountains in the KwaZulu-Natal province of South Africa (Jubb, 1966) (Fig. 2.1) where they were apparently fairly plentiful in 1938 (Pike & Tedder, 1973). Barnard (1938a) described it as *Labeo quathlambae*, but Greenwood & Jubb (1967) assigned it to its own monospecific genus *Oreodiamon*, when they failed to establish its relationship to other African cyprinids. The name *Oreodiamon* meaning "spirit of the mountains" was appropriate at the time as it was thought to be extinct as a result of predation and competition from trout (*Salmo trutta* L. and *Onchorhynchus mykiss* (Walbaum)). The latter was introduced into the Mkhomazana River around 1910-1920 and again in 1926 and 1927 (Jubb, 1966). Subsequent surveys failed to record *P. quathlambae* in the Mkhomazana River (Pike & Karssing, 1995) and the probable explanation seems to be that they have been extirpated from that system. Skelton (1974b; 1988) finally recognised that the Maloti minnow belongs in the genus *Pseudobarbus*, allied with the redfins of the Cape Floristic Region (southwest South Africa).

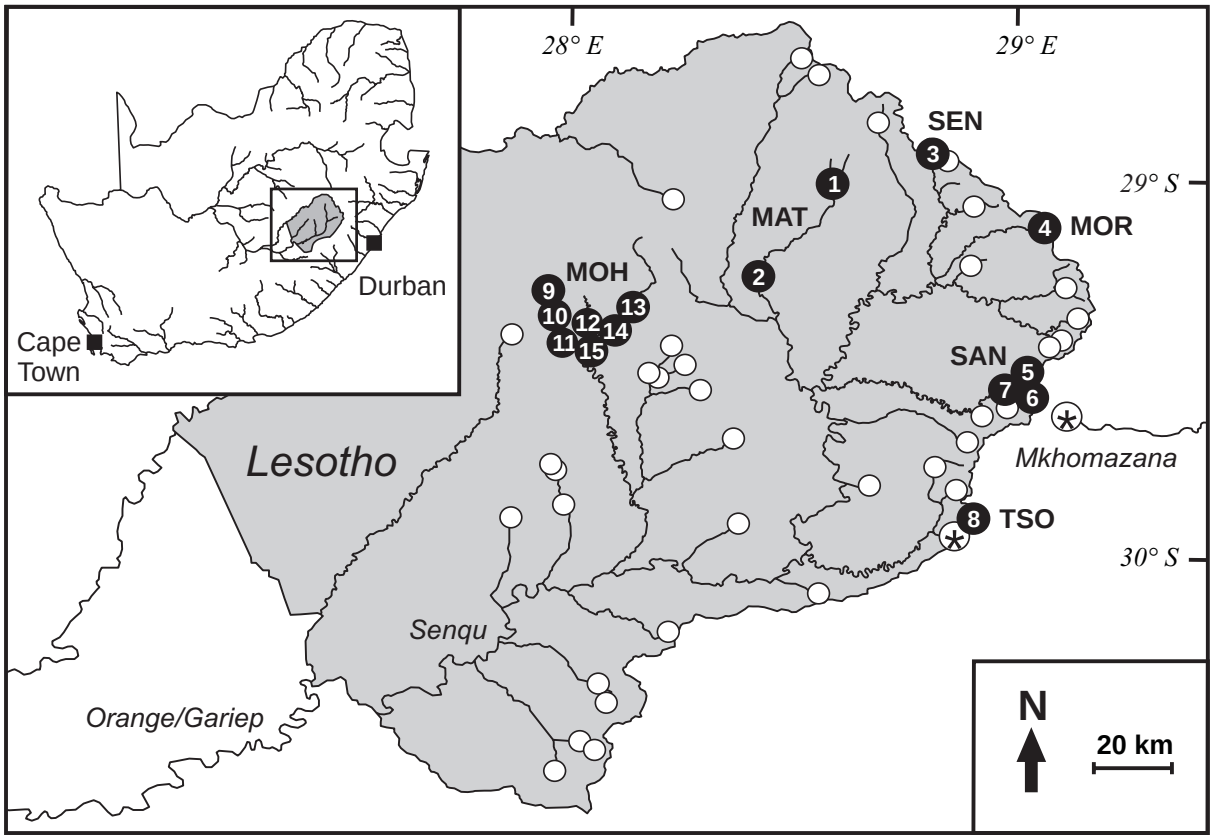


Fig. 2.1. Map of Lesotho and surrounding areas of South Africa where *P. quathlambae* were successfully collected (solid numbered circles) (Skelton *et al*., 2001; Rall *et al*., 2002). Matsoku (MAT), Senqu (SEN), Moremoholo (MOR), Sani (SAN), Tsoelikane (TSO) and Mohale (MOH) are the only remaining populations of this species. Open circles show localities where Skelton *et al*. (2001) failed to record *P. quathlambae*. The open circles with asterisks show where known extinctions have occurred (Skelton *et al*., 2001; Pike & Karssing, 1995; Jubb, 1966).

Pike & Tedder (1973) rediscovered *P. quathlambae* in the Tsoelikane River (Sethlabathebe National Park, eastern Lesotho) in 1970. These authors introduced 56 *P. quathlambae* individuals above the Tsoelikane waterfall in 1973 (Fig. 2.1). This was done to ensure the survival of this population in the face of the threat from rainbow trout (*O. mykiss*). Gephard (1978) suggested that this translocated population became quickly established above the Tsoelikane waterfall. He also suggested that the population was coexisting with the rainbow trout and occasional smallmouth yellowfish *Labeobarbus aeneus* (Burchell) below the waterfall. Further populations of *P. quathlambae* were discovered in 1975 in the upper Senqu and Moremoholo rivers (Rondorf, 1976), followed by the Mohale population (Senqunyani catchment) in 1987 (Cambray & Meyer, 1987), the Sani population in 1988 (Skelton, 2000) and the Matsoku River in 2000 (SAIAB 61857) (see Fig. 2.1). Waterfalls protect the Mohale, Senqu, Moremoholo and translocated Tsoelikane populations from invasion by trout, whereas the Matsoku, Sani and original Tsoelikane populations occur with trout and are vulnerable to further invasion.

The distribution of *P. quathlambae* presents an anomaly within the genus. It is the only redfin species that is not associated with the Cape Floristic Region, being isolated in the Drakensberg Alpine Floral Centre. This geographic isolation is reflected in its phylogenetic relationships, because it is genetically the most divergent *Pseudobarbus* species (Chapter 6). Compared to the other redfins, it occurs at much higher altitudes of about 1950 to 2760 meter above sea level (Rall, 1993; Rondorf, 1976). At these altitudes, *P. quathlambae* is exposed to extreme daily temperature shifts in the shallow low order streams that often freeze over in winter. Only the extinct Mkhomazana population occurred in a less extreme environment at altitudes around the approximate 1615 meter above sea level of the type locality (Barnard, 1938a). All the Lesotho populations occur in clear rivers flowing over Drakensberg basalt,

except for the Tsoelikane River that is also clear but flows over sandstone of the Clarens Formation (Keyser, 1998).

The Mohale population represents 70 % of the extent of occurrence of the species (Skelton *et al.*, 2001). Unfortunately, a large impoundment called Mohale dam was to be built in this catchment, allowing trout to move through a tunnel built between the latter and Katze dam, as part of the Lesotho Highlands Water Project (LHWP). In response to the threat posed by the LHWP, a major survey was planned to assess the distribution and conservation status of *P. quathlambae* and the importance of the Mohale population for conservation management purposes (Skelton *et al.*, 2001). Localities for the survey were selected based on the habitat requirements of this species (Rall, 1993). A strong emphasis was placed on localities with gradients less than 1: 40 (P. H. Skelton & S. Mashapa, unpublished) and where waterfalls have potentially prevented invasion by trout (Skelton *et al.*, 2001). A helicopter was used to reach the remote localities and a total of 39 rivers and 47 localities were surveyed in Lesotho's Senqu River system (called Orange/Gariep in South Africa).

The survey improved knowledge regarding the range of the six extant populations, but not a single new *P. quathlambae* population was discovered (Fig. 2.1). The persistence of the translocated population above the Tsoelikane waterfall was confirmed. However, only a single *P. quathlambae* individual was found below this waterfall amongst a population of rainbow trout. The Tsoelikane population has therefore, for all practical reasons, been extirpated from their natural range. The conservation status of *P. quathlambae* changed from Endangered (Skelton, 1977; 1987) to Critically Endangered (Skelton *et al.*, 2001) in view of the survey results and the additional threat that the LHWP poses. It also raised the question of

how important the Mohale population was in terms of its contribution towards the overall genetic diversity of the species.

A basic step in conservation management should be to allow evolutionary processes that have shaped current intraspecific diversity, to continue into the future (Crandall *et al.*, 2000; Moritz, 1999; 2002). The introduction of trout during the first half of the 20th century (Pike & Tedder, 1973) has fragmented and isolated *P. quathlambae* populations, making it impossible to directly assess migration patterns among populations. Therefore indirect approaches such as genetics have to be used. Intraspecific variation of *P. quathlambae* has only been studied to a limited extent by Gephard (1978) who showed pigment pattern differences among the Senqu, Moremoholo and Tsoelikane populations and by Van der Bank *et al.* (2001) who reported minor allozyme differentiation within the Senqunyani catchment (Mohale population). The objective of the present study was to assess genetic differentiation among all extant *P. quathlambae* populations and to infer potential evolutionary processes that have been shaping the genetic diversity.

Materials and Methods

Sampling

Specimens were collected using Deca electro-shockers during the surveys of Skelton *et al.* (2001) and Rall *et al.* (2002). Muscle or whole fish samples were stored in liquid nitrogen in the field and transferred to a –70 °C freezer upon returning to the laboratory or whole fish samples were placed in EtOH (Department of Genetics, University of Pretoria). In addition, whole fish samples and samples from which muscle tissue was dissected, were fixed in

formalin and deposited in the South African Institute for Aquatic Biodiversity collection as voucher specimens (SAIAB 62656, 63392, 63394, 63399, 63408-9, 63414 and 63416-17).

DNA extraction, primer design, amplification and sequencing

Total genomic DNA was isolated from frozen muscle tissue using standard protocols of chemical digestion and phenol/chloroform extraction (Sambrook *et al.*, 1989). Two cyprinid specific primers were designed, namely L16560 (5' CCAAAGCCAGAATTCTAAC 3') in the tRNA (Thr) on the 5' side of mtDNA control region and H677 (5' GTCGCGCAAAAACCAAAG 3') within the 3' side of control region. These primer names were given according to the positions of the 3' base of each primer in the complete mtDNA genome sequence of *Cyprinus carpio* (Chang *et al.*, 1994). The widely used vertebrate primers L14724 (Kocher *et al.*, 1989) and H15499 (Avise, 1994), of which the former was modified (5' TGAYATGAAAAAYCATCGTTG 3'), were used to amplify 500 base pairs of the mtDNA cytochrome *b* gene.

PCR and cycle sequencing were performed in a Geneamp® PCR System 9700 (Applied Biosystems). Amplification was performed in 50 µl volumes, each containing 1 x buffer, 2 mM $MgCl_2$, 0.2 mM of each of the four nucleotides (Promega), 25 pmol of each primer and 1.5 U of Super-Therm DNA polymerase (Southern Cross Biotechnology) and approximately 100-200 ng template DNA. PCR cycling conditions for the mtDNA reactions involved an initial denaturation of 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 54°C and 45 seconds at 72°C and a final extension of 5 minutes at 72°C. PCR products were purified using the High Pure™ PCR Product Purification Kit (Boehringer Mannheim) followed by elution in ddH_2O.

Cycle sequencing was performed in 10 µl volumes with the reaction mix containing 100 ng of purified PCR template, 1.6 pmol of one of the above-mentioned primers and 2 µl of ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Nucleotide sequences were determined through an ABI 377 automated sequencer. Consensus sequences were obtained from the forward and reverse sequences through alignment and inspection in Sequence Navigator 1.01 (Applied Biosystems). These sequences were aligned using Clustal X (Thompson *et al.*, 1997) and checked manually. Mitochondrial control region and cytochrome *b* sequences are available from GenBank under the accession numbers AY791701 to AY791807 and AY791808 to AY791833 respectively.

A portion of the Major Histocompatibility gene (MH) (nomenclature of Stet *et al.*, 2003) that codes for the class II β chain, was amplified using the primers OL92-139 and OL93-23 described by Dixon *et al.* (1996). Amplification was performed in the same volumes and concentrations as for the mtDNA markers, but with conditions for the reactions being 5 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 52°C and 2 minutes at 72°C and finally 5 minutes at 72°C. After failure to detect single-strand conformation polymorphism (SSCP) variation across all six populations according to the methods outlined by Sunnucks *et al.* (2000), seven individuals were sequenced from the Matsoku (1), Moremoholo (1), Sani (1), Tsoelikane (2) and Mohale (2) populations according to methods mentioned above. These sequences are available from GenBank under accession numbers AY791834 to AY791840.

The intron 1 section of the sequence did not show variation among the individuals that were sequenced. Only two variable sites were detected in the protein coding exon 2 section. One of these sites resulted in an amino acid change between leucine and valine and also formed part of the restriction site for the enzyme *Rsa* I (Promega). After digestion with this enzyme, fragments were separated on an agarose gel and scored for homo- or heterozygosity for two alleles (two amino acids) that resulted from the variable site. Control samples were included on all gels.

Substitution model, diversity and structure analysis

A hierarchical likelihood ratio test as performed in MODELTEST version 3.06 (Posada & Crandall, 1998) was used to select one of the 56 models of nucleotide substitution that best fits the control region and cytochrome *b* data. This program was also used to estimate the Ti:Tv ratio, proportion of invariable sites (I) and the α value of the gamma distribution (rate variation among sites).

Gene diversity (δ) and nucleotide diversity (π) and their standard errors, which takes sampling variance into account in the former and both sampling and stochastic variance into account in the latter, were calculated for each population for the control region data, using ARLEQUIN version 2.000 (Schneider *et al.*, 2000). An exact test of population differentiation was performed in the same program among all the populations for the control region and MH alleles. The latter test and program were used to test whether the MH alleles deviated from Hardy-Weinberg equilibrium.

ARLEQUIN was also used to perform an AMOVA (Excoffier *et al*., 1992) on the control region alleles to generate F_{ST} and ϕ_{ST}, both analogues to Wright's (1965) *F*-statistics. These *F*-statistics were tested for significance through permutation tests (10 000 replicates). Four hierarchical structures were defined: (1) Mohale and eastern populations of *P. quathlambae*; (2) Mohale, Tsoelikane and the rest of the eastern populations; (3) individual populations except for lumping Senqu and Moremoholo; (4) Tsoelikane and the rest of the eastern populations (excluding Mohale).

Pairwise F_{ST} and ϕ_{ST} among all the populations were also calculated for the control region data. ϕ_{ST} not only takes allele frequencies into account to estimate variance, as is the case with F_{ST}, but also considers nucleotide differences between alleles. The Tamura-Nei model of substitution with the gamma correction found in MODELTEST 3.06 was used to calculate genetic distances on which ϕ_{ST} was based. Applying different models or gamma corrections did not seem to affect the ϕ_{ST} estimates.

Nested clade analysis (NCA)

Parsimony based cladogram estimation of the control region sequences was done according to Templeton *et al.* (1992) in the program TCS (Clement *et al*., 2000). Alleles in this cladogram were then grouped into hierarchical nesting levels from the tips to the interior of the cladogram without nesting interiors until they could be nested with tip clades (Cunningham, 2002). Hierarchical nesting was also done according to the rules described by Templeton *et al.* (1987) and Templeton & Sing (1993) to compare to the above design. An exact contingency test was performed on each nested clade to test whether a null hypothesis of no association between clades or alleles and geographic location could be rejected, without

taking geographic distance into consideration (Templeton & Sing, 1993). This was done by comparing observed χ^2 values to distributions of χ^2 values generated from 10000 random permutations of the original data in the program GEODIS version 2.0 (Posada *et al.*, 2000).

With the same program, clade distances (D_c), nested clade distances (D_n), average interior versus tip clade distances (IT_c) and average interior versus tip nested clade distances (IT_n) were calculated through the use of the nested design and geographic distances along river courses among localities, as were measured from 1: 50 000 maps. D_c measures how geographically widespread individuals within a particular clade are, whereas D_n is a measure of the distribution of individuals in a particular clade compared to all individuals within the nested clade (Templeton *et al.*, 1995). Templeton *et al.* (1995) proposed that different evolutionary processes affect these geographic distance statistics (D_c, D_n, IT_c and IT_n) in different ways. This suggests that the evolutionary process can be inferred. The inference key of Templeton (2004) was used to assist in interpreting these distance patterns. More conservatively, the distance parameters were also used to classify the evolutionary processes as migration-type or isolation-type processes.

Mismatch distribution

Observed frequencies of pairwise nucleotide differences (mismatch distribution) were calculated for the control region sequences to test their distribution against a Poisson distribution (Rogers & Harpending, 1992), using ARLEQUIN. Mismatch distributions that approximate a Poisson distribution can suggest rapid population expansion (Harpending, 1994; Slatkin & Hudson, 1991), whereas stable populations are expected to show a ragged or erratic mismatch distribution. A raggedness index (r) (Harpending *et al.*, 1993) was calculated

using ARLEQUIN to quantify the "smoothness" of mismatch distributions. A lower value of *r* is indicative of a "smooth" mismatch distribution, which in turn could suggest population expansion.

With the same program, a sum of square deviation test was used to test for a fit to a model of sudden expansion. Using the infinite sites model, both the latter tests were tested for significance using bootstrapping (Schneider & Excoffier, 1999). Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) tests were performed and tested for significance using 1000 bootstrap replicates. Apart from neutrality of the gene, significant negative values for these tests may also be interpreted as indicative of population expansions (Pereira *et al.*, 2001; Tajima, 1989).

Markov chain Monte Carlo approach (MCMC)

In an attempt to distinguish between the processes of isolation and migration using a MCMC approach, the simple three-parameter model as performed in the program MDIV (Nielsen & Wakeley, 2001) was used to estimate M, T and θ, which are given by $2Nm$, $t/2N$ and $4N\mu$ respectively from the control region sequences (N = effective population size, m = migration rate, t = divergence time and μ = mutation rate). This model assumes selective neutrality, a constant mutation rate over time, no subdivision within populations, that the effective population size of the ancestor and the two descendant populations are equal in size and remain constant over time, and that migration rate between the two descendant populations are equal. Since effective population sizes are assumed to be equal and constant over time, M and T are assumed to be indicative of migration rate and divergence time respectively.

The model under consideration is simple and several of the assumptions may be violated. The results are therefore only interpreted in terms of whether a model of migration or isolation was more likely. In the pairwise population runs, the HKY85 substitution model was used with a Markov chain of five million cycles and a burn-in time of 0.5 million. Likelihood ratio tests were performed to estimate minimum and maximum likelihood confidence intervals of the posterior distributions of parameters M, T and θ. An upper limit of $M = 10$ and $T = 10$ was allowed.

Results

Surveys

Samples were successfully collected from all known extant populations. The combination of the Matsoku, Senqu, Moremoholo and Sani populations will be referred to as the north-eastern populations and with Tsoelikane included, they will be referred to as the eastern populations. Samples were collected from more than one locality within the Matsoku (2), Sani (3) and Senqunyani (Mohale population) (7) catchments (Table 2.1; Fig. 2.1). Since these catchments each have a single continuously distributed population, these localities were only analysed separately in the NCA. Progeny of the introduced population above the Tsoelikane waterfall were included in the present study as representatives of the original Tsoelikane population. Geographic distances among the sampled localities along the river drainages varied between 4 and 481 km (Table 2.1).

Table 2.1. Distances along the river courses among sampled localities of the Matsoku (MAT), Senqu (SEN), Moremoholo (MOR), Sani (SAN), Tsoelikane (TSO) and Mohale (MOH) populations of *P. quathlambae*. Localities 1-8 and 15 were collected from 27 September to 6 October 2000 (Skelton *et al.*, 2001) and localities 9-14 were collected from 27 June to 30 July 2002 (Rall *et al.*, 2002). Latitudes and longitudes of the localities are given at the bottom of the table (see Fig. 1 for map).

Code	Locality	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1) MAT 1	Upper Matsoku														
2) MAT 2	Lower Matsoku	69													
3) SEN	Senqu	219	151												
4) MOR	Moremoholo	208	141	157											
5) SAN 5	Mangaung	264	197	224	214										
6) SAN 6	Sani	264	197	224	214	6									
7) SAN 7	Linakeng	260	192	220	209	4	4								
8) TSO	Tsoelikane	349	281	309	298	354	354	350							
9) MOH 9	Upper Jordane	475	408	436	425	481	481	477	346						
10) MOH 10	Middle Jordan	470	402	430	419	475	475	471	340	6					
11) MOH 11	Lower Jordane	455	387	415	404	460	460	456	325	21	15				
12) MOH 12	Bokong	455	387	415	404	460	460	456	325	37	32	17			
13) MOH 13	Upper Senqunyani	467	399	427	416	472	472	468	337	50	44	29	15		
14) MOH 14	Middle Senqunyani	457	390	418	407	463	463	459	328	40	35	20	6	10	
15) MOH 15	Lower Senqunyani	449	381	409	398	454	454	450	319	31	26	11	6	18	9

MAT 1 = 29° 00’ 27” S & 28° 45’ 46” E; MAT 2 = 29° 15’ 35” S & 28° 33’ 29” E; SEN = 28° 55’ 32” S & 29° 01’ 27” E; MOR = 29° 07’ 29” S & 29° 19’ 38” E; SAN 5 = 29° 31’ 53” S & 29° 15’ 34” E; SAN 6 = 29° 33’ 47” S & 29° 16’ 20” E; SAN 7 = 29° 33’ 22” S & 29° 14’ 34” E; TSO = 29° 53’ 51” S & 29° 07’ 14” E; MOH 9 = 29° 21’ 19” S & 28° 02’ 12” E; MOH 10 = 29° 22’ 52” S & 28° 02’ 23” E; MOH 11 = 29° 25’ 19” S & 28° 04’ 54” E; MOH 12 = 29° 23’ 32” S & 28° 06’ 35” E; MOH 13 = 29° 22’ 27” S & 28° 10’ 13” E; MOH 14 = 29° 24’ 20” S & 28° 08’ 16” E; MOH 15 = 29° 25’ 25” S & 28° 06’ 24” E

Control region sequence variation and divergence

The 107 individuals analysed for control region variation yielded 29 alleles (Table 2.2). Only five of these alleles were shared among populations and also only among the north-eastern populations. The nine alleles found from Mohale and the six alleles found from Tsoelikane were unique to these populations. Alleles private to a single population were also detected in the Matsoku (3), Moremoholo (4) and Sani (2) populations. All alleles found in the Senqu population were also found among other north-eastern populations. Of the 601 nucleotide sites, 35 were variable, including 24 parsimony informative sites (three with indels) and 11 sites with autapomorphic mutations (four with indels). HKY85 (Hasegawa *et al.*, 1985) was determined as the substitution model that best fits the control region data, with a Ti:Tv ratio of 7.950, I = 0.769 and $\alpha = 0.295$.

Table 2.2. Frequency of mtDNA control region alleles among sampled localities of *P. quathlambae*. See Table 1 for locality descriptions.

		MAT		SEN	MOR	SAN			TSO	MOH						
Allele number	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	26	-	1	12	13	-	-	-	-	-	-	-	-	-	-	-
2	12	-	1	2	-	2	6	1	-	-	-	-	-	-	-	-
3	8	-	6	-	-	-	2	-	-	-	-	-	-	-	-	-
4	5	-	-	1	-	-	4	-	-	-	-	-	-	-	-	-
5	4	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-
6	2	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-
7	2	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-
8 - 11	4	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-
12 & 13	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
15	9	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-
16	2	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
17 - 20	4	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-
21	10	-	-	-	-	-	-	-	-	1	1	-	-	1	-	7
22	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
23	5	-	-	-	-	-	-	-	-	-	-	1	-	-	1	3
24	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
25 - 29	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
Total	**107**	**3**	**12**	**15**	**17**	**3**	**15**	**1**	**15**	**1**	**1**	**1**	**1**	**1**	**1**	**22**

Genetic distances were based on this model and are presented by branch lengths in the unrooted neighbour-joining phylogram shown in Fig. 2.2A. It shows a large divergence between Mohale and the eastern populations ($0.024 < D < 0.07$), compared to relatively low divergence within Mohale ($0.002 < D < 0.012$) and within the eastern populations ($0 < D < 0.019$). Differentiation between Tsoelikane and the north-eastern populations ($0.006 < D < 0.019$) was similar to the differentiation within Tsoelikane ($0 < D < 0.008$) or within the north-eastern populations ($0 < D < 0.019$). Gene diversity did not differ significantly between the Matsoku, Sani and Mohale populations (Table 2.3). These populations, however, showed significantly higher gene diversities compared to the Senqu and Moremoholo populations. The Tsoelikane population was not significantly different from any of the other populations, except for significantly higher gene diversity than that of the Senqu population. Nucleotide diversity was not significantly different among any of the populations (Table 2.3).

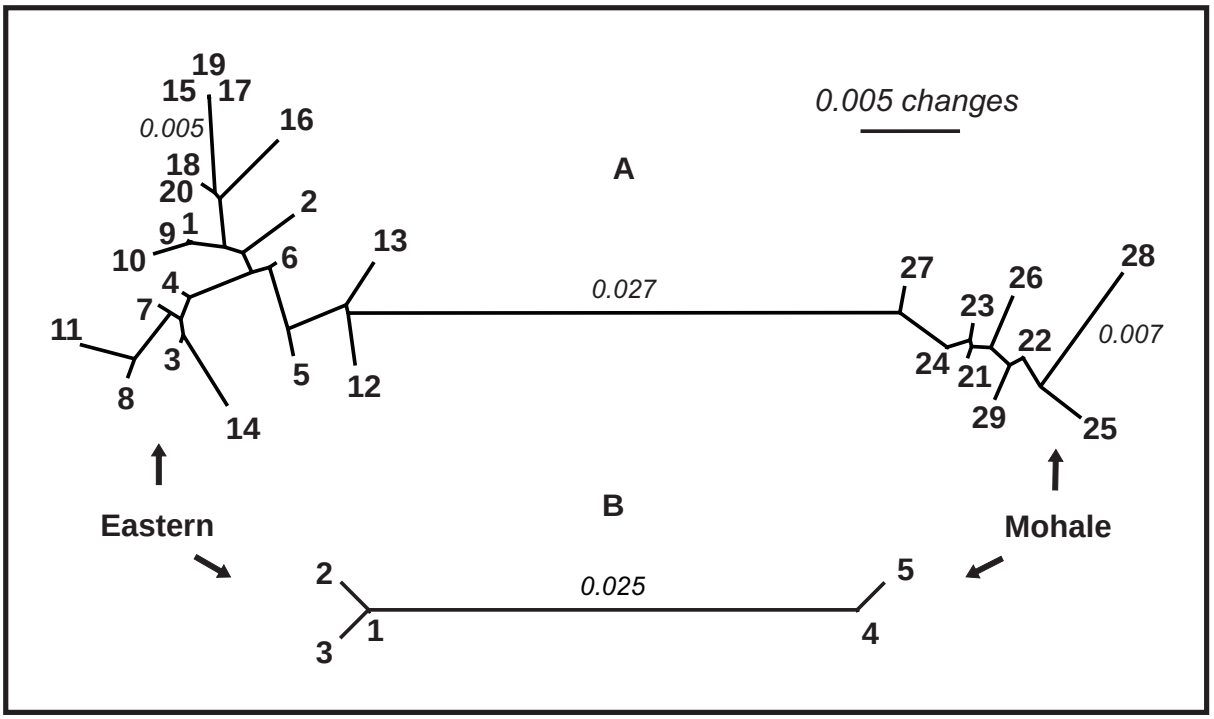


Fig. 2.2. Neighbour-joining phylogram showing HKY85 (Hasegawa *et al.*, 1985) genetic distances among control region (A) and cytochrome *b* (B) alleles. Only genetic distances greater than 0.005 are indicated.

Table 2.3. Pairwise population F_{ST} (above the diagonal), ϕ_{ST} (below the diagonal) and per population gene diversity (δ ± SE) and nucleotide (π ± SE) diversity of *P. quathlambae*. For the *F*-statistics, only the comparisons between the Senqu and Moremoholo populations were not significant ($p > 0.05$).

	F-statistics						Diversity indices	
	1	2	3	4	5	6	δ	π
1) MAT		0.381	0.360	0.165	0.276	0.200	0.800 (0.083)	0.003 (0.002)
2) SEN	0.435		-0.016^{NS}	0.390	0.495	0.391	0.362 (0.145)	0.001 (0.001)
3) MOR	0.398	-0.019^{NS}		0.411	0.467	0.369	0.427 (0.147)	0.002 (0.001)
4) SAN	0.259	0.417	0.426		0.303	0.227	0.743 (0.085)	0.002 (0.002)
5) TSO	0.728	0.738	0.718	0.738		0.269	0.648 (0.134)	0.003 (0.002)
6) MOH	0.889	0.920	0.915	0.906	0.896		0.800 (0.057)	0.003 (0.002)

MAT = Matsoku; SEN = Senqu; MOR = Moremoholo; SAN = Sani; TSO = Tsoelikane; MOH = Mohale

Control region structuring

Failure to reject the hypothesis of random distribution of alleles between pairs of populations with exact tests only occurred between Senqu and Moremoholo (p = 0.490). In all other pairwise population comparisons this hypothesis was rejected (p < 0.001). A similar result was found with *F*-statistics, with all the populations showing significant levels of differentiation, except for the Senqu and Moremoholo comparison (Table 2.3). Most of the variation as measured with AMOVA, was explained by differentiation among groups (62.5 - 86.1 %) in all of the structures specified *a priori* (Table 2.4). However, there was low confidence in the values for the comparisons among groups, since few populations exist with which to do this test. Variation among populations within groups (-0.5 – 12.7%) and variation within populations (8.4 - 24.8%) explained much less of the diversity compared to the differentiation among groups. The overall ϕ_{ST} values were all large and significant, indicating high levels of structuring in all *a priori* structures.

Table 2.4. AMOVA results for the *a priori* structures among *P. quathlambae* populations. Asterisks indicate significant results ($p < 0.05$).

	Variance components			
Source of variation	Mohale vs "Eastern"	Mohale vs Tsoelikane vs rest	Only Moremoholo and Senqu grouped	Tsoelikane vs rest (excl. Mohale)
Among groups	6.592 (80.4%)	5.397 (84.3%)	4.102 (86.1%)	1.580 (62.5%)
Populations within groups	0.917 (11.2%)*	0.318 (5.0%)*	-0.026 (-0.5%)	0.322 (12.7%)*
Within populations	0.688 (8.4%)*	0.688 (10.7%)*	0.688 (14.4%)*	0.626 (24.8%)*
Overall ϕ_{ST}	0.916*	0.893*	0.856*	0.752*

Nested clade analysis

Two ambiguous branches (between allele 4 and the missing allele connected to allele 2 and between allele 6 and allele 3) were broken, in order to keep branches elsewhere in the cladogram that connected control region alleles of higher frequency (Fig. 2.3). Alleles from the Mohale and eastern populations could not be connected by the program TCS, since there were more than ten mutational steps between them. Association between clades and their geographic distance was not tested in clade 4-2 or any of the clades within it, since all the samples were from a single locality (Tsoelikane). In addition, clades 1-1, 1-4, 1-5, 1-10, 1-12 and 2-7 represented only a single locality each.

Clades 1-2, 1-9, 1-11, 2-1, 2-5, 2-6, 3-1, 3-2, 3-4 and 4-3 did represent more than one locality, but did not show a significant association between their clades or alleles and geographic position ($0.467 < \chi^2 < 8.651$; $0.091 < p < 1.000$). Clades 1-3, 2-2, 4-1, 5-1, 6-1 and 7-1 showed a significant association between the clades or alleles within them and geographic position ($9.937 < \chi^2 < 107.000$; $0.000 < p < 0.038$). Three higher-level geographically restricted clades were identified, namely 5-1 (north-eastern populations), 4-2 (Tsoelikane) and 4-3 (Mohale). Processes inferred from the NCA inference key of Templeton (2004) are shown in Table 2.5.

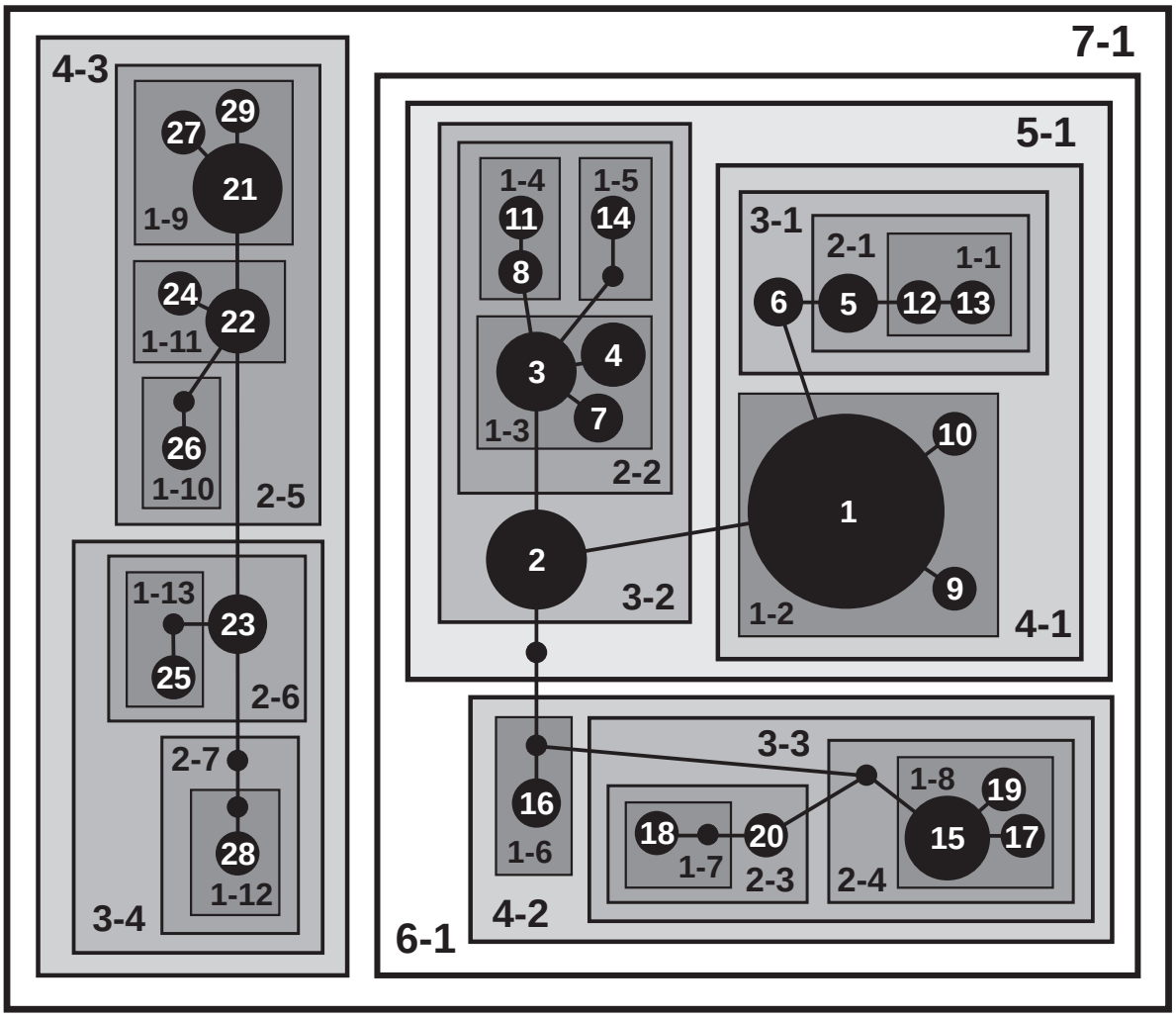


Fig. 2.3. Nested clade design for control region alleles (numbered circles) of *P. quathlambae*. The size of the circles indicates the relative frequency of the alleles (see also Table 2.2). Smaller circles that are not numbered indicate missing alleles.

Table 2.5. Evolutionary processes identified through NCA and the inference key of Templeton (2004).

Clade	Populations in clade	Inference chain	Migration or isolation	Inferred evolutionary pattern
1-3	MAT, SEN & SAN	1, 2, 11, 17, 4, No	Migration	Restricted gene flow with isolation by distance
2-2	North-eastern	1, 2, 11, 17, No	Inconclusive	Inconclusive
4-1 & 5-1	North-eastern	1-2-11-12-13-14-21-No	Isolation	Long distance colonization and/or past fragmentation or gradual movement during past range expansion and subsequent fragmentation
6-1	Eastern populations	1, 19, No	Isolation	Historical isolation
7-1	All populations	1, 19, No	Isolation	Historical isolation

MAT = Matsoku; SEN = Senqu; SAN = Sani

Only one of the clades that showed a significant association between the clades within it and geographic position in the nesting design according to Templeton *et al.* (1987) and Templeton & Sing (1993) differed from equivalent clades in the design shown in Fig. 2.3. The tests done for this clade were done across the same alleles to those of clade 5-1 shown in Fig. 2.3, but with different nested clades specified within it. However, the same chain of inference and conclusion was reached.

Mismatch distribution

Apart from the Mohale population, sample sizes were too low to interpret mismatch distributions for individual populations. The Mohale population, a combination of the Senqu and Moremoholo populations, a combination of the north-eastern populations and a combination of the eastern populations did not show significant *SSD* or *r*, or significant and negative Tajima's *D* or Fu's F_S values (Table 2.6). The non-significant Tajima's *D* and Fu's F_S tests confirmed that the control region is selectively neutral among the *P. quathlambae* populations.

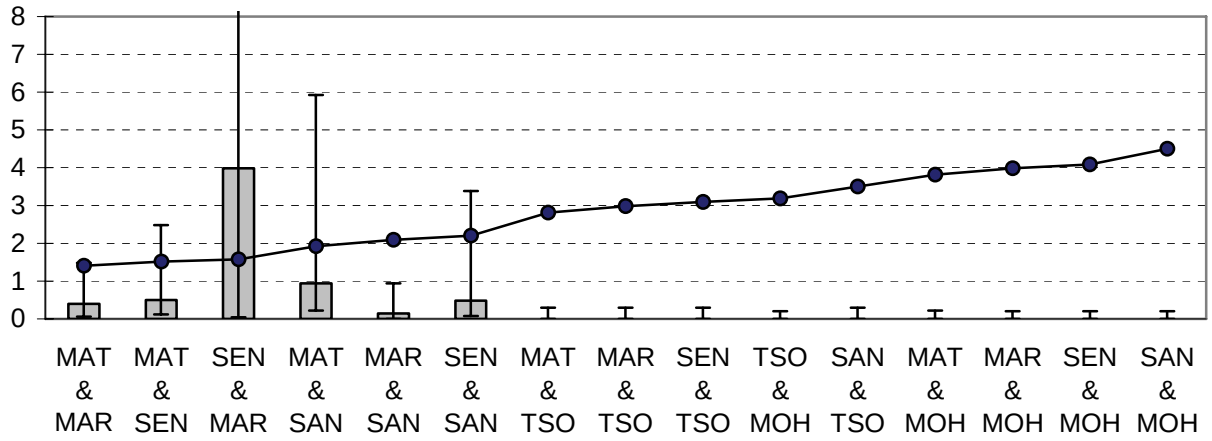
Table 2.6. Raggedness index (*r*) and sum of square deviation tests for population expansion, based on mismatch distribution, and Tajima's *D* (1989) and Fu's *FS* (1997) tests of population expansion and neutrality of the gene. Only *p*-values of *FS* that ≤ 0.02 can be considered as significant, as it corresponds to the 5% significance cut-off value of most statistical inference (Fu, 1997). Therefore, none of these results were significant.

	Eastern populations	North-eastern populations	Senqu-Moremoholo populations	Mohale population
SSD	0.001 (*p*= 0.920)	0.001 (*p*= 0.765)	0.013 (*p*= 0.376)	0.001 (*p*= 0.801)
r	0.009 (*p*= 0.994)	0.029 (*p*= 0.837)	0.209 (*p*= 0.658)	0.040 (*p*= 0.772)
Tajima's *D*	-0.059 (*p*= 0.395)	-0.857 (*p*= 0.214)	-1.384 (*p*= 0.066)	-1.376 (*p*= 0.079)
Fu's *FS*	-3.434 (*p*= 0.108)	-4.279 (*p*= 0.033)	-2.432 (*p*= 0.041)	-3.237 (*p*= 0.021)

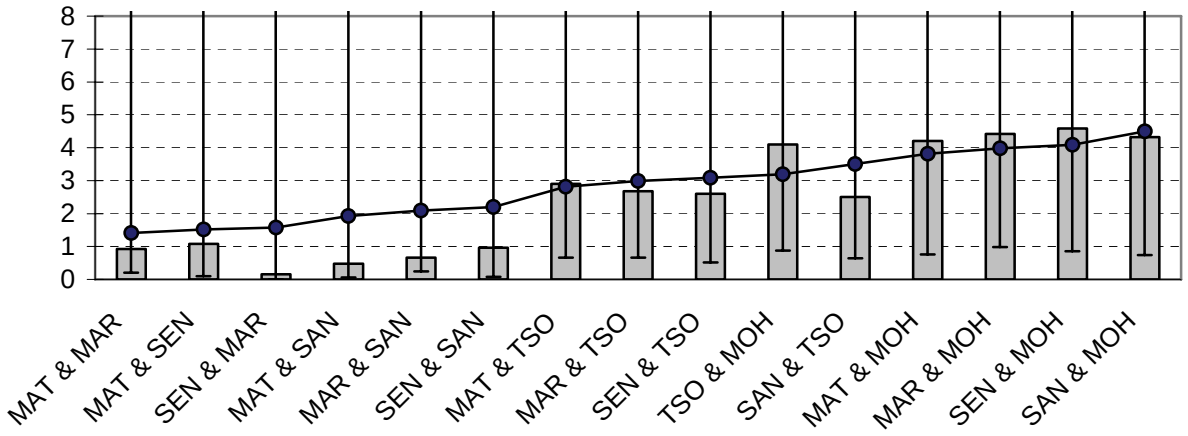
MCMC approach

Based on control region, confidence limits for the posterior distribution of the parameter *M* ranged between zero and 5.9 (Fig. 2.4A). The only exception was the comparison between Senqu and Moremoholo where the maximum limit of *M* could not be established. The value of $M = 0$ could not be rejected in the comparison between Moremoholo and Sani. In addition, $M = 0$ was not only within the confidence limits, but also the most likely value for all the comparisons that included the Tsoelikane or Mohale populations. In all other comparisons, zero fell outside the confidence limits. The parameter *T* was more problematic, since the maximum value of the confidence interval could not be established for any of the pairwise population comparisons (Fig. 2.4B). An estimate of zero divergence time was rejected in all comparisons, except where this value was included in the confidence interval of the Senqu and Moremoholo comparison. In all comparisons involving Mohale, θ was greater than 2.3, whereas in all other pairwise comparisons θ was estimated to be less than 1.5. This possibly indicates large effective population size of the Mohale population if one assumes mutation rate to be constant (Fig. 2.4C).

A (Parameter *M*)



B (Parameter *T*)



C (Parameter θ)

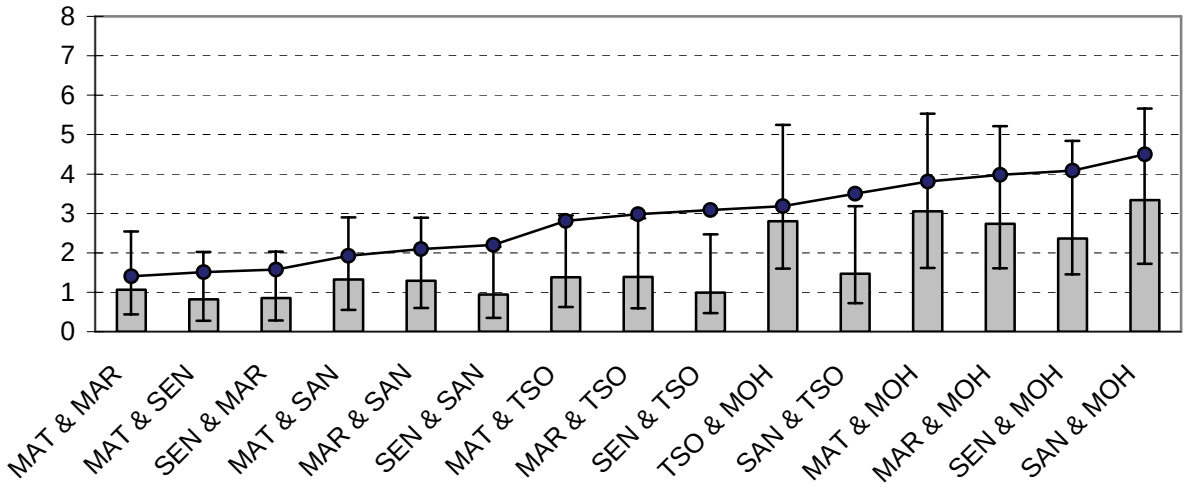


Fig. 2.4. Histograms of the parameters *M*, *T* and θ in pairwise population comparisons of *P. quathlambae* as simulated with a MCMC approach, plotted against the pairwise geographic distances between populations (circles connected with a line), shown in figures A, B and C respectively. "Error bars" refer to the minimum and maximum likelihood confidence intervals of the posterior distributions of the parameters *M*, *T* and θ as estimated with likelihood ratio tests (MAT = Matsoku; SEN = Senqu; MAR = Moremoholo; SAN = Sani; TSO = Tsoelikane; MOH = Mohale).

Cytochrome b *differentiation*

Twenty-six individuals were analysed for cytochrome *b* variation, but only five alleles were detected. None of these were shared between Mohale and the eastern populations (Table 2.7). Fifteen of the 500 nucleotide sites were variable, including 12 parsimony informative and three autapomorphic mutations. All these nucleotide differences were synonymous at amino acid level. Genetic distances were based on the selected HKY85 substitution model (Hasegawa *et al.*, 1985), with a Ti:Tv ratio of 6.599 and equal rates among sites. A divergence, similar to the control region results, was detected between Mohale and the eastern populations ($0.025 < D < 0.029$) (Fig. 2.2B).

MH alleles

Two MH alleles were detected from the restriction profiles of 60 *P. quathlambae* individuals, but only one of these occurred in the Mohale population (Table 2.7). Mohale therefore showed significant allele frequency differences compared to all the eastern populations based on exact tests ($p < 0.001$). Tsoelikane did not show significant heterogeneity of allele frequencies in a comparison where all the north-eastern populations were grouped ($p = 0.616$) or in comparison to the Matsoku population ($p = 0.285$). There were significant frequency differences between Tsoelikane and Moremoholo ($p = 0.007$) and between the latter and Matsoku ($p < 0.001$). Sample sizes, however, were too low to detect a clear genetic pattern among the eastern populations. The MH alleles were in Hardy-Weinberg equilibrium in all of the populations ($0.125 < p < 1.000$).

Table 2.7. Frequency of mtDNA cytochrome *b* and MH alleles among sampled localities of *P. quathlambae*. See Table 2.1 for locality descriptions.

Allele number	N	MAT1	MAT2	SEN	MOR	SAN2	TSO	MOH7
Mitochondrial cytochrome b								
1	16	1	3	3	2	2	5	-
2	1	-	-	-	1	-	-	-
3	1	-	-	-	-	1	-	-
4	7	-	-	-	-	-	-	7
5	1							1
Total	**26**	**1**	**3**	**3**	**3**	**3**	**5**	**8**
MH class II gene								
Leucine allele	61	4	14	4	7	9	23	-
Valine allele	59	-	2	-	13	1	7	36
Total individuals	**60**	**2**	**8**	**2**	**10**	**5**	**15**	**18**

Discussion

Genetic differentiation patterns

The most striking genetic pattern among existing *P. quathlambae* populations is the differentiation between Mohale and the five eastern populations. This differentiation is evident from the divergence in control region, cytochrome *b*, and from the absence of one of the two MH alleles in the Mohale population. Much of the genetic structuring observed with the AMOVA analysis is explained by differentiation between Mohale and the eastern populations.

The differentiation between Tsoelikane and north-eastern populations is reflected in the lack of sharing of alleles, the AMOVA results and the F_{ST} estimates, but to a much lesser extent in the ϕ_{ST} estimates. This is due to the latter taking genetic distance into account and there was low control region differentiation between Tsoelikane and the north-eastern populations. However, there was a lack of cytochrome *b* differentiation between the Tsoelikane and the north-eastern populations. This is in contrast to the large cytochrome *b* divergence between Mohale and the eastern populations (the latter including Tsoelikane). Genetic patterns among the north-eastern populations were complex. There were several control region alleles that were restricted to certain localities. However, all the localities shared several control region alleles, showed a lack of cytochrome *b* differentiation and had inconclusive MH allele frequency differences.

Evolutionary history and processes

Both the Mohale and Tsoelikane populations have been historically isolated. The lack of sharing of control region alleles and inferences from *F*-statistics, NCA and MCMC provided evidence for isolation rather than migration-type processes when either the Mohale or Tsoelikane populations were involved in population comparisons. Coalescence occurred among alleles of the Mohale lineage and among alleles of the eastern lineage long before these two lineages connected. Therefore, apart from recognising that historical isolation and subsequent differentiation occurred, the specific historical event that led to the current distributions cannot be inferred (Templeton *et al.*, 1995).

Similarly, it is unsure whether fragmentation of a larger eastern population or distant colonization preceded isolation between Tsoelikane and the north-eastern populations. The Mohale population, however, was isolated much earlier compared to the relatively recent isolation of Tsoelikane. This is evident from low control region and a lack of cytochrome *b* differentiation in the latter, compared to large divergences at both mitochondrial markers in the former. The potential elimination of internal alleles in the Tsoelikane population (see clade 4-2, Fig. 2.3) may be related to the bottleneck of 56 translocated individuals in 1973 (Pike & Tedder, 1973). The latter may be the only case where recent anthropogenic disturbance is reflected at mtDNA level in *P. quathlambae*.

There was evidence for both isolation and migration-type processes among the north-eastern popultions. Past migration-type processes cannot be rejected among any of the north-eastern populations, apart from possibly the Moremoholo and Sani comparison. This suggests that the north-eastern populations share a more recent history of migration and/or colonization, as

opposed to comparisons where Tsoelikane or Mohale were involved. The alleles that were private to some populations, nonetheless suggest that some level of isolation also played a role. The Senqu and Moremoholo populations (only 157 km apart) apparently shared a more recent history of migration or more historical gene flow than any of the other pairwise population comparisons. A recent history of migration cannot be rejected between these two populations with exact tests of heterogeneity of allele frequencies, *F*-statistics, NCA or MCMC analysis of migration versus isolation. In the case of the latter analysis, $M = 10$ fell within the confidence interval of the Senqu and Moremoholo comparison. In re-runs of the data, even the maximum value of $M = 20$ fell within the confidence interval, suggesting a wide range of possible migration rates.

Stable population size cannot be rejected as a demographic scenario with mismatch distribution analysis and neutrality tests for the Mohale population, a combination of the Senqu and Moremoholo populations, the north-eastern populations or the eastern populations. This is surprising, since one would expect major demographic instability within *P. quathlambae* populations due to the harsh environmental conditions that they are exposed to. However, vicariance within and among populations, probably have a major effect on these analyses, especially where different populations were grouped because of low sample size.

The large size of the Mohale population was reflected by the larger estimates of θ (MCMC analysis) and the relatively large gene diversity estimate in all the pairwise comparisons in which this population was included. Matsoku, Sani and Tsoelikane, however, did not differ significantly from Mohale in terms of gene diversity, possibly suggesting larger historical population size. This result is surprising for at least the Tsoelikane population, considering that it has gone through a recent bottleneck of only 56 individuals. Genetic divergence times

are notoriously difficult to assess (Graur & Martin, 2004) and this was also evident from the MCMC analysis of the parameter *T*. A maximum *T* = 20 fell within the confidence limit of all the population comparisons and a wide range of possibilities cannot be excluded.

Isolation mechanisms

The waterfall that marks the lower limit of the Mohale population would have prevented upstream migration for a considerable period of time. In addition, the large geographic distances between Mohale and the eastern populations (319 - 481 km) may have led to isolation by distance. The interruption of gene flow was probably exaggerated by the lack of continuous suitable habitat and the occurrence of large native cyprinids and clariid and bagrid catfish, especially in mainstream areas. The explanation for the isolation of the Tsoelikane population is probably similar to the Mohale scenario. Large geographic distances (281 – 354 km) with intermittent suitable habitat and potential competition from large native mainstream fishes, probably interrupted gene flow, although to a lesser extent or later in the history of the Tsoelikane population compared to the Mohale population.

The hypotheses of competition avoidance and habitat preference cannot be tested directly, because of the fragmentation and isolation caused by trout and the deterioration of mainstream habitats. However, genetic structuring among redfin populations in different tributaries of the same river system, seems to be the rule rather than the exception (Bloomer & Impson, 2000; Swartz *et al.*, 2004). This suggests that *P. quathlambae*, like other redfin species studied thus far, naturally prefers tributary streams. Considering the harsh environment that *P. quathlambae* have to endure and because they are mostly the only fish species where they occur, a hypothesis of competition avoidance might apply.

Isolation factors must have been less severe for *P. quathlambae* in north-eastern Lesotho. Both colonization and migration would have occurred over relatively shorter distances among the currently isolated north-eastern populations (141 – 260 km), compared to larger distances between the latter populations, Tsoelikane and Mohale (281 – 481 km). Distances among the north-eastern populations must have been much smaller because of larger downstream population sizes, before the recent fragmentation and isolation caused by trout. The inference of migration-type processes among north-eastern populations therefore makes sense in terms of geographical proximity, but other isolation factors such as habitat availability, competition from mainstream fish and the occurrence of waterfalls must also have been less severe.

It is possible that there were high levels of gene flow between the Senqu and Moremoholo populations, or that they were a single population before the introduction of trout. However, a wide range of possible migration rates was inferred between these populations. A reason for this might be because of unidirectional gene flow from the Senqu to the Moremoholo population, as all of the control region alleles that occur in the Senqu population also occur in the Moremoholo population. Waterfalls that would have prevented upstream gene flow mark the lower limits of both the Senqu and Moremoholo populations. The waterfall on the Moremoholo, however, is much smaller and may have become insurmountable for small fish relatively recently.

Value of the Mohale population and conservation of overall evolutionary processes

The most important evolutionary processes that must be allowed to continue within *P. quathlambae* are relatively simple to manage. The differentiation of the Mohale and Tsoelikane populations requires only that they remain isolated, provided that population size

remains large. Securing the survival of the Mohale population will be difficult, however, due to the impact of the Mohale dam. This population represents a unique and divergent lineage within the species. It therefore contributes significantly to the overall genetic diversity and it is also at present the largest population. It can be considered to be an Evolutionarily Significant Unit (ESU) compared to the eastern populations according to Moritz's (1994) definition (historically isolated and divergent) and criteria (monophyly of mtDNA and nuclear DNA frequency differences). Based on the findings of the present study and the survey of Skelton *et al*. (2001), several measures, including transplantation to sanctuary sites, were undertaken to conserve the Mohale population (Rall *et al*., 2002). Lack of differentiation at cytochrome *b* and inconclusive MH allele frequency differences suggest that the Tsoelikane population may not be a different ESU compared to the north-eastern populations. It should be managed in a similar way to Mohale, however, since the underlying evolutionary process is isolation. Eradication of trout downstream will allow *P. quathlambae* to re-occupy its former natural distribution range in this catchment.

According to Moritz's (1994) definition, each of the six populations can be considered to be current management units, because of recent fragmentation and isolation. Only the combination of the Senqu and Moremoholo populations is possibly a single historical management unit, before the current isolation caused by trout. Under present circumstances, it will be difficult to allow a process of restricted gene flow to continue between the Senqu and Moremoholo populations. Major trout eradication programs will be needed to re-connect such historical corridors. If these were to happen, the effect on migration between these two populations would be interesting to investigate. A more realistic goal would be to secure all the existing populations by increasing their range and size by eradicating trout to a downstream barrier to prevent re-invasion. This is needed urgently for the Matsoku and Sani

populations that co-occur with trout. The present study provided a unique opportunity to assess the value of a single population of a threatened fish species and to directly influence conservation actions, before a major impact occurred.

Acknowledgements

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Chapter 3

Sea level changes, river capture and the evolution of populations of the Eastern Cape and fiery redfins (*Pseudobarbus afer* and *P. phlegethon*, Cyprinidae) across multiple river systems in South Africa

Abstract

Lower sea levels than at present, most notably a – 130 m sea level during the last glacial maximum about 18 000 years ago, seems to have played a dominant role over river capture in allowing currently isolated river systems on the south coast of South Africa to share common confluences before reaching the sea, possibly forming only a few palaeoriver systems. This may have allowed the primary freshwater species, *Pseudobarbus afer*, and its unique genetic lineages to have a wide distribution across at least 22 such river systems, possibly expanding its range during times of lower sea level. Four major lineages, identified through analysis of mtDNA control region sequences, show a strong association with the proposed palaeoriver systems. A western "Forest" lineage, however, is widespread across two such proposed systems and is closely related to *P. phlegethon* on the west coast of South Africa. The Gourits River system is the only pathway that can realistically explain this relationship. Both the "Krom" and "St. Francis" lineages were identified in the single palaeoriver system proposed for St. Francis Bay. A fourth "Algoa" lineage is restricted to the one or two palaeoriver systems proposed for Algoa Bay. Four minor lineages (Klein Brak, Wilderness Lakes region, Plettenberg Bay and Tsitsikamma) were identified within the "Forest" lineage and two (Swartkops and Sundays) within the "Algoa" lineage. The dominant evolutionary process of isolation should be allowed to continue, whilst keeping local population size large enough to ensure continued survival of these unique lineages.

Introduction

The Eastern Cape redfin (*Pseudobarbus afer*) has been recognised as a polytypic species with several morphological characters that vary within and among its populations (Skelton, 1988). The species was described from an eastern population within its distribution, probably from the Swartkops River system (Jubb, 1965). Smith (1936) described *Barbus senticeps* from the Krom River system near the centre of the current distribution of *P. afer*, but did not compare this species to *P. afer* (in *Barbus* at that stage) or any other described species of redfin. Jubb (1965) later synonimised *B. senticeps* with *B. afer*, but the taxonomic confusion persisted.

There was confusion especially between *P. afer* and *P. asper* with western coastal populations of *P. afer* initially included in *P. asper* (Barnard, 1943; Jubb, 1965). When Skelton (1988) described the genus *Pseudobarbus*, however, he concluded that these western coastal populations belong to *P. afer* and that *P. asper* was a distinct species that is restricted to the Gourits and Gamtoos River systems. In addition, he showed that scale counts (reflecting scale size) varied among different populations of *P. afer* from the western coastal area, the more central Gamtoos River system and from the eastern part of its range. The notion that *P. afer* is a polytypic has been strengthened by the phylogenetic analyses based on mitochondrial DNA in Chapter 6. Apart from the analysis that was only based on morphological characters, all the other analyses based on the genetic dataset or combined genetic and morphological datasets showed that *P. phlegethon* groups within *P. afer*. This suggests that *P. afer* is polyphyletic and that *P. asper* is not the sister species of *P. afer*.

Pseudobarbus afer as it is defined today is the most widespread redfin species, occurring between the Klein Brak River system that flows into Mossel Bay in the west to the Sundays River system near Port Elizabeth in the east (Skelton, 1988), a distance of approximately 380 km (Fig. 3.1) They have been recorded in as many as 22 river systems (Rippon, 1996; Russell, 1999; Skelton, 1988; 1994b). Most of these are small coastal river systems that do not penetrate the coastal mountain ranges of the southern Cape Fold Mountains that run parallel to the coast. Three of the river systems, the Keurbooms, Krom and Swartkops, penetrate these coastal mountain ranges and two of the river systems, namely the Gamtoos and Sundays, are even larger and drain from the interior of the Western and Eastern Cape Provinces of South Africa. *Pseudobarbus phlegethon* is restricted to tributaries of the Olifants and Doring catchments of the Olifants River system on the west coast (insert in Fig. 3.1).

The majority of the rivers in which *P. afer* occur, originate in or almost entirely flow over sediments consisting of Table Mountain sandstones in the west and Witteberg shales in the east (Keyser, 1998), that yield low mineral and suspension loads (Day *et al.*, 1998). In the west, rivers drain through Southtern Afrotemperate Forest (Mucina & Rutherford, 2004) and are generally acidic and dark peat stained (Russell, 1999). In the central areas the rivers are also acidic, but are clearer, since they drain areas with mostly Kouga and Tsitsikamma Sandstone Fynbos, Kouga Grassy Sandstone Fynbos and Groot and Gamtoos Thicket (Mucina & Rutherford, 2004). In the east, rivers are clear or slightly turbid, more alkaline and drain areas with Kouga Grassy Sandstone Fynbos and Suurberg Shale or Quartzite Fynbos (Mucina & Rutherford, 2004; Russell, 1998/1999). *Pseudobarbus phlegethon* occur in clear mountain streams that flow over Table Mountain sandstone (Keyser, 1998) in catchments with Olifants and Cederberg Sandstone Fynbos (Mucina & Rutherford, 2004) that therefore yield low mineral and suspension loads.

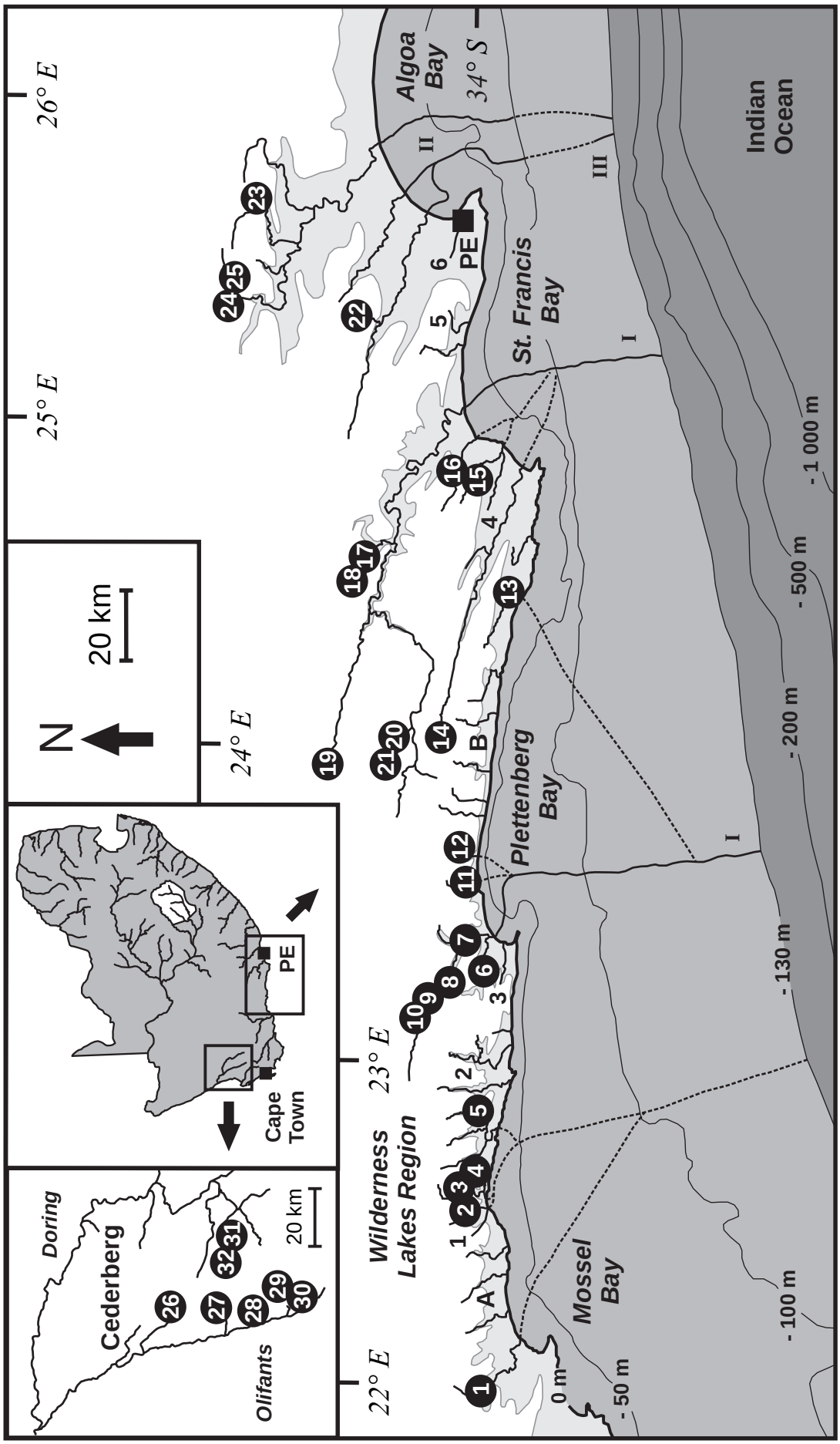

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Fig. 3.1. (Previous page) Localities indicated in circled numbers show from where *P. afer* (Klein Brak [1], Kaaimans [2], Touws [3], Duiwe [4], Karatara [5], Bitou [6], Keurbooms [7-10], Groot [11], Bloukrans [12] and Tsitsikamma [13]; Krom lineage: Krom [14]; St. Francis lineage: Swart [15], Kabeljous [16] and Gamtoos [17-21]; Algoa lineage: Swartkops [22] and Sundays [23-25]) and *P. phlegethon* (Rondegat [26], Boskloof [27], Noordhoeks [28], Thee [29], Oudste [30] and Breekkrans [31]) were collected and analysed. Un-circled numbers show other river systems where *P. afer* have been recorded (Swart (George) [26], Goukamma and Knysna [27], Piesang [28], Seekoei [29], Maitlands [30] and Baakens [31]). Distribution gaps are across the Groot Brak, Malgas and Gwaing River systems (A) and from the Bloukrans to Tsitsikamma River systems (B). The light shaded area that may have been unavailable for *P. afer* during the early Pliocene transgression. Possible LGM palaeoriver courses based on the geological literature are shown in solid lines in the moderate shaded area (I = Dingle & Rogers (1972); II = Bremner & Day (1991); III = Dingle *et al.* (1987)) and those inferred from mostly the available bathymetry are shown in dashed lines. Inserts show the Olifants River system with circled numbers from where *P. phlegethon* was collected and the study areas within South Africa (PE = city of Port Elizabeth).

Pseudobarbus afer is not considered to be threatened, but rather is listed as near threatened (IUCN, 2003), because of its wide distribution. However, certain local populations are seriously threatened, which could result in the loss of unknown unique lineages. The *P. afer* populations are mainly affected by the introduction of alien fishes and agricultural activities such as excessive water extraction. Two puzzling gaps have been identified in the distribution of *P. afer* across three river systems in the west of its distribution and it has not been recorded from at least 11 small river systems close to the central area of its distribution (Skelton, 1988). In contrast, *P. phlegethon* has been under threat of extinction since the first red data lists were compiled for South Africa (Skelton, 1977). It is currently listed as endangered (IUCN, 2003), mainly because of the introduction of North-American bass species, particularly *Micropterus dolomieu* that has been able to penetrate tributary streams (Bills, 1999; Swartz *et al.*, 2004).

Historically, major climatic and geological changes would have influenced the distribution and evolution of *P. afer* and *P. phlegethon*. River capture is often presented as a way for fishes from different river systems to share a recent history of gene flow (Brito *et al.*, 1997; Mesquita *et al.*, 2001; Waters & Wallis, 2000). However, in the case of *P. afer*, the confluence of rivers during low sea levels possibly played a more important role in shaping gene flow patterns. Sea level has ranged between more than 400 m above (Dingle *et al.*, 1983) to less than 400 m below (Siesser & Dingle, 1981) current sea level in relation to the South African coastline. The southern coastal area where *P. afer* occurs, has been rising tectonically and only became stable in Quaternary times (Maud, 1990).

The last major transgression occurred during the early Pliocene (about 3.4 - 5.2 MYA) and reached levels of around + 200 m (Butzer & Helgren, 1972) to over + 300 m (Siesser & Dingle, 1981) along the south coast of South Africa. During this time many river systems would have been drowned, but ever since the early Pliocene transgression, sea levels have not risen more than + 30m above present sea levels (Butzer & Helgren, 1972; Rogers, 1985) and thus would only have affected the smallest and lower altitude river systems. Several major regressions, however, have occurred since the major transgression of the early Pliocene, most notably a regression of approximately – 130 m as recently as the last glacial maximum (LGM) around 18 000 years ago (Ramsay & Cooper, 2002; Rogers, 1985; Tankard, 1976). These regressions would certainly have allowed several different river systems to have a common confluence before reaching the sea. Since bays can probably be extended seawards as natural valleys, one would expect that river systems that flow into the same bay are more likely to share a common confluence. Such river systems are therefore more likely to have fish populations that share a more recent history of colonization, migration, introgression or hybridisation, than those occurring between such bays.

The first aim of this paper was to construct a map of the possible offshore drainage patterns during the last major regression event based on available bathymetry and geological studies. This map was used to assess which river systems were likely to have been connected during sea level regressions. This would allow for more realistic geographic distances to be measured among sampling localities for use in geographic genetic analysis. Secondly, the aim was to assess the geographic genetic structuring and differentiation within the *P. afer* and *P. phlegethon* complex by analysing the mtDNA control region variation among as many of the existing population as possible.

The mtDNA control region lacks structural genes and mitochondrial DNA is only maternally inherited (Moritz *et al.*, 1987). However, mitochondrial DNA in general and control region specifically has been successfully employed in intraspecific phylogeographic studies because of its relatively fast mutation rate and genealogical history that is free of recombination (Brown *et al.*, 1993; Moritz, 1994; Moritz *et al.*, 1987; Suárez *et al.*, 2001; Waters & Wallis, 2000). Thirdly, an assessment was made to determine which evolutionary processes (migration and isolation type processes) played an important role in the genetic patterns we see today. Finally, an attempt was made to associate these evolutionary processes with the geological and climatic processes of river capture and confluence of river systems during low sea levels.

Materials and Methods

Sampling

Pseudobarbus afer and *P. phlegethon* specimens were caught with a 3m seine net or by snorkelling with a hand net. Muscle or whole fish samples were stored in liquid nitrogen in the field and transferred to a –70 °C freezer upon returning to the laboratory or muscle, fin-clips or whole fish samples were placed in EtOH (Department of Genetics, University of Pretoria). The source specimen and/or additional specimens were fixed in 10% formaldehyde and deposited in the South African National fish collection (South African Institute for Aquatic Biodiversity, Grahamstown) as voucher specimens.

Map reconstructions and geographic distance measurement

Maps of possible palaeoriver courses were constructed based on the bathymetry of the South African Navy Charts, the bathymetry proposed by Birch *et al.* (1978) for the Wilderness Lakes region, seismic profiling of offshore sediments by Birch (1980), Birch *et al.* (1978) and Bremner & Day (1991) and reviews published on offshore stratigraphical, sedimentological and bathymetric studies by Dingle *et al.* (1987) and Dingle & Rogers (1972). Geographic distance between sampled localities were measured along current river courses from a GIS layer (South African Department of Environmental Affairs and Tourism) and where necessary, along the hypothetical palaeoriver courses.

If it was assumed that certain river systems did not connect before reaching the continental shelf at any stage, then the geographic distance measurement followed the – 200 m contour line between the proposed palaeoriver systems, since this contour line is very close to the edge of the continental shelf on the south coast and relatively close to the – 130 m contour that is used as a surrogate for the LGM's coastline. For comparison in the genetic analysis, geographic distances among sampled localities were also measured along current river courses and along the current coastline (Fig. 3.1). The + 200 m contour was used as a surrogate for the high sea level relative to land of the early Pliocene transgression (Butzer & Helgren, 1972).

DNA extraction, amplification and sequencing

Total DNA was isolated from frozen or EtOH preserved tissue using standard protocols of chemical digestion and phenol/chloroform extraction (Sambrook *et al.*, 1989), followed by amplification (PCR) with primers specially designed for amplification in *Pseudobarbus* (Chapter 2), namely L16560 (5' CCAAAGCCAGAATTCTAAC 3') in the tRNA (Thr) on the 5' side of control region and H677 (5' GTCGCGCAAAAACCAAAG 3') at the 3' side of control region. Primer names are according to sequence positions of the common carp (*Cyprinus carpio*) published by Chang *et al.* (1994). Amplification was performed in 50 µl volumes containing 1 x buffer, 2 mM $MgCl_2$, 0.2 mM of each of the four nucleotides (Promega), 25 pmol of each primer, 1.5 U of Super-Therm DNA polymerase (Southern Cross Biotechnology) and 100-200 ng template DNA. Conditions for amplification were 2 minutes at 94°C, then 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 45 seconds at 72°C, finishing with 5 minutes at 72°C. PCR products were purified using the High Pure™ PCR Product Purification Kit (Boehringer Mannheim), followed by elution in ddH_2O.

Cycle sequencing was performed in 10 µl volumes, containing 100 ng of purified DNA as template, 1.6 pmol primer (either L16560 or H677 mentioned above) and 2 µl of ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). PCR and cycle sequencing was performed in a Geneamp® PCR System 9700 (Applied Biosystems) and nucleotide sequences were determined through an ABI 377 automated sequencer. Consensus sequences of a total of 605 base pairs were obtained from the forward and reverse sequences and by comparing these to other sequences through alignment and inspection in Sequence Navigator 1.01 (Applied Biosystems). Consensus sequences were aligned using Clustal X (Thompson *et al.*, 1997) and checked manually.

Genetic analysis

The model of nucleotide substitution that best fits the data was selected from 56 models with a hierarchical likelihood ratio test in MODELTEST version 3.06 (Posada & Crandall, 1998). With the same program, base frequencies, Ti:Tv ratio, proportion of invariable sites (I) and the α value of the gamma distribution (rate variation among sites) were estimated. Genetic distances among the alleles were based on these parameters. These parameters were also used in a neighbour-joining estimation of phylogenetic relationships (Saitou & Nei, 1987), which was done in the program PAUP* (Swofford, 2002). In addition, a genealogy was estimated with the program TCS (Clement *et al.*, 2000), based on 95% confidence of connections among alleles (Templeton *et al.*, 1992).

Gene (δ) and nucleotide diversity (π) and their standard errors were calculated for each lineage, using ARLEQUIN version 2.000 (Schneider *et al.*, 2000). The standard errors take sampling variance into account in the case of gene diversity and both sampling and stochastic variance into account in the case of nucleotide diversity. In the same program, an AMOVA (Excoffier *et al.*, 1992) was performed and tested for significance with permutation tests (10 000 replicates) on the major lineages defined as a hierarchical structure, with (1) *P. afer* from the Klein Brak to Tsitsikamma River systems, (2) the Krom River system, (3) the Swart to Gamtoos River systems, (4) the Swartkops and Sundays River systems and *P. phlegethon* from the Olifants River system as groups (Fig. 3.1).

Regions within these groups were defined as the Klein Brak, Wilderness lakes region (Kaaimans to Karatara), Plettenberg Bay (Keurbooms to Bloukrans), Tsitsikamma, Krom, rest of St. Francis Bay (Swart, Kabeljous and Gamtoos), Swartkops, Sundays and the Olifants and Doring catchments of the Olifants River system. Pairwiseϕ_{ST}, which takes allele frequencies and nucleotide differences between alleles into account, was also calculated among all regions using the same program. The Tamura-Nei model of substitution with the gamma correction found in MODELTEST 3.06 was used to calculate distances on which ϕ_{ST} was based.

Alleles in the genealogy were nested hierarchically from the tips to the interior without nesting interiors until they could be nested with tip clades (Cunningham, 2002). Exact contingency tests were performed on each nested clade to test whether a scenario of no association between alleles or clades and their geographic location could be rejected (Templeton & Sing, 1993) by comparing observed χ^2 values to distributions of χ^2 generated from 10000 random permutations of the original data in the program GEODIS version 2.0 (Posada *et al.*, 2000).

With the same program, clade distances (D_c), nested clade distances (D_n), average interior versus tip clade distances (IT_c) and average interior versus tip nested clade distances (IT_n) were calculated based on the nested design and the geographic distances as explained above. According to Templeton *et al.* (1995), D_c is a measure of how geographically widespread individuals in a clade are, and D_n is a measure of the geographic distribution of individuals in a clade compared to all individuals in the nested clade. Different historical processes influence these geographic distance measures (D_c, D_n, IT_c and IT_n) in particular ways and can possibly indicate what type of process has occurred (Templeton *et al.*, 1992). Templeton's

(2004) inference key was used to assist in interpreting these distance patterns and to help classify evolutionary processes as either migration-type or isolation-type processes.

Results

Survey

A river and all its tributaries was considered to be a river system if it flows directly into the sea or an estuary or one of the brackish Wilderness coastal lakes, in other words, redfins would not be able to travel to other river systems under normal circumstances, since they are primary freshwater species. A total of 49 *P. afer* specimens was collected and analysed from 16 such river systems and 25 localities, namely the Klein Brak (4), Kaaimans (2), Touws (1), Duiwe (2), Karatara (2), Bitou (2), Keurbooms (4), Groot (2), Bloukrans (2), Tsitsikamma (2), Krom (7), Swart (2), Kabeljous (3), Gamtoos (5), Swartkops (2) and Sundays (7) River systems. More than one locality was analysed from the Keurbooms (4), Gamtoos (5) and Sundays (3) River systems (Table 3.1; circled numbers in Fig. 3.1).

Apart from these, *P. afer* was not recorded from four localities in the Maitlands River system or at single localities each in the Swart (near George) and Piesang River systems, despite previous records of their presence (Rippon, 1996; Skelton, 1988). It might still occur in the Seekoei and Baakens River systems (Skelton, 1988), but these river systems were not visited during recent surveys. In addition, *P. afer* was recorded from the Knysna and Goukamma River systems but were not included in the analysis. This species was not recorded, however, from a further six river systems and 14 localities within its possible range, in the Groot Brak (4), Gwaing (2), Malgas (1), Hoogekraal (west of Karatara) (1), Noetsie (east of Knysna) (2),

Groot-Klip (west of Tsitsikamma) (2) and Van Stadens (west of Maitlands) (1) River systems (Fig. 3.1).

In contrast to *P. afer*, the distribution of *P. phlegethon* has been well established (Bills, 1999; Swartz, 2000; Swartz *et al.*, 2004; E. R. Swartz *et al*., unpublished). Of the seven remaining populations, only the Driehoeks population was not included in the present study. A total of 11 specimens were analysed from the Rondegat (1), Boskloof (1), Noordhoeks (2), Thee (1), Oudste (2) and Breekkrans (4) tributaries of the Olifants River system.

Table 3.1. Localities from where *P. afer* and *P. phlegethon* specimens were collected and analysed.

Map	Locality	System	Code	N	Lattitude	Longitude	Altitude (m)	Date
1	Klein Brak	Klein Brak	KLE	4	33° 57’ 10” S	21° 58’ 45” E	210	22/04/2000
2	Kaaimans	Kaaimans	KAA	2	33° 58’ 14” S	22° 33’ 30” E	65	16/04/2000
3	Touws	Touws	TOU	1	33° 56' 44" S	22° 36' 46" E	125	12/09/1998
4	Duiwe	Duiwe	DUI	2	33° 59’ 00” S	22° 39’ 00” E	10	13/04/2000
5	Karatara	Karatara	KAR	2	33° 58’ 55” S	22° 50’ 11” E	10	16/04/2000
6	Bitou	Bitou	BIT	2	34° 01’ 29” S	23° 16’ 12” E	140	08/04/2000
7	Lower Keurbooms	Keurbooms	KEU 1	1	33° 56’ 17” S	23° 22’ 00” E	18	09/04/2000
8	Middle Keurbooms	Keurbooms	KEU 2	1	33° 53’ 22” S	23° 15’ 46” E	135	10/04/2000
9	Kwaai	Keurbooms	KEU 3	1	33° 48’ 50” S	23° 10’ 47” E	300	11/04/2000
10	Voogt’s	Keurbooms	KEU 4	1	33° 48’ 34” S	23° 10’ 30” E	300	11/04/2000
11	Groot	Groot	GRO	2	33° 58’ 08” S	23° 33’ 35” E	1	05/04/2000
12	Bloukrans	Bloukrans	BLO	2	33° 57’ 10” S	23° 39’ 30” E	80	05/04/2000
13	Tsitsikamma	Tsitsikamma	TSI	2	34° 05’ 49” S	24° 26’ 52” E	18	02/04/2000
14	Krom	Krom	KRO	7	33° 51’ 48” S	23° 58’ 46” E	480	06/05/2000
15	Swart	Swart	SWA	2	33° 58’ 39” S	24° 46’ 46” E	225	01/04/2000
16	Kabeljous	Kabeljous	KAB	3	33° 56’ 26” S	24° 48’ 17” E	185	01/04/2000

Table 3.1. Continued.

Locality		System	Code	N	Lattitude	Longitude	Altitude (m)	Date
17	Ys	Gamtoos	GAM 1	1	33° 39’ 58” S	24° 34’ 25” E	375	08/05/2000
18	Witte	Gamtoos	GAM 2	1	33° 39’ 13” S	24° 32’ 06” E	560	09/05/2000
19	Baviaanskloof	Gamtoos	GAM 3	1	33° 32’ 14” S	23° 56’ 03” E	510	09/05/2000
20	Opkoms	Gamtoos	GAM 4	1	33° 46’ 26” S	24° 01’ 14” E	200	08/05/2000
21	Braam	Gamtoos	GAM 5	1	33° 43’ 58” S	23° 57’ 40” E	145	07/05/2000
22	Swartkops	Swartkops	SKO	2	33° 40’ 20” S	25° 19’ 00” E	330	03/03/2001
23	Otto’s Pool	Sundays	SUN 1	2	33° 20’ 08” S	25° 41’ 33” E	235	10/05/2000
24	Kaboega *	Sundays	SUN 2	3	33° 15’ 43” S	25° 22' 45” E	325	21/03/2003
25	Kaboega tributary *	Sundays	SUN 3	3	33° 15’ 58” S	25° 25’ 19” E	415	21/03/2003
26	Rondegat	Olifants	OLI 1	1	32° 21’ 05” S	19° 02’ 00” E	380	16/02/1998
27	Boskloof	Olifants	OLI 2	1	32° 33’ 29” S	19° 03’ 32” E	255	23/02/1998
28	Noordhoeks	Olifants	OLI 3	2	32° 43’ 19” S	19° 04’ 14” E	245	18/02/1998
29	Thee	Olifants	OLI 4	1	32° 47’ 40” S	19° 05’ 50” E	290	24/03/1998
30	Oudste	Olifants	OLI 5	2	32° 49’ 33” S	19° 5’ 48” E	280	24/03/1998
31	Lower Breekkrans	Olifants	OLI 6	3	32° 33’ 34” S	19° 17’ 57” E	720	23/03/2002
32	Upper Breekkrans	Olifants	OLI 7	1	32° 33’ 43” S	19° 16’ 41” E	670	25/03/1998

All collections were made by E. R. Swartz and fellow collectors (see acknowledgements), apart from the Kaboega samples that were collected by J. A. Cambray and A. Bok*.

Map reconstructions

Four major historical river systems possibly can be inferred on the south coast from the reconstructions of a – 130 m sea level (light shaded area in Fig. 3.1). It is possible that the two western river systems may have had a common confluence before reaching the latter low sea level, but apart from the direction of the Keurbooms (Dingle & Rogers, 1972), very little information is available regarding the offshore palaeoriver courses of these rivers. From reconstructions based on Bremner & Day (1991) and Dingle *et al.* (1987) it is unclear whether a historical Baakens-Swartkops-Coega River system (Algoa Bay region in Fig. 3.1) would have had a common confluence with the Sundays River system before reaching the – 130 m LGM coastline. It seems as if all the river systems flowing into St. Francis Bay, however, would have had a common confluence before reaching the latter coastline.

Specimens of *P. afer* were collected at altitudes ranging from almost sea level to 560 m above sea level (Table 3.1). Only nine of these 25 localities were at or above an altitude of 300 m (two in the Keurbooms, the Krom locality, three in the Gamtoos, the Swartkops locality and two in the Sundays), which was possibly the maximum level to which the early Pliocene sea level would have transgressed (Dingle *et al.*, 1983) and well above the + 200 m level that is used as a surrogate for this high sea level (Butzer & Helgren, 1972) (insert of Fig. 3.1). The areas now occupied by *P. phlegethon* in the Olifants River system on the west coast would have been unaffected by changes in sea level. The Olifants River system would not have had a common confluence with any other major river system during the LGM. “River distances” among all the sampled localities, ranged from 1 – 1796 km and 1 – 2014 km, depending on whether the – 200 m contour (and proposed palaeoriver courses) or the current sea level was used in the measurements respectively (Table 3.2).

Table 3.2. Geographic distances (km) among sampled localities along inland river courses, proposed palaeoriver courses and the – 200 m contour line (below diagonal) or along inland river courses and the current coastline (above diagonal).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	KLE		84	91	91	121	202	197	211	233	233	203	213	294	444	371	377
2	KAA	173		18	18	47	129	124	138	160	160	130	140	221	370	297	303
3	TOU	178	17		14	50	131	126	140	162	162	132	142	223	373	300	306
4	DUI	178	17	14		50	131	126	140	162	162	132	142	223	373	300	306
5	KAR	166	56	61	61		114	109	123	145	145	114	125	205	355	282	288
6	BIT	360	329	334	334	322		35	49	71	71	41	51	132	282	208	214
7	KEU1	355	324	329	329	317	35		14	36	36	36	46	127	277	203	209
8	KEU2	369	338	343	343	331	49	14		22	22	50	60	141	291	217	223
9	KEU3	391	360	365	365	353	71	36	22		0	72	82	163	313	239	245
10	KEU4	392	360	365	365	353	71	36	22	0		72	82	163	313	239	245
11	GRO	335	303	308	308	296	54	49	63	85	85		14	95	245	172	178
12	BLO	339	307	312	312	300	58	53	67	89	89	32		90	240	167	173
13	TSI	365	333	338	338	326	192	187	201	223	223	166	170		166	93	99
14	KRO	563	531	536	536	524	464	459	473	495	495	438	442	468		135	141
15	SWA	482	451	456	456	444	384	379	393	415	415	358	362	388	190		50
16	KAB	481	450	455	455	443	383	378	392	414	414	357	361	387	189	61	
17	GAM1	560	529	534	534	522	462	457	471	493	493	436	440	466	268	188	187
18	GAM2	565	533	538	538	527	467	462	476	498	498	441	445	471	273	193	192
19	GAM3	645	613	618	618	607	547	542	556	578	578	521	525	551	353	273	272
20	GAM4	649	617	622	622	611	551	546	560	582	582	525	529	555	357	277	276
21	GAM5	658	626	631	631	620	560	555	569	591	591	534	538	564	366	286	285
22	SKO	593	562	567	567	555	495	490	504	526	526	469	473	499	385	305	304
23	SUN1	639	607	612	612	601	541	536	550	572	572	515	519	545	431	351	350
24	SUN2	657	625	630	630	619	559	554	568	590	590	533	537	563	449	369	368
25	SUN3	665	633	638	638	627	567	562	576	598	598	541	545	571	457	377	376
26	OLI1	1345	1314	1319	1319	1307	1367	1362	1376	1398	1398	1341	1345	1371	1569	1489	1488
27	OLI2	1370	1339	1344	1344	1332	1392	1387	1401	1423	1423	1366	1370	1396	1594	1514	1513
28	OLI3	1385	1353	1358	1358	1347	1407	1402	1416	1438	1438	1381	1385	1411	1609	1529	1528
29	OLI4	1392	1361	1366	1366	1354	1414	1409	1423	1445	1445	1388	1392	1418	1616	1536	1535
30	OLI5	1396	1365	1370	1370	1358	1418	1413	1427	1449	1449	1392	1396	1422	1620	1540	1539
31	OLI6	1467	1436	1441	1441	1429	1489	1484	1498	1520	1520	1463	1467	1493	1691	1611	1610
32	OLI7	1469	1438	1443	1443	1431	1491	1486	1500	1522	1522	1465	1469	1495	1693	1613	1612

Table 3.2. Continued.

		17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	KLE	476	481	561	565	574	508	581	599	607	1355	1380	1395	1402	1406	1477	1479
2	KAA	403	408	488	492	501	435	508	526	534	1367	1392	1406	1414	1418	1489	1491
3	TOU	405	410	490	494	503	437	510	528	536	1374	1399	1414	1421	1425	1496	1498
4	DUI	405	410	490	494	503	437	510	528	536	1374	1399	1414	1421	1425	1496	1498
5	KAR	388	393	473	477	486	419	493	511	519	1404	1429	1443	1451	1455	1526	1528
6	BIT	314	319	399	403	412	346	419	437	445	1485	1510	1525	1532	1536	1607	1609
7	KEU1	309	314	394	398	407	341	414	432	440	1480	1505	1520	1527	1531	1602	1604
8	KEU2	323	328	408	412	421	355	428	446	454	1494	1519	1534	1541	1545	1616	1618
9	KEU3	345	350	430	434	443	377	450	468	476	1516	1541	1556	1563	1567	1638	1640
10	KEU4	345	350	430	434	443	377	450	468	476	1516	1541	1556	1563	1567	1638	1640
11	GRO	277	282	362	366	375	309	382	400	408	1486	1511	1526	1533	1537	1608	1610
12	BLO	273	278	358	362	371	304	378	396	404	1496	1521	1536	1543	1547	1618	1620
13	TSI	198	203	283	287	296	230	303	321	329	1577	1602	1617	1624	1628	1699	1701
14	KRO	241	246	326	330	339	272	346	364	372	1727	1752	1766	1774	1778	1849	1851
15	SWA	150	155	235	239	248	182	255	273	281	1654	1679	1693	1701	1705	1776	1778
16	KAB	136	141	221	225	234	168	241	259	267	1660	1685	1699	1707	1711	1782	1784
17	GAM1		5	115	119	128	240	313	331	339	1759	1784	1799	1806	1810	1881	1883
18	GAM2	5		120	124	133	245	318	336	344	1764	1789	1804	1811	1815	1886	1888
19	GAM3	115	120		130	139	325	398	416	424	1844	1869	1884	1891	1895	1966	1968
20	GAM4	119	124	130		9	329	402	420	428	1848	1873	1888	1895	1899	1970	1972
21	GAM5	128	133	139	9		338	411	429	437	1857	1882	1897	1904	1908	1979	1981
22	SKO	383	388	468	472	481		177	195	203	1791	1816	1831	1838	1842	1913	1915
23	SUN1	429	433	513	517	526	318		90	98	1864	1889	1904	1911	1915	1986	1988
24	SUN2	447	451	531	535	544	336	90		8	1882	1907	1922	1929	1933	2004	2006
25	SUN3	455	460	540	544	553	344	98	8		1890	1915	1930	1937	1941	2012	2014
26	OLI1	1567	1572	1652	1656	1665	1600	1646	1664	1672		59	74	81	85	268	270
27	OLI2	1592	1597	1677	1681	1690	1625	1671	1689	1697	59		29	36	40	293	295
28	OLI3	1607	1611	1691	1695	1704	1640	1685	1703	1711	74	29		13	17	308	310
29	OLI4	1614	1619	1699	1703	1712	1647	1693	1711	1719	81	36	13		12	315	317
30	OLI5	1618	1623	1703	1707	1716	1651	1697	1715	1723	85	40	17	12		319	321
31	OLI6	1689	1694	1774	1778	1787	1722	1768	1786	1794	268	293	308	315	319		2
32	OLI7	1691	1696	1776	1780	1789	1724	1770	1788	1796	270	295	310	317	321	2	

River systems: KLE = Klein Brak, KAA = Kaaimans, TOU = Touws, DUI = Duiwe, KAR = Karatara, BIT = Bitou, KEU = Keurbooms, GRO = Groot, BLO = Bloukrans, TSI = Tsitsikamma, KRO = Krom, SWA = Swart, KAB = Kabeljous, GAM = Gamtoos, SKO = Swartkops, SUN = Sundays and OLI = Olifants.

Sequence variation and diversity

The total of 605 base pairs analysed yielded 73 variable sites, with 57 parsimony informative (6 with gaps) and 16 autapomorphic characters (3 with gaps). The variable sites defined 29 alleles in the 60 individuals that were analysed. The HKY85 substitution model (Hasegawa *et al.*, 1985) with a Ti:Tv ratio of 4.378, I = 0.697 and α = 0.592 was selected with MODELTEST.

Five major lineages (Forest, Krom, St. Francis, Algoa and Cederberg) are evident from the neighbour-joining tree (Fig. 3.2), which shows the genetic distances among lineages according to the parameters found in MODELTEST. The range of genetic distances within the identified historical lineages (*D* = 0 - 2.3%) did not overlap with those between lineages (*D* = 3.1 - 10.8%). The largest range of genetic distances among alleles within lineages was found in the Forest lineage (*D* = 0.2 - 2.3%), mainly because *P. afer* specimens from the Klein Brak River system, the Wilderness Lakes region, the Plettenberg Bay area and the Tsitsikamma River system respectively, can be considered as minor lineages even though there is a slight overlap in genetic distances within these minor lineages (*D* = 0 – 1.3%) compared to between them (*D* = 0.9 – 2.3%). The range of genetic distances within the Algoa lineage was also large as a result of differentiation between the Swartkops and Sundays River systems (*D* = 1.3 - 1.7%), with little differentiation within them (*D* = 0 - 0.3%). The range of genetic distances was lowest within the St. Francis lineage (*D* = 0 - 0.3%) and the minor genetic distances measured within the Krom lineage is understandable (*D* = 0 - 0.5%), since this lineage was only recorded from a single locality. The circled numbers in Fig. 3.1 show the localities from where the Forest (1-13), Krom (14), St. Francis (15-21), Algoa (22-25) and Cederberg (26-32) lineages were collected.

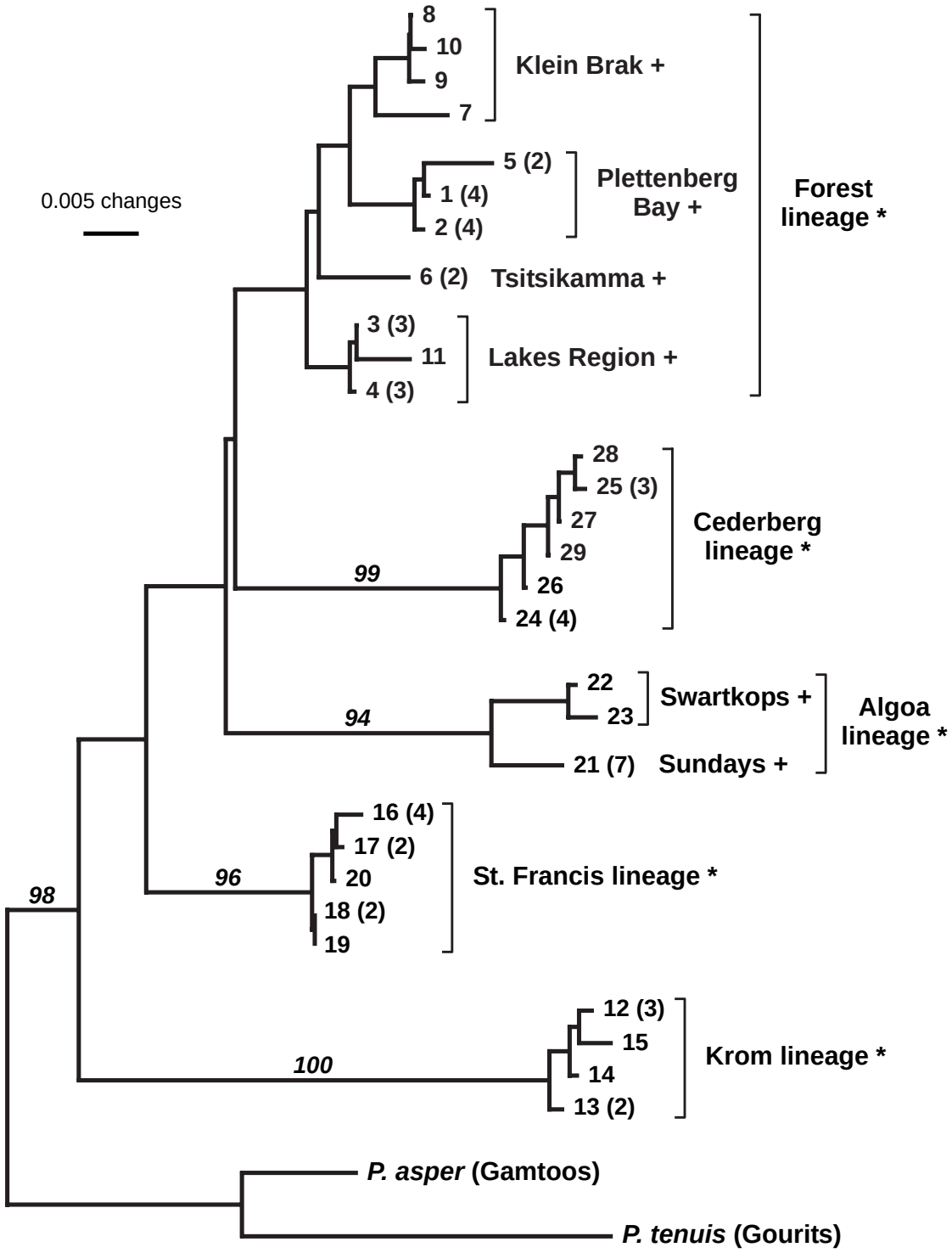
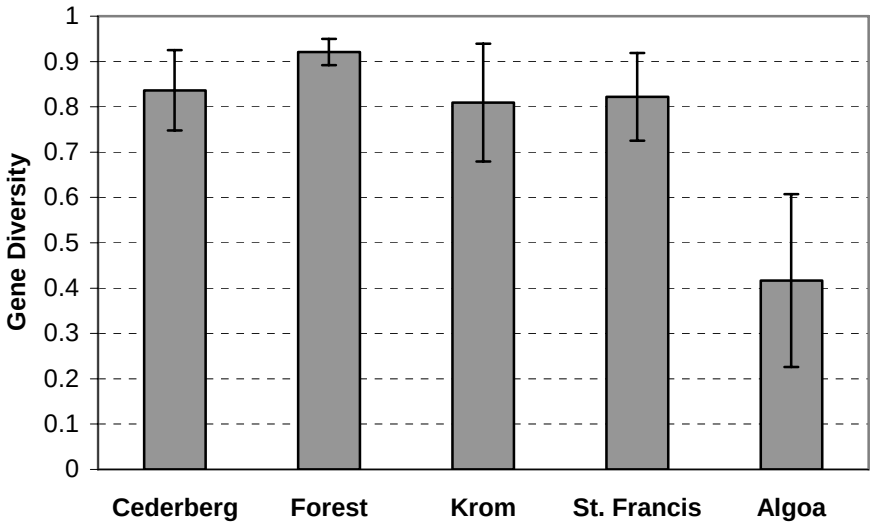


Fig. 3.2. Neighbor-joining tree that shows the differentiation among four major (*) and six minor (+) lineages within *P. afer*. The tree is based on the genetic HKY85 substitution model of Hasegawa *et al.* (1985) with a Ti/Tv ratio of 3.968, I = 0.685 and α = 0.659. Allele numbers (regular text), allele sample size (in brackets) and bootstrap support (italic text) are also shown.

Gene diversity did not differ significantly between the Cederberg, Forest, Krom and St. Francis lineages, but all of these lineages showed significantly larger gene diversity in comparison to the Algoa lineage (Fig. 3.3A). Nucleotide diversity was lowest in the Krom and St. Francis lineages. The Cederberg and Algoa lineages did not differ significantly from any of the other lineages, but the Forest lineage showed significantly larger nucleotide diversity compared to the Krom and St. Francis lineages (Fig. 3.3B).

A



B

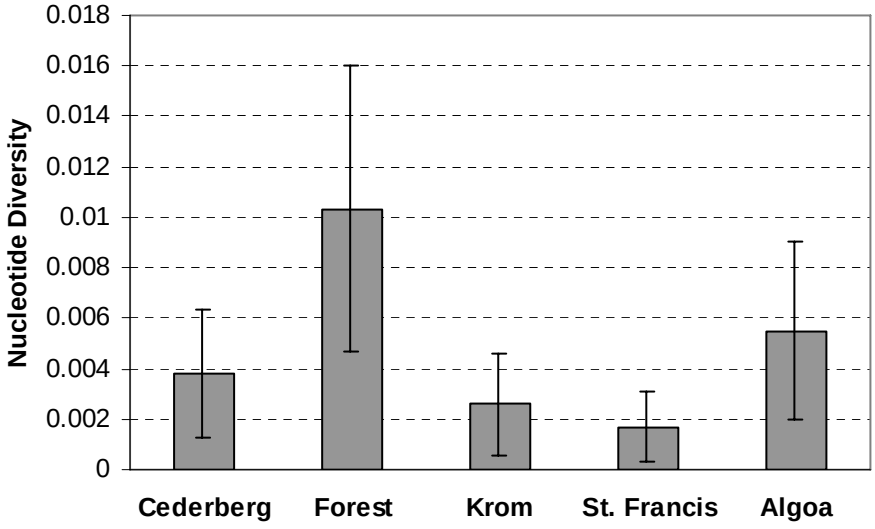


Fig. 3.3. Gene diversity ± SE (A) and nucleotide diversity ± SE (B) within the one *P. phlegethon* (Cederberg) and four *P. afer* lineages (Forest, Krom, St. Francis and Algoa).

Genetic structuring

Only four of the alleles were shared between river systems, namely allele 3 between the Kaaimans and Karatara River systems, allele 4 between the Touws and Duiwe River systems, allele 2 between the Groot and Bloukrans River systems and allele 16 between the Gamtoos and Swart River systems. A further four alleles were shared among different localities, but within the same river system, namely allele 1 that was the only allele across all four sites in the Keurbooms River system, allele 17 between the Opkoms (GAM 4) and Braam (GAM 5) localities in the Kouga section of the Gamtoos River system, allele 21 across all three sites in the Sundays River system and allele 25 between the Thee and Oudste tributaries of the Olifants River system (Table 3.3; Fig. 3.1).

Table 3.3. Frequency of alleles among sampled localities of 49 *P. afer* and 11 *P. phlegethon* individuals.

Allele No.	N	Forest												
		KLE (4)	KAA (2)	TOU (1)	DUI (2)	KAR (2)	BIT (2)	KEU 1 (1)	KEU 2 (1)	KEU 3 (1)	KEU 4 (1)	GRO (2)	BLO (2)	TSI (2)
1	4	-	-	-	-	-	-	1	1	1	1	-	-	-
2	4	-	-	-	-	-	-	-	-	-	-	2	2	-
3	3	-	2	-	-	1	-	-	-	-	-	-	-	-
4	3	-	-	1	2	-	-	-	-	-	-	-	-	-
5	2	-	-	-	-	-	2	-	-	-	-	-	-	-
6	2	-	-	-	-	-	-	-	-	-	-	-	-	2
7	1	1	-	-	-	-	-	-	-	-	-	-	-	-
8	1	1	-	-	-	-	-	-	-	-	-	-	-	-
9	1	1	-	-	-	-	-	-	-	-	-	-	-	-
10	1	1	-	-	-	-	-	-	-	-	-	-	-	-
11	1	-	-	-	-	1	-	-	-	-	-	-	-	-

Table 3.3. Continued.

Allele No.	N	Krom	Gamtoos							Algoa			
		KRO (7)	SWA (2)	KAB (3)	GAM 1 (1)	GAM 2 (1)	GAM 3 (1)	GAM 4 (1)	GAM 5 (1)	SKO (2)	SUN 1 (1)	SUN 2 (3)	SUN 3 (3)
12	3	3	-	-	-	-	-		-	-	-	-	-
13	2	2	-	-	-	-	-		-	-	-	-	-
14	1	1	-	-	-	-	-		-	-	-	-	-
15	1	1	-	-	-	-	-		-	-	-	-	-
16	4	-	2	-	1	-	1	-	-	-	-	-	-
17	2	-	-	-	-	-	-	1	1	-	-	-	-
18	2	-	-	2	-	-	-	-	-	-	-	-	-
19	1	-	-	1	-	-	-	-	-	-	-	-	-
20	1	-	-	-	-	1	-	-	-	-	-	-	-
21	7	-	-	-	-	-	-	-	-	-	1	3	3
22	1	-	-	-	-	-	-	-	-	1	-	-	-
23	1	-	-	-	-	-	-	-	-	1	-	-	-

Table 3.3. Continued.

Allele No.	N	Cederberg						
		OLI 1 (1)	OLI 2 (1)	OLI 3 (2)	OLI 4 (1)	OLI 5 (2)	OLI 6 (3)	OLI 7 (1)
24	4	-	-	-	-	-	3	1
25	3	-	-	-	1	2	-	-
26	1	1	-	-	-	-	-	-
27	1	-	1	-	-	-	-	-
28	1	-	-	1	-	-	-	-
29	1	-	-	1	-	-	-	-

Locality names (see Table 3.1 for full locality descriptions): KLE = Klein Brak; KAA = Kaaimans; TOU = Touws; DUI = Duiwe; KAR = Karatara; BIT = Bitou; KEU 1 = Lower Keurbooms; KEU 2 = Middle Keurbooms; KEU 3 = Kwaai; KEU 4 = Voogt's; GRO = Groot; BLO = Bloukrans; TSI = Tsitsikamma; KRO = Krom; SWA = Swart; KAB = Kabeljous; GAM 1 = Braam; GAM 2 = Opkoms; GAM 3 = Witte; GAM 4 = Ys; GAM 5 = Baviaanskloof; SKO = Swartkops; SUN 1 = Otto's Pool; SUN 2 = Kaboega; SUN 3 = Kaboega tributary; OLI 1 = Rondegat; OLI 2 = Boskloof; OLI 3 = Noordhoeks; OLI 4 = Thee; OLI 5 = Oudste; OLI 6 = Breekkrans

When the five lineages were specified as the groups, differentiation among the groups accounted for 73.64% of the variation (overall ϕ_{ST} = 0.952), whereas only 21.53% and 4.83% of the variation was explained by differentiation among regions within groups and within regions respectively (all *p* values were < 0.002). Several other structures (e.g. using the bays or breaks in the distribution as groups) were also tested, but all of them explained lower percentages of the variation among groups compared to the structure above (Table 3.4). High and significant pairwise ϕ_{ST} values among the specified regions, indicated that much more structuring exists within *P. afer* than just the five major lineages, with only the comparisons among Klein Brak, Tsitsikamma and Swartkops and between the Doring and Swartkops that were not significant due to low sample size (Table 3.5).

Table 3.4. AMOVA results for the *a priori* structures among *P. afer* populations. All the values were significant (*p* > 0.05).

	Variance components		
Source of variation	Three regions	Five lineages	Five bays
Among groups	6.563 (50.26%)	9.729 (73.64%)	3.865 (31.76%)
Among populations within groups	5.857 (44.85%)	2.844 (21.53%)	7.666 (62.99%)
Within populations	0.639 (4.89%)	0.639 (4.83%)	0.639 (5.25%)
Overall ϕ_{ST}	0.951	0.952	0.948

Table 3.5. Pairwise ϕ_{ST} for the *P. afer* and *P. phlegethon* localities, grouped in "regions". The comparisons that were significant ($p > 0.05$) are indicated with asterisks. Sample sizes are given in brackets.

	1	2	3	4	5	6	7	8	9
1) Klein Brak (4)									
2) Lakes Region (7)	0.755*								
3) Plettenberg Bay (10)	0.729*	0.795*							
4) Tsitsikamma (2)	0.747	0.879*	0.849*						
5) Krom (7)	0.923*	0.956*	0.947*	0.954*					
6) Gamtoos (10)	0.919*	0.944*	0.943*	0.950*	0.959*				
7) Swartkops (2)	0.868	0.946*	0.931*	0.958	0.958*	0.962*			
8) Sundays (7)	0.952*	0.975*	0.958*	1.000*	0.978*	0.980*	0.964*		
9) Olifants (7)	0.892*	0.927*	0.931*	0.946*	0.958*	0.949*	0.939*	0.972*	
10) Doring (4)	0.901*	0.957*	0.942*	1.000	0.971*	0.965*	0.982	1.000*	0.679*

Nested clade analysis

Although an ambiguous branch among missing alleles within clade 3-2 had to be broken to resolve the cladogram, it had no effect on the nesting design, since all the alleles within this clade are from a single locality (Klein Brak) (Fig. 3.4). None of the four major lineages could be connected by the program TCS, since there were more than ten mutational steps between them. All the alleles within clades 1-11, 2-7 and 2-9 are from single localities (Kabeljous, Krom and Swartkops respectively) and therefore exact contingency tests for geographic association could not be done. Using the –200 m contour for geographic distances, clades 1-2, 1-8, 1-9, 1-12, 2-1, 2-5, 2-7, 3-1and 3-3 did not show a significant association between their clades or alleles and geographic position, even though more than one locality was represented ($2.400 < \chi^2 < 13.000$; $0.112 < p < 1.000$). A significant association between the clades or alleles within them and geographic position was detected in clades 2-9, 3-4, 3-5, 3-6, 4-1, 4-2, 5-1, 6-1 and 7-1 ($9.0000 < \chi^2 < 59.0000$; $0.0000 < p < 0.0272$). The four divergent lineages identified in the neighbour-joining tree were restricted to clades 2-7 (Krom *P. afer*), 2-8 (St. Francis *P. afer*), 3-4 (*P. phlegethon*), 3-5 (Algoa *P. afer*) and 5-1 (Forest *P. afer*). Most of the inferences based on the key of Templeton (2004) refer to isolation type processes and more specifically historical isolation (Table 3.6). When the current coastline was used as a surrogate for undersea river distances, only the inference of clade 5-1 changed. The conclusion changed from an inference where long-distance colonization, fragmentation and range expansion could have played a role to a more simple inference of the migration-type process of restricted gene flow with isolation by distance. The difference in the two conclusions is as a result of much shorter geographical distances (Table 3.2) when the current coastline is used compared to when the – 200 m contour line is used.

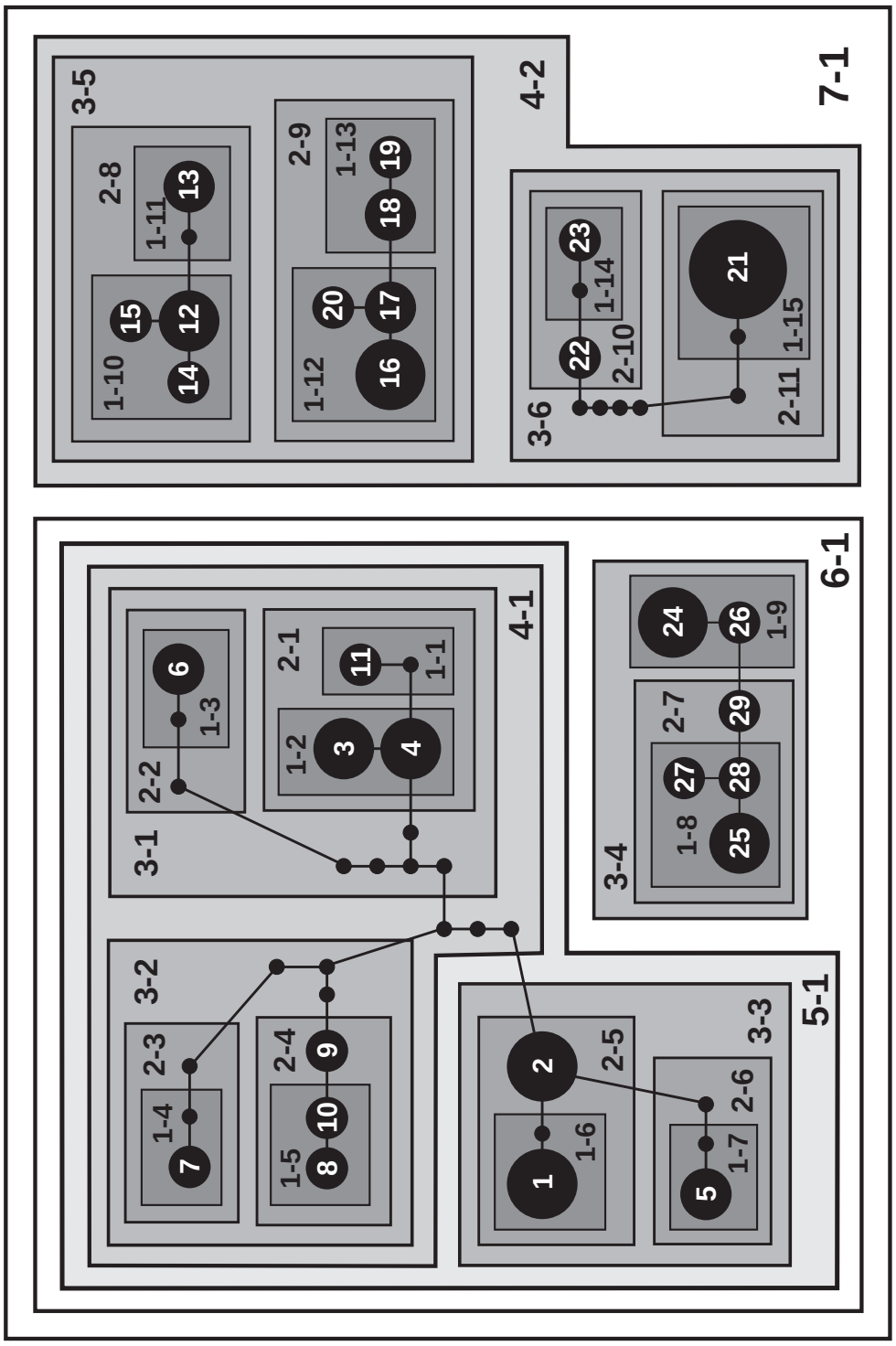


Fig. 3.4. Nested clade design of the control region alleles of *P. afer* and *P. phlegethon*. The five major lineages are restricted to clades 5-1 (Forest), 2-8 (Krom), 2-9 (St. Francis) and 3-6 (Algoa) and 3-4 (*P. phlegethon*). The six minor lineages are restricted to clades 2-1 (Wilderness Lakes region), 2-2 (Tsitsikamma), 3-2 (Klein Brak), 3-3 (Plettenberg Bay), 2-10 (Swartkops) and 1-15 (Sundays).

Table 3.6. Evolutionary processes identified through nested clade analysis and with the inference key of Templeton (2004).

Clade	Populations in clade	Inference chain	Migration or isolation	Inferred evolutionary pattern
River distances assuming a –200m sea level:				
2-9	St. Francis Bay lineage	1, 19, No	Isolation	Allopatric fragmentation
3-4	*P. phlegethon*	1, 19, No	Isolation	Allopatric fragmentation
3-5	Krom and St. Francis Bay lineages	1, 19, No	Isolation	Historical isolation
3-6	Algoa Bay lineage	1, 19, No	Isolation	Allopatric fragmentation
4-1	KLE, KAA, TOU, DUI, KAR and TSI	1, 19, No	Isolation	Allopatric fragmentation
4-2	Krom, St. Francis Bay and Algoa Bay lineages	1, 19, No	Isolation	Historical isolation
5-1	Forest lineage	1, 2, 3, 5, 6, 13, Yes	Migration/ Isolation	Long-distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion
6-1	Forest lineage and *P. phlegethon*	1, 19, No	Isolation	Historical isolation
7-1	All localities	1, 19, No	Isolation	Historical isolation
Alternative conclusions using river distances along the current coastline:				
5-1	Forest lineage	1, 2, 3, 4, No	Migration/ Isolation	Restricted gene flow with isolation by distance

KLE = Klein Brak; KAA = Kaaimans; TOU = Touws; DUI = Duiwe; KAR = Karatara; TSI = Tsitsikamma.

Discussion

Lineages and their distribution

The most striking result is that four divergent lineages exist within *P. afer*. The expectation that different river systems that flow into the same bays are more likely to share a more recent history of connection and therefore migrants, is partly supported, especially in the eastern section of the range. Within Algoa Bay, the Sundays and Swartkops River systems share a close relationship, as do the Gamtoos, Kabeljous and the Swart River systems that flow into St. Francis Bay, but surprisingly in the latter bay, the Krom River system is divergent. Also surprising is the minor levels of differentiation that is detected across the 10 river systems analysed from the Mossel Bay to the Plettenberg Bay area, especially considering the two large gaps in distribution (spanning the Groot Brak, Gwaing and Malgas River systems and the at least 11 small river systems between the Bloukrans and Tsitsikamma River systems). This Forest lineage is the most widespread lineage in *P. afer* and the populations from the Swart (George), Goukamma, Knysna and Piesang River systems probably also form part of this lineage. More surveys will have to be done, but from our current knowledge, the two large gaps in distribution seem to be real and probably because of historic reasons rather than recent anthropogenic disturbance.

There was little differentiation within *P. phlegethon* and can therefore be considered as a single mitochondrial DNA lineage, despite seven fixed allelic differences that was found in a total of 25 allozyme loci by Swartz *et al.* (2004). The distribution of *P. phlegethon* has been well established. Only seven populations remain (Bills, 1999; Swartz *et al.*, 2004). At least one population has been extirpated in the Jan Dissels River system because of the introduction

of North-American bass species (E. R. Swartz *et al*., unpublished) and Barnard (1943) collected specimens of *P. phlegethon* in the mainstream Olifants River at Keerom. Bass now dominate the mainstream areas to the complete exclusion of *P. phlegethon* and some other smaller indigenous fish species.

The Krom lineage is the most restricted and it seems as if the species is only surviving in a very short section (< 1 km) of the upper Krom River between two waterfalls that protect the population from the impact of alien bass (*Micropterus* sp.). All the other river systems of St. Francis Bay may share the same lineage, which would include the Swart, Kabeljous and Gamtoos River systems (confirmed) and possibly also the Seekoei and Maitlands River systems. Due to the differentiation of the Krom River system, care must however be taken in making such an assumption. Recent surveys, failed to find *P. afer* in the Maitlands, raising fears that the species has been eliminated from this river system. The Seekoei River system is likely to have shared migrants in recent times with the Swart River system, since they share a common estuary. The Algoa lineage may also be more widespread than what has been confirmed by the present study. Apart from the Swartkops and Sundays River systems, there are also records of *P. afer* occurring in the Baakens River system (Skelton, 1988).

Cederberg and Forest lineages

The close relationship between this widespread Forest lineage of *P. afer* and *P. phlegethon* is difficult to explain. It suggests that these two lineages diverged from each other relatively recently. Alternatively, it is possible that the mitochondrial DNA genealogy does not reflect the species tree, if introgression occurred at some stage. The mitochondrial DNA alleles of *P. phlegethon*, for example, may have been replaced by *P. afer* mitochondrial DNA alleles.

Nuclear DNA will have to be sequenced to resolve this question, but whatever the case may be, the two species must have been in contact at some stage or recent speciation occurred. This suggests that representatives of one or both species or a common ancestor occurred in the Gourits River system that was extirpated either after introgression or hybridisation or during or after speciation. The Gourits River system is the only logical pathway that can explain a connection between these two lineages.

The isolation between Doring and Olifants populations of *P. phlegethon* that was inferred from allozyme electrophoresis (Swartz *et al.*, 2004), was not as evident from the mitochondrial DNA analysis of the present study. Only one mutational step separates the allele that was found in the Breekkrans tributrary of the Doring River from the allele that was identified in the Rondegat tributary of the Olifants River. Historical isolation was nonetheless inferred as an evolutionary process, since alleles were only shared between the close-by Thee and Oudste tributaries of the Olifants River. The evolutionary processes within *P. phlegethon* and apparent discrepancies between the allozyme and mitochondrial DNA datasets will have to be investigated further with later sample sizes and different genetic makers.

Within the Forest lineage, the most complex evolutionary scenario is inferred for the Klein Brak to Tsitsikamma area where the bays are less well defined and where there was probably a more complex drainage pattern during low sea levels, compared to the other lineages. Historical isolation or allopatric fragmentation was inferred as a possible evolutionary process within the Forest lineage (Fig. 3.4 and Table 3.6). It seems as if four minor lineages are restricted to the Klein Brak River system, the Wilderness Lakes region, the Plettenberg Bay area and the Tsitsikamma River system respectively. No alleles were shared among any of these minor lineages with divergence of between 0.9 and 2.3% (at least seven mutational

steps). Other possible processes identified with NCA within the Forest lineage are quite complex, with long-distance colonization and subsequent fragmentation or past fragmentation followed by range expansion or even restricted gene flow with isolation by distance as further possible explanations of the genetic patterns. The long distance colonization can refer to a river capture event or events or confluence with subsequent extinction of intermediate populations.

It remains uncertain whether connectivity between the Klein Brak, Lakes region, Plettenberg Bay and Tsitsikamma was maintained solely by confluence during low sea levels, or whether river captures also played a role. It is certainly possible that the Klein Brak joined the Wilderness Lakes Region's river systems during the LGM's – 130 m sea level and that the Tsitsikamma joined Plettenberg Bay's river systems during the same time. The distribution of *P. afer* would have been much wider during the LGM and they would have been able to colonise other river systems such as the Groot Brak, Gwaing, Malgas and the smaller river systems between the Bloukrans and Tsitsikamma River systems, provided that these were not isolated with natural barriers like waterfalls. It is therefore likely that *P. afer* was eliminated in many of these smaller river systems, possibly due to the fragmentation and shrinking of available habitat since the LGM and during the transgression towards present sea levels. The fragmentation followed by range expansion, long-distance colonization followed by fragmentation or restricted gene flow with isolation by distance that were inferred with the NCA could be simplified and interpreted as referring to a process of major expansion during low sea levels and fragmentation during high sea levels. Therefore the only clear possibility where river capture could have played a role is if there was no common confluence between the two historical western river systems that is hypothesized for the LGM and possibly also during earlier low sea levels.

The Krom and St. Francis lineages

In contrast to the relatively low divergence of 0.9 to 2.3% among the 10 currently isolated river systems of the Forest lineage, a major divergence was revealed within the St. Francis Bay area (6.1- 7.3%), between the Krom River systems and other river systems of this bay (Krom and St. Francis lineages). From the offshore map reconstructions, it would appear that the Krom had a common confluence with the other river systems of St. Francis Bay, before reaching the – 130 m LGM sea level. The genetic data indicates that this confluence either did not occur or no migrants were exchanged possibly due to ecological separation mediated by barriers such as waterfalls. It is also possible that these two lineages date back to a common ancestor that was separated by a river capture event. The geomorphology indicates that the Kouga section of the Gamtoos River system captured the upper reaches of the Krom River, but no dating exists for this event.

Other such cases of restricted lineages exist in the Cape Fold Mountains. For example *B. erubescens* is restricted to a single catchment with a 12 m waterfall below their distribution (Marriott, 1998; Skelton, 1974a) that would have prevented upstream migration and contact with their sister species (*B. calidus*) that occupies most of the remainder of the Olifants River system. Why *B. erubescens* has not spread throughout the river system remains unexplained (Skelton, 1974a; Swartz *et al.*, 2004), unless some level of ecological speciation occurred. More recently, a similar scenario was discovered in the Breede River system, where a unique lineage of *P. burchelli* is restricted to the Tradou catchment, with a much more widespread lineage occupying the rest of the Breede River system, as well as two neighbouring river systems (Chapter 5). Apart from the major differentiation between the Krom and St. Francis lineages, apparent allopatric fragmentation has resulted in a lack of sharing of alleles between

the Gamtoos-Swart and the Kabeljous River systems within the St. Francis lineage, which could be due to recent genetic drift since the LGM.

Algoa lineage

The divergence between *P. afer* from the Swartkops and Sundays River systems of at least seven mutations (*D* = 1.3 - 1.7%) suggests a process of allopatric fragmentation or historical isolation. The expectation is that the Baakens fish should share a very recent history of contact with the Swartkops, since these two river systems would have had a common confluence within Algoa Bay before reaching the – 130 m LGM sea level. It is, however, unclear whether a combined Baakens, Swartkops and Coega River system would have had a common confluence with the Sundays River system before reaching the – 130 m LGM sea level. These two historic river systems may have remained separate within Algoa Bay due to the Riy Bank (Bremner & Day, 1991). If confluence did not occur, river capture may offer a better explanation for the close relationship between the Swartkops and Sundays River systems.

Overall biogeography

A process of recent and historical fragmentation and isolation was inferred as the dominant process that shaped the genetic patterns in *P. afer* and *P. phlegethon*. In the case of *P. afer*, low sea levels appear to have played a very important role in historical colonisation and migration among currently isolated river systems (see also Ketmaier *et al.*, 2004). Because of the early Pliocene transgression, most of the river systems as we know them today would not have been available to populations of *P. afer*. The + 200 m contour gives an indication of

which river systems may have been available for occupation by freshwater fish. Butzer & Helgren (1972) suggested that only the larger river systems such as the Keurbooms, Krom and Gamtoos pre-date the last major transgression. Divergence between the major lineages, however, suggests a possible age of differentiation in the range of 1 to 3.6 million years if one assumes the relatively slow mutation rate of about 3% per million years that has been suggested for the salmonid mtDNA control region (Bernatchez & Danzmann, 1993). The age estimate of these lineages would be significantly more recent if one assumes the much faster rates of control region mutation that has been suggested for other fish (Brown *et al.*, 1993). Therefore, the current control region diversity between lineages of *P. afer* and *P. phlegethon* probably reflect coalescence during the late Pliocene.

Differentiation among the major lineages therefore probably reflects isolation within major historical river systems that would have formed during low sea levels. Exceptions to this pattern are the differentiation of the Krom lineage within the St. Francis Bay suite of catchments (differentiation within a possible historical river system) and the low levels of differentiation that is found across the range of the Forest lineage (lack of major divergence between possible historic river systems). Alternatively, connections between river systems may have occurred in different ways to what is proposed in Fig. 3.1. The divergence of control region alleles between between *P. phlegethon* and the Forest lineage of *P. afer* is the lowest of all the major lineages. Introgression or colonization between these two lineages must therefore have occurred at the earliest during the late Pleistocene or as recently as the Holocene.

Conservation and taxonomic implications

Conservation considerations should take the intraspecific lineage diversity of *P. afer* as the latter is currently defined by Skelton (1988) into account and should seek to retain the evolutionary processes that have shaped the genetic diversity within the *P. afer* and *P. phlegethon* complex. The four major lineages described here for *P. afer* can be considered as Evolutionarily Significant Units (Moritz, 1994) and should be conserved separately. Translocations should not be allowed between populations that are currently isolated in different river systems, especially not where divergence exist between the minor lineages within the Forest lineage (Klein Brak, Wilderness Lakes Region, Plettenberg Bay and Tsitsikamma) and the two minor lineages within the Algoa lineage (Swartkops and Sundays). The same can be said for Olifants and Doring populations of *P. phlegethon*, until their evolutionary history is fully understood. Whereas a natural process of isolation should be allowed to continue, efforts to ensure as large a population size as possible within systems should be made to ensure continued local survival.

The Krom lineage needs very urgent conservation attention if it is to survive. Its current range is far too small to ensure even short-term survival. If there are ecological differences between the fish from the Krom and those from the remainder of the St. Francis Bay river systems, then there would be a strong case for the resurrection of the species that Smith (1936) described from the Krom River system as *Barbus senticeps* (therefore *Pseudobarbus senticeps*). According to phylogenetic analyses based on mitochondrial DNA, *P. afer* as it is currently defined is not monophyletic, since *P. phlegethon* groups with the Forest lineage of *P. afer*. The taxonomic and conservation status of all the *P. afer* lineages will therefore have to be reviewed.

Acknowledgements

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Chapter 4

Geographic genetic structure of *Pseudobarbus asper* and *P. tenuis* (Teleostei, Cyprinidae), two co-occurring sister species with differing habitat preferences in southern South Africa

Abstract

Differentiation at mtDNA control region showed that two lineages of *P. tenuis* exist. The one occurs, in the Gourits River system often in sympatry with *P. asper*, and the other occurs in the Keurbooms and Bitou River systems in southern South Africa. *Pseudobarbus asper* does not share alleles between the Gourits and the neighbouring Gamtoos River systems, but divergence between alleles from these systems was low. Similarly, there was a lack of sharing of alleles but with low divergence between alleles of *P. tenuis* from the Keurbooms and Bitou River systems that share a common estuary on the south coast. There were therefore more recent opportunities for exchange between Gourits and Gamtoos River systems for *P. asper* and between the Keurbooms and Bitou River systems for *P. tenuis*, compared to the Gourits versus the Keurbooms/Bitou River systems for *P. tenuis*. The river capture of south-eastern tributaries of the Gourits River system by the Keurbooms River would have resulted in unidirectional colonization of the latter by *P. tenuis*. Two-way exchange between the Keurbooms and Bitou River systems would have been possible for *P. tenuis* during lower sea levels. Speciation between *P. asper* and *P. tenuis* probably occurred within the Gourits River system with or without the Gamtoos River system playing a role. The absence of *P. asper* in the Keurbooms and Bitou River systems and *P. tenuis* in the Gamtoos River system probably relates to their different habitat preferences.

Introduction

The redfins (*Pseudobarbus*) is an endemic southern African group of cyprinid minnows associated with the Cape Floristic Region in South Africa, with one species in the highlands of Lesotho. Of the seven described species, the only confirmed case of sympatry between different *Pseudobarbus* species is between the smallscale redfin (*P. asper*) and the slender redfin (*P. tenuis*) and only in the Gourits River system (Skelton, 1988) (Fig. 4.1). *Pseudobarbus asper* (Boulenger, 1911) was described from the neighbouring Gamtoos River system (Fig. 4.1). *Pseudobarbus tenuis* (Barnard, 1938b) was described from the Gourits River system. They have since also been recorded from the Keurbooms, Bitou and Noetsie River systems (Skelton, 1994a) (Fig. 4.1). Mitochondrial DNA evidence suggests, however, that the Noetsie population has been introduced (E. R. Swartz, unpublished) and was therefore not included in the present study.

There was confusion between *P. asper* and *Pseudobarbus* populations from the Afrotemperate Forest region to the east of the mouth of the Gourits River system (Barnard, 1943; Jubb, 1965). During his revision of the genus, Skelton (1988) concluded that these populations belong to *P. afer* and that *P. asper* and *P. afer* are sister species. Skelton (1988) placed *P. tenuis* with *P. quathlambae* as sister species. Allopatric speciation has been sufficient to explain these relationships. The phylogenetic investigation in Chapter 6, however, has shown that *P. asper* and *P. tenuis* are sister species. This raises the possibility that the species arose through sympatric speciation within the Gourits River system.

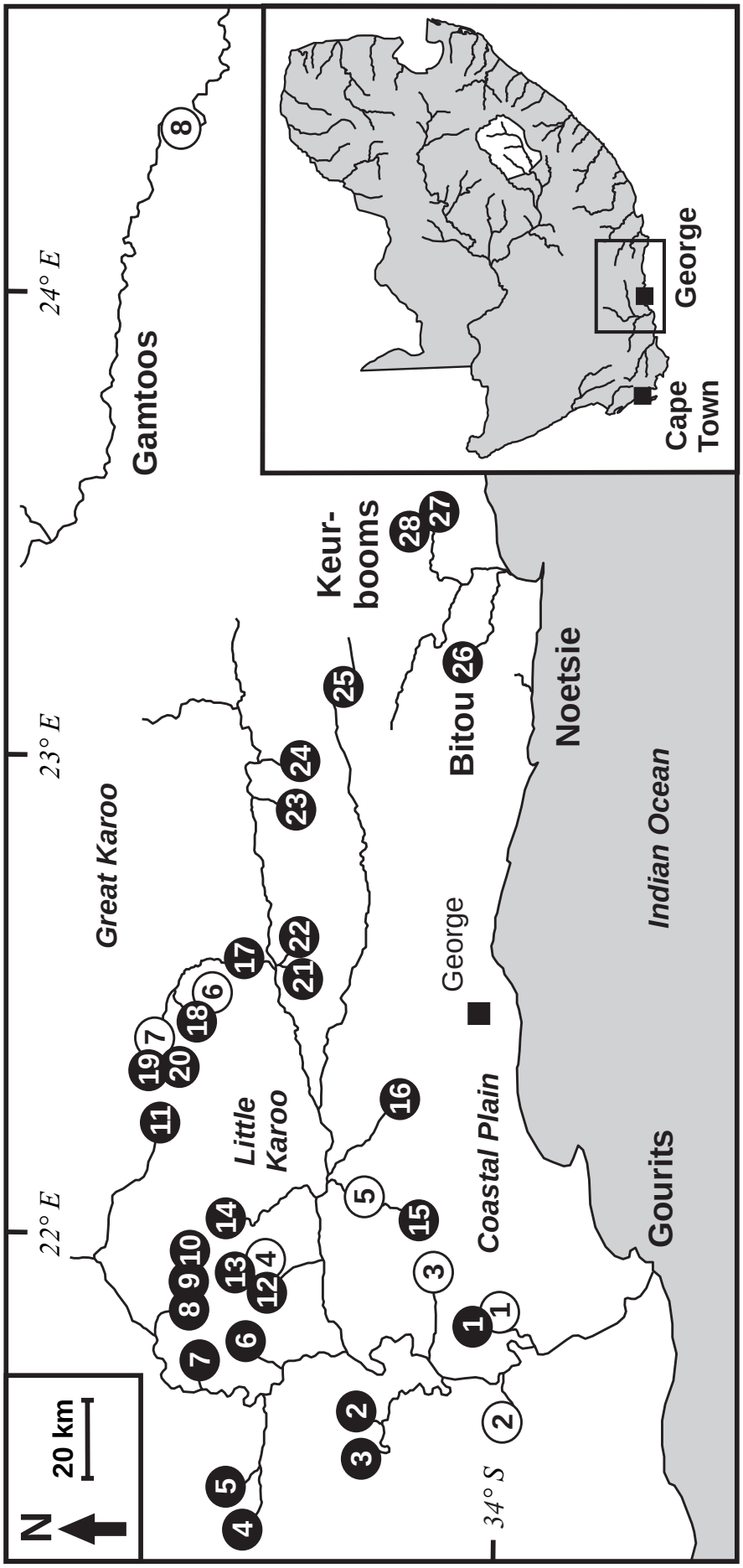

Fig. 4.1. Map of the Gourits, Gamtoos, Keurbooms and Bitou River systems showing localities that were analysed for *P. asper* (open circles) and *P. tenuis* (solid circles). Localities 1, 4 and 6-7 for *P. asper* were collected in sympatry with *P. tenuis* (localities 1, 12 and 18-19).

There are clear differences in habitat preferences between *P. asper* and *P. tenuis*. *Pseudobarbus tenuis* prefers mountain tributary streams. These streams drain over mostly Table Mountain sandstone (Keyser, 1998), which are oligotrophic with low dissolved minerals. *Pseudobarbus tenuis* do not occur in mainstream environments (Skelton, 1988). In *Pseudobarbus,* preference for tributary streams seems to be the rule rather than the exception, which results in differentiation among populations in the same river system due to low levels of gene flow through mainstream areas (Bloomer & Impson, 2000; Swartz *et al.*, 2004; Chapter 2). In contrast, *P. asper* is a more mainstream species and can tolerate eutrophic rivers. They occur in areas that drain mostly over Bokkeveld marine sediments (Keyser, 1998), yielding water with high mineral content that are often turbid (Skelton, 1988). They are also known to go on mass breeding migrations after rains in summer months (Cambray, 1990). Therefore, one would expect *P. asper* to have been able to maintain higher levels of gene flow across its range in the Gourits River system compared to *P. tenuis*.

Sympatry between *P. asper* and *P. tenuis* is especially prevalent in areas of overlap between oligotrophic and eutrophic habitats, therefore at the foot of mountains and near the transition between Table Mountain sandstone and Bokkeveld Marine sediments. In comparing *P. asper* and *P. afer*, based on Skelton's (1988) conclusion that they are sister species, it was found that their growth (Cambray & Hecht, 1995), sex ratio (Cambray, 1994) and egg size and egg number (Cambray & Bruton, 1994) reflect evolutionary adaptations to their different environments. In these studies, *P. asper* was found to have more but smaller eggs, higher relative fecundity, a longer breeding season, smaller first feeding larval fish and that they matured earlier and had a shorter life span compared to *P. afer*. *Pseudobarbus tenuis* probably has a life history strategy that is more similar to *P. afer* than *P. asper*, because of its preference for oligotrophic tributary streams.

The Keurbooms and Bitou Rivers flow into the same estuary, but since all *Pseudobarbus* spp. are primary freshwater species, this estuary would be a barrier to migration between these two systems except possibly during floods or during lower sea levels in the past. The Keurbooms and Bitou are therefore treated as separate river systems. The connection of different river systems during sea level regressions played an important role in the wide distributions of lineages of *P. afer* (Chapter 3) and *P. burchelli* (Chapter 5). The more inland distribution of *P. asper* and *P. tenuis*, however, suggests that river capture may be a better explanation for the occurrence of these two species in different river systems (see Brito *et al.*, 1997; Mesquita *et al.*, 2001; Waters & Wallis, 2000). Skelton (1980) suggested that the occurrence of *P. asper* in both the Gourits and Gamtoos River systems can be explained through exchange in the low gradient inland areas of the Great Karoo, whereas *P. tenuis* probably reached the Keurbooms River system through low order tributary river capture. The first aim of the present study was to investigate how potential connections between different river systems influenced the genetic structure of *P. asper* and *P. tenuis*. The second aim was to infer potential evolutionary processes that have been shaping genetic diversity of these two species, especially in relation to their different habitat preference.

Materials and Methods

Sampling

Pseudobarbus asper and *P. tenuis* specimens were collected with a 3m seine net or by snorkelling with a handnet. Muscle or whole fish samples were either stored in liquid nitrogen in the field and transferred to a –70 °C freezer upon returning to the laboratory, or muscle, fin-clips or whole fish samples were placed in EtOH (Department of Genetics, University of Pretoria). Voucher specimens (dissected specimens and/or additional whole fish samples) were fixed in formalin and deposited in the South African Institute for Aquatic Biodiversity collection (SAIAB, Grahamstown).

DNA extraction, amplification and sequencing

Total genomic DNA was isolated using standard protocols of chemical digestion and phenol/chloroform extraction (Sambrook *et al.*, 1989). The primers L16560 (5’ CCAAAGCCAGAATTCTAAC 3’) and H677 (5’ GTCGCGCAAAAACCAAAG 3’) (Chapter 2) were used to PCR amplify the 5’ end of the mitochondrial control region. PCR was performed in 50 µl volumes containing 1 x buffer, 2 mM $MgCl_2$, 0.2 mM of each of the four nucleotides (Promega), 25 pmol of each primer, 1.5 U of Super-Therm DNA polymerase (Southern Cross Biotechnology) and 100-200 ng template DNA. Conditions for PCR cycling was an initial denaturation of 2 minutes at 94°C, then 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 45 seconds at 72°C, finishing with a final extension of 5 minutes at 72°C.

PCR products were purified using the High Pure™ PCR Product Purification Kit (Boehringer Mannheim), followed by elution in ddH_2O. Cycle sequencing was performed in 10 µl volumes, containing 100 ng of purified DNA as template, 1.6 pmol primer (either L16560 or H677) and 2 µl of ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). PCR and cycle sequencing were performed in a Geneamp® PCR System 9700 (Applied Biosystems). Nucleotide sequences were determined through ABI 377 or ABI 3100 automated sequencers. Consensus sequences were obtained from forward and reverse sequences through alignment and inspection in Sequence Navigator 1.01 (Applied Biosystems). Consensus sequences were aligned using Clustal X (Thompson *et al.*, 1997) and checked manually.

Genetic analysis

MODELTEST version 3.06 (Posada & Crandall, 1998) was used to select a nucleotide substitution model that best fits the data from 56 such models, using a hierarchical likelihood ratio test. The same program was used to estimate the Ti:Tv ratio, proportion of invariable sites (I) and the α value of the gamma distribution (rate variation among sites). PAUP* (Swofford, 2002) was used to estimate phylogenetic relationships and genetic distances among the alleles, based on these parameters.

ARLEQUIN version 2.000 (Schneider *et al.*, 2000) was used to calculate gene (δ) and nucleotide diversity (π). The calculated standard errors for gene diversity take sampling variance into account and standard errors for nucleotide diversity take both sampling and stochastic variance into account. In the same program, AMOVA (Excoffier *et al.*, 1992) was performed on *a priori* hierarchical structures of *P. asper* and *P. tenuis*. Significance was

tested with permutation tests (10 000 replicates). For *P. asper*, the Gamtoos River system and southern localities (1-3) and northern localities (4-7) of the Gourits River system were defined as regions, with the Gamtoos and Gourits River systems as groups (Fig. 4.1). For *P. tenuis* three regions (localities 1-15; 16-20; 21-23) and nine regions (localities 1-3; 4-6; 7-11; 12-14; 15-16 and 25; 17-20; 21-24; 26; 27-28) were defined. The Gourits River system and the Keurbooms/Bitou River system were treated as two groups. In addition, the Keurbooms/Bitou River system was excluded from the analysis, with western localities (1-11), central localities (12-16 and 25) and eastern localities (17-24) of the Gourits River system as the remaining groups. The Tamura-Nei model of substitution with the gamma correction found in Modeltest 3.06 was used to calculate distances on which ϕ_{ST} values were based.

Genealogies based on 95% confidence of connections among alleles (Templeton *et al.*, 1992) were determined with the program TCS (Clement *et al.*, 2000). Alleles in these genealogies were nested hierarchically from the tips to the interior without nesting interiors until they could be nested with tip clades (Cunningham, 2002). Exact contingency tests were performed on each nested clade to test whether a lack of association between alleles or clades and their geographic location can be rejected (Templeton & Sing, 1993). This was done by comparing observed χ^2 values to distributions of χ^2, generated from 10000 random permutations of the original data in the program GEODIS version 2.0 (Posada *et al.*, 2000).

Also using GEODIS, clade distances (D_c), nested clade distances (D_n), average interior versus tip clade distances (IT_c) and average interior versus tip nested clade distances (IT_n) were calculated based on the nested design and geographic distances. The latter were measured from GIS layers in ArcView 3 among localities along river courses, or along the current coastline between localities of the Gourits and Keurbooms River Systems. According to

Templeton *et al.* (1995), D_c is a measure of how geographically widespread individuals in a clade are, and D_n is a measure of the geographical distribution of individuals in a clade compared to all individuals in the nested clade. Different historical processes influence these geographic distance measures (D_c, D_n, IT_c and IT_n) in different ways. This may indicate which type of process has occurred (Templeton *et al.*, 1992). Templeton's (2004) inference key was used to assist in interpreting these distance patterns and to identify potential evolutionary processes.

Results

Survey

Eleven *P. asper* individuals were analysed from the Langtou (1), Weyers (1), Kamma (2), Kruis (1), Moeras (1), Groot (1) and De Aap (1) tributaries of the Gourits River system and a single locality in the Gamtoos River system (3). Twenty-five *P. tenuis* individuals were analysed from the Gourits River system, each from a separate tributary. In addition, ten *P. tenuis* individuals were analysed from the Kransbos (5) tributary in the Bitou River system and the Langbos (3) and Diep (2) tributaries of the Keurbooms River system (Table 4.1; Fig. 4.1). Geographic distances among samples localities were 51-901 km for *P.asper* (Table 4.2) and 1-553 km for *P. tenuis* (Table 4.3).

Table 4.1. Localities where *P. asper* and *P. tenuis* specimens were collected successfully. Asterisks show localities where both species were collected. All collections were made by E. R. Swartz and fellow collectors (see acknowledgements), apart from two Keurbooms samples that were collected by I. A. Russell+.

Code	Locality	Lattitude & Longitude	Date
Both *P. asper* and *P. tenuis* (Gourits River system)			
A1, T1	Langtou	33° 58’ 30” S 21° 47’ 20” E	23/04/2000
A4, T12	Lower Kruis	33° 28’ 45” S 21° 54’ 10” E	04/02/2000
A6, T18	Aaps	33° 19’ 39” S 22° 27’ 44” E	03/02/2000
A7, T19	Lower Groot	33° 16’ 20” S 22° 21’ 15” E	03/02/2000
Only *P. asper* (Gourits River system)			
A2	Weyers	34° 01’ 29” S 21° 35’ 00” E	25/04/2000
A3	Kamma	33° 52’ 20” S 21° 54’ 30” E	04/05/2000
A5	Moeras	33° 43’ 19” S 22° 02’ 21” E	02/05/2000
***P. asper* (Gamtoos River system)**			
A8	Groot	33° 19’ 05” S 24° 20’ 50” E	06/02/2001 & 23/09/1998
Only *P. tenuis* (Gourits River system)			
T2	Assegaaibos	33° 43’ 39” S 21° 33’ 50” E	03/05/2000
T3	Bos	33° 43’ 50” S 21° 30’ 22” E	07/05/2000
T4	Kobus	33° 27’ 47” S 21° 19’ 57” E	02/02/2000
T5	Seweweekspoort	33° 24’ 54” S 21° 24’ 13” E	11/09/1998
T6	Nels	33° 28’ 03” S 21° 44’ 09” E	04/02/2000
T7	Mooifontein	33° 21’ 17” S 21° 44’ 53” E	12/10/2002
T8	Huis	33° 20’ 47” S 21° 49’ 05” E	15/10/2002
T9	Waterkloof	33° 20’ 40” S 21° 50’ 52” E	15/10/2002
T10	Trib. Waterkloof	33° 20’ 44” S 21° 53’ 10” E	15/10/2002
T11	Wilgerdal	33° 17’ 15” S 22° 15’ 21” E	03/02/2000
T13	Upper Kruis	33° 26’ 15” S 21° 53’ 17” E	15/10/2002

Table 4.1. Continued.

Code	Locality	Lattitude & Longitude	Date
Only *P. tenuis* (Gourits River system) continued.			
T14	Vinknes	33° 24’ 20” S 21° 59’ 45” E	15/10/2002
T15	Saffraan	33° 50’ 50” S 21° 59’ 10” E	04/05/2000
T16	Groot Doring	33° 47’ 47” S 22° 15’ 30” E	02/05/2000
T17	Meiringspoort	33° 29’ 00” S 22° 33’ 50” E	11/09/1998
T20	Upper Groot	33° 16’ 24” S 22° 20’ 53” E	03/02/2000
T21	Vermaaks	33° 34’ 39” S 22° 32’ 07” E	10/10/2002
T22	Marnevicks	33° 34’ 32” S 22° 34’ 56” E	10/10/2002
T23	Buffelsklip	33° 34’ 10” S 22° 54’ 00” E	05/05/2000
T24	Wilge	33° 34’ 00” S 22° 58’ 00” E	05/05/2000
T25	Holdrif	33° 40’ 23” S 23° 08’ 40” E	08/10/2002
***P. tenuis* (Keurbooms and Bitou River systems)**			
T26	Kransbos	33° 55’ 20” S 23° 13’ 15” E	11/04/2000
T27	Langbos +	33° 51’ 15” S 23° 29’ 26” E	27/02/2001
T28	Diep +	33° 52’ 07” S 23° 29’ 52” E	27/02/2001

Table 4.2. Geographic distances (km) among sampled *P. asper* localities along the current coastline (between river systems) and along inland river courses. See Table 4.1 for locality descriptions.

	1	2	3	4	5	6	7
1							
2	51						
3	85	56					
4	155	126	114				
5	160	131	119	53			
6	243	214	202	136	103		
7	253	224	212	146	113	70	
8	684	699	733	803	808	891	901

Table 4.3. Geographic distances (km) among sampled *P. tenuis* localities along the current coastline (between river systems) and along inland river courses. See Table 4.1 for locality descriptions.

	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	26	27
1																											
2	96																										
3	106	18																									
4	171	153	163																								
5	168	150	160	29																							
6	138	120	130	59	56																						
7	176	158	168	75	72	64																					
8	203	185	195	102	99	91	51																				
9	207	189	199	106	103	95	55	6																			
10	211	193	203	110	107	99	59	10	6																		
11	255	237	247	154	151	143	103	82	86	90																	
12	155	137	147	110	107	77	115	142	146	150	194																
13	161	143	153	116	113	83	121	148	152	156	200	54															
14	177	159	169	132	129	99	137	164	168	172	216	70	76														
15	176	158	168	131	128	98	136	163	167	171	215	69	75	63													
16	177	159	169	132	129	99	137	164	168	172	216	70	76	64	53												
17	213	195	205	168	165	135	173	200	204	208	252	106	112	100	89	86											
18	243	225	235	198	195	165	203	230	234	238	282	136	142	130	119	116	30										
19	253	235	245	208	205	175	213	240	244	248	292	146	152	140	129	126	40	70									
20	254	236	246	209	206	176	214	241	245	249	293	147	153	141	130	127	41	71	1								
21	213	195	205	168	165	135	173	200	204	208	252	106	112	100	89	86	14	44	54	55							
22	213	195	205	168	165	135	173	200	204	208	252	106	112	100	89	86	14	44	54	55	10						
23	257	239	249	212	209	179	217	244	248	252	296	150	156	144	133	130	58	88	98	99	56	56					
24	269	251	261	224	221	191	229	256	260	264	308	162	168	156	145	142	70	100	110	111	68	68	32				
25	282	264	274	237	234	204	242	269	273	277	321	175	181	169	158	155	159	189	199	200	159	159	203	215			
26	305	365	375	440	437	407	445	472	476	480	524	424	430	446	445	446	482	512	522	523	482	482	526	538	551		
27	306	366	376	441	438	408	446	473	477	481	525	425	431	447	446	447	483	513	523	524	483	483	527	539	552	69	
28	307	367	377	442	439	409	447	474	478	482	526	426	432	448	447	448	484	514	524	525	484	484	528	540	553	70	3

Sequence variation and diversity

The region of control region analysed in the present study corresponds with positions 17 – 615 in *Cyprinus carpio* (Chang *et al.*, 1994). For *P. asper*, this region yielded nine variable characters with four being parsimony informative and five being autapomorphic (one with indels). In the case of *P. tenuis*, 28 variable sites, with 18 parsimony informative (two with indels) and 10 autapomorphic (two with indels) characters were found. In addition, one site was parsimony informative for an indel and autapomorphic for a nucleotide substitution. Seven and 24 alleles were detected in *P. asper* (Table 4.4) and *P. tenuis* (Table 4.5) respectively. The HKY85 substitution model (Hasegawa *et al.*, 1985) was selected for both species with the Modeltest analysis, with equal rates among sites for *P. asper* and a Ti:Tv ratio of 4.433, I = 0.861 and α = 0.950 for *P. tenuis*.

Table 4.4. Frequency of alleles among sampled localities of *P. asper*. See Table 4.1 for locality descriptions.

Allele		Localities of *P. asper*							
number	N	A1	A2	A3	A4	A5	A6	A7	A8
1	3	-	-	-	-	-	-	-	3
2	2	-	1	-	1	-	-	-	-
3	2	-	-	2	-	-	-	-	-
4	1	1	-	-	-	-	-	-	-
5	1	-	-	-	-	1	-	-	-
6	1	-	-	-	-	-	-	1	-
7	1	-	-	-	-	-	1	-	-

Table 4.5. Frequency of alleles among sampled localities of *P. tenuis*. See Table 4.1 for locality descriptions.

Allele number	N	Localities of *P. tenius*													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	5	-	-	-	-	1	-	-	1	1	-	-	-	1	-
2	2	-	-	-	-	-	-	-	-	-	1	-	1	-	-
3	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
4	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-
5	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-
6	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-
7	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-
8	1	-	-	-	-	-	-	-	-	-	-	1	-	-	-
9	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-
10	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
		15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
11	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
12	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-
13	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-
14	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-
15	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-
16	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-
17	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-
18	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-
19	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-
20	1	-	-	-	-	-	-	-	-	-	-	1	-	-	-
21	5	-	-	-	-	-	-	-	-	-	-	-	-	3	2
22	3	-	-	-	-	-	-	-	-	-	-	-	3	-	-
23	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-
24	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-

The neighbour-joining tree (Fig. 4.2) shows the genetic distances according to parameters found in Modeltest among both *P. asper* and *P. tenuis* alleles with *P. afer* from the Swartkops River system and *P. phlegethon* from the Olifants River system as outgroups (HKY85 model with a Ti:Tv ratio of 4.049, I = 0.668 and α = 0.745). A single *P. asper* and two *P. tenuis* lineages (Gourits and Keurbooms-Bitou) are evident from this tree. Differentiation within *P. asper* was low (*D* = 0-1%). The differentiation within the Gourits lineage of *P. tenuis* (*D* = 0-2.3%) and within the Keurbooms-Bitou lineage (*D* = 0-0.7%) was lower than the differentiation between these two lineages (1.4-3.4%) for *P. tenuis*. The allele from the Saffraan River (site 15 in Fig. 4.1; allele 11 in Fig. 4.3 B) showed a high divergence to other alleles of the Gourits River system**.** When the Saffraan allele was excluded, divergence within the Gourits River system was only 0-1.5%. The differentiation between the Gourits and Gamtoos River systems for *P. asper* was similar to the differentiation between the Keurbooms and Bitou River systems for *P. tenuis* (0.2 - 0.8% and 0.5-0.7% respectively).

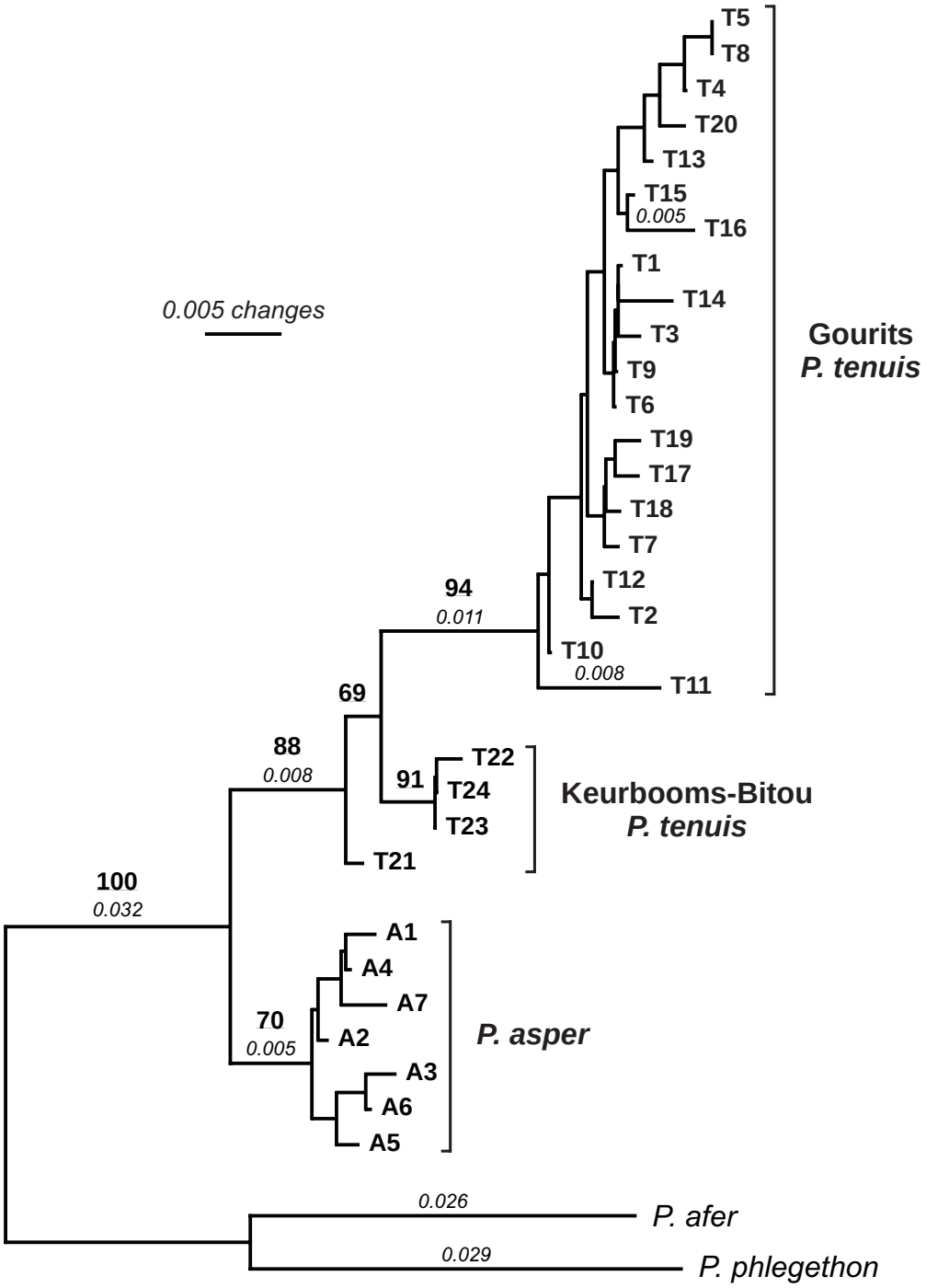

Fig. 4.2. Neighbor-joining tree that shows the differentiation among a single lineage of *P. asper* and the two lineages of *P. tenuis*. The tree is based on the genetic HKY85 substitution model of Hasegawa *et al.* (1985) with a Ti:Tv ratio of 4.049, I = 0.668 and α = 0.74. Allele numbers for *P. asper* (A1-7) and *P. tenuis* (T1-24), allele sample size (in brackets), bootstrap support (regular text) and HKY85 distances (italic text) are also shown. Only genetic distances greater than 0.005 are indicated. *Pseudobarbus afer* and *P. phlegethon* were chosen as outgroups based on the analyses of Chapter 6.

Gene diversity was high and not significantly different between *P. asper* (δ = 0.9091; SE = 0.066) and *P. tenuis* (δ = 0.954; SE = 0.021). The three *P. asper* individuals analysed from the Gamtoos locality showed a lack of variation. *Pseudobarbus asper* and *P. tenuis* from the Gourits River system showed high gene diversity (*P. asper*: δ = 0.929, SE = 0.084; *P. tenuis*: δ = 0.951; SE = 0.035), both being significantly higher compared to *P. tenuis* from the Keurbooms/Bitou River system (δ = 0.711; SE = 0.118). Nucleotide diversity was not significantly different between *P. asper* as a whole (π = 0.005; SE = 0.003), *P. tenuis* as a whole (π = 0.010; SE = 0.006), *P. asper* from the Gourits (π = 0.005; SE = 0.003), *P. tenuis* from the Gourits River system (π = 0.005; 0.003) and *P. tenuis* from the Keurbooms and Bitou River systems (π = 0.004; SE = 0.002).

Genetic structuring

Only one *P. asper* allele was shared between localities, namely the Weyers and Kruis Rivers (localities 2 and 4 respectively in Fig. 4.1). Two *P. tenuis* alleles were shared between localities. The first was shared between the Seweweekspoort, Huis, Waterkloof, upper Kruis and Wilge Rivers (localities 5, 8-9, 13 and 24) within the Gourits River system. The second was shared between the Langbos and Diep Rivers (localities 27 and 28) within the Keurbooms River system. No alleles were shared between river systems for either species. When the Gourits and Gamtoos river systems were specified as groups for *P. asper*, 59.3% of the variation was explained by differentiation within the specified regions (Table 4.6). Only 43.2% of the variation is explained by differentiation between the two river systems, with a negligible contribution from differentiation among the specified regions within the river systems (-2.5%).

With the three and nine *a priori* specified regions for *P. tenuis*, differentiation between the Gourits River system and Keurbooms and Bitou River systems explained most of the variation. However, only the structure where nine regions specified was significant. Differentiation among regions specified within the Gourits explained very little of the variation, compared to differentiation within the regions. Despite the lack of sharing of alleles between the Gourits and Gamtoos River systems for *P. asper*, overall ϕ_{ST} was not very high due to the low divergence of the Gamtoos allele compared to the Gourits alleles. For *P. tenuis*, overall ϕ_{ST} was high when the Gourits River system and Keurbooms and Bitou river systems were treated as two groups, but ϕ_{ST} was low when regions were defined within the Gourits River system.

Table 4.6. AMOVA results for the *a priori* structures among *P. asper* and *P. tenuis* populations. Asterisks indicate significant results ($p < 0.05$).

A priori structures:	*P. asper*	*P. tenuis* (3 regions)	*P. tenuis* (9 regions)	
Variation	Gourits vs Gamtoos	Gourits vs Keurbooms	Gourits vs Keurbooms	Within Gourits
Among groups	0.893 (43.2%)	4.046 (73.1%)	3.956 (71.8%)*	0.165 (10.1%)*
Among populations in groups	-0.05 (-2.5%)	0.090 (1.6%)	0.582 (10.6%)*	0.135 (8.3%)
Within populations	1.22 (59.3%)*	1.401 (25.3%)*	0.970 (17.6%)*	1.330 (81.6%)*
Overall ϕ_{ST}	0.407*	0.747*	0.824*	0.184*

Nested clade analysis

With one ambiguous branch in *P. asper*, three different cladograms were inferred (one of these is shown in Fig. 4.3 A). The second and third cladograms differ from the one shown in Fig. 4.3 A only in that allele 2 is linked to the missing allele between clades 2-1 and 2-2, instead of linking with the missing allele in clade 1-1. The latter scenario changes the nesting design. Significant association between alleles or clades and geographic position was not inferred within any of the clades in either the cladogram shown in Fig. 4.3 A ($3.000 < \chi^2 < 10.000$; $0.112 < p < 1.000$) or the ones that are not shown ($0.004 < \chi^2 < 6$; $0.254 < p < 1.000$).

Several ambiguous branches within clades 3-1 and 3-2 (Fig. 4.3 B), made it difficult to resolve the cladogram for *P. tenuis*. As a result, several different cladograms were inferred that had to be tested with the nested clade analysis (one of these is shown in Fig. 4.3 B). None of the clades in any of the inferred cladograms that only involved Gourits River system samples, showed a significant association between their alleles or clades and geographic position (cladogram in Fig. 4.3 B: $2.000 < \chi^2 < 39.000$; $0.121 < p < 1.000$). The three alleles of clade 3-5 (unchanged in all the cladograms) were from a single locality and therefore exact contingency tests for geographic association was not done. Clades 4-3 (Keurbooms versus Bitou River system samples) and 8-1 (Gourits River system versus Keurbooms and Bitou River system samples) that was unchanged in all the inferred cladograms, showed significant association between their clades and geographic position ($10.000 < \chi^2 < 35.000$; $0.000 < p < 0.009$). Allopatric fragmentation was inferred for both these clades.

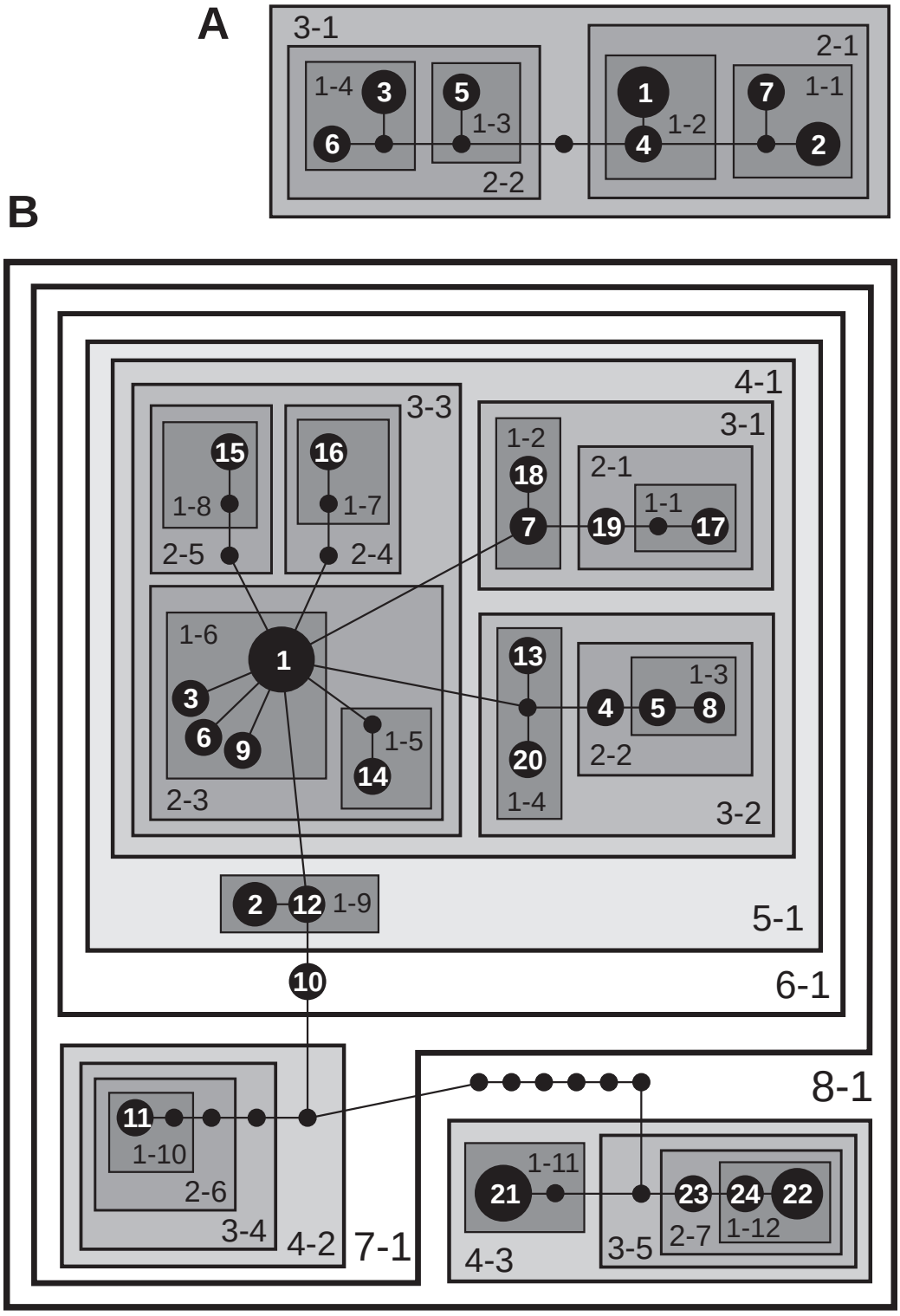

Fig. 4.3. Nested clade designs of the control region alleles of *P. asper* (A) and *P. tenuis* (B). For *P. asper*, an ambiguous branch also linked allele 2 to the missing allele between clades 2-1 and 2-2. Several ambiguous branches occurred for *P. tenuis* that resulted in alternative nested designs. However, the only clades that showed an association between their clades or alleles and geographic position (clades 4-3 and 8-1), were unchanged in all these cladograms.

Discussion

Differentiation patterns and evolutionary processes

A clear differentiation in genetic structure was only found between *P. tenuis* samples from the Gourits versus the Keurbooms and Bitou River systems, indicating that historical isolation of the species has occurred between these localities. Although there was a lack of sharing of alleles between *P. asper* from the Gourits and Gamtoos River systems, divergence was low. This suggests that the two *P. tenuis* lineages were separated much earlier than the isolation of *P. asper* populations from the Gourits and Gamtoos River systems. The type of evolutionary processes involved could not be inferred with confidence from NCA for *P. asper*. Too few *P. asper* individuals were collected from the Gamtoos River system.

The similar level of differentiation between Gourits and Gamtoos River systems for *P. asper* and that between the Keurbooms and Bitou River systems for *P. tenuis* may suggest a similarly recent isolation of populations from these different river systems. Sample sizes were large enough to infer a process of historical isolation or allopatric fragmentation between *P. tenuis* localities from the Gourits versus those from the Keurbooms/Bitou River systems, as well as between localities of the Keurbooms and Bitou River systems. In both these cases coalescence of alleles occurred within the nested clades before the different clades were connected. Apart from recognising that isolation has occurred and, in the case of the two lineages, also divergence, the specific historical event that led to the current distributions cannot be inferred (Templeton *et al.*, 1995).

The small amount of sharing of alleles among localities for both *P. asper* and *P. tenuis* may indicate differentiation among populations. There was, however, little regional structuring for both species within the Gourits River system. There was also no discernable difference in genetic structure between the species within this river system. This suggests that historic isolation of populations has not occurred and that both species have been able to maintain relatively recent and/or low levels of gene flow among different regions of this river system. The only possible exception from the current analysis may be the Saffraan population of *P. tenuis*. The relatively high divergence between the allele from the Saffraan River compared to the alleles of other Gourits *P. tenuis*, may indicate that the former population has been isolated relatively recently in the high altitudes of the Attakwas mountains.

Biogeographic routes between river systems

The present analysis indicates a relatively longer isolation between the Gourits lineage as opposed to the Keurbooms/Bitou lineage of *P. tenuis* compared to the Gourits and Gamtoos populations of *P. asper* and this probably reflects more recent opportunities for gene flow in the latter. Tributaries of the Gourits and Gamtoos River systems are in close proximity to each other in the interior regions of the Great Karoo, where they drain low gradient valleys and dry marshlands (Fig. 4.1). Skelton (1980) proposed that drainages in some of these areas may have been connected during wet periods or floods allowing *P. asper* to migrate between the Gourits and Gamtoos River systems. Because of the low gradient of the Great Karoo valleys, gene flow could have occurred in either direction.

The river capture event that Skelton (1980) proposed to explain the occurrence of *P. tenuis* in both the Gourits and Keurbooms River systems, however, was probably unidirectional. The higher altitude of the south-eastern tributaries, the angle at which the rivers drain and the fact that the Keurbooms River is on the wetter coastal side of the Outeniqua-Tsitsikamma mountains suggests that the south-eastern tributaries of the Gourits River system was captured by the Keurbooms River. The Bitou River system would have then been colonised from the Keurbooms River system, probably during lower sea levels. Even though sea levels were 130 m below current levels during the last glacial maximum, only about 18 000 years BP (Ramsay & Cooper, 2002; Rogers, 1985; Tankard, 1976), the divergence between the Gourits and Keurbooms-Bitou lineages of *P. tenuis* suggests that the species was already present in the Keurbooms River system before this time.

Two-way gene flow between the Keurbooms and Bitou River systems would have been possible with lower sea levels. Currently gene flow between populations in the Keurbooms and Bitou River systems would only be possible if the common estuary shared by these systems became fresh, for example during severe floods. *Pseudobarbus tenuis* would not have been able to reach the Gamtoos River system via low sea levels from the Keurbooms River system because these systems were never connected (Chapter 3). It is surprising, however, that *P. tenuis* did not become established in several other coastal river systems that were connected to the Keurbooms and Bitou River systems.

Role of ecology in differentiation

The reason for the absence of *P. asper* in the Keurbooms and Bitou River systems and the absence of *P. tenuis* in the Gamtoos River system seems to be related to their respective habitat preferences. There is no highly mineralised habitat for *P. asper* in either the Bitou or the Keurbooms River systems. If *P. asper* was included in a river capture event or events, it is possible that they were not able to adapt to these coastal river systems. It is also possible that they were not included in a river capture event or events. Only *P. tenuis* (and no *P. asper*) was recorded in recent surveys in the south-eastern mountain tributary streams of the Gourits River system (E. R. Swartz, unpublished). The habitat in these streams seems to be more suitable for *P. tenuis*. This species also tends to occur much higher in mountain tributary streams and are therefore more likely to be included in river capture events in the Cape Fold Mountains.

It is more difficult to explain why *P. tenuis* does not occur in the Gamtoos River system. In the mountainous sections of this river system, there are several tributary streams that have possible suitable oligotrophic habitat. One possibility is that *P. afer* may have excluded *P. tenuis* through competition. Both *P. tenuis* and *P. afer* occur in the Keurbooms and Bitou River systems, but not in sympatry (Skelton, 1994a). *Pseudobarbus tenuis* occurs much higher in the tributary streams compared to *P. afer*, which is similar to the situation between *P. tenuis* and *P. asper* in the Gourits River system. The difference, however, is that the habitat lower down in the Keurbooms and Bitou River systems are typically peat stained Afrotemperate Forest rivers as opposed to the highly mineralised Karoo streams in which *P. asper* occur. Therefore, *P. tenuis* and *P. afer* might have slightly different habitat preferences.

Another consideration is that *P. afer* from the Gamtoos River system might more directly compete with *P. tenuis* compared to *P. afer* from the Afrotemperate Forest region. *Pseudobarbus afer* from the Keurbooms and Bitou River systems fall within a different lineage compared to *P. afer* from the Gamtoos River system and may have a different habitat preference (Chapter 3). The more likely scenario, however, is that *P. tenuis* was not included in exchanges between the Gourits and Gamtoos River systems. They have never been recorded in the northern Great Karoo tributaries of the Gourits River system. *Pseudobarbus asper*, however, has been recorded in these areas and is known to migrate into upland areas where there are greater chances for exchange (Cambray, 1990).

Speciation between P. asper *and* P. tenuis

It is unlikely that *P. tenuis* would have been able to re-invade the Gourits River system from the Keurbooms or Bitou River systems, since the river capture event or events seems to have been unidirectional from the former to the latter. It is possible, however, that speciation between *P. asper* and *P. tenuis* occurred between the Gourits and Gamtoos and that *P. asper* then re-invaded the Gourits River system at a later stage. Alternatively, the speciation between *P. asper* and *P. tenuis* occurred within the Gourits River system, possibly in sympatry due to the markedly different habitat types that has been available. They would then have been pre-adapted to colonise the other river systems with possible continued or stochastic gene flow between the Gourits and Gamtoos River systems for *P. asper*. Barriers to gene flow such as waterfalls could have reinforced ecological speciation.

Taxonomic and conservation implications

Skelton (1988) found that *P. tenuis* from the Keurbooms and Bitou River systems differed from *P. tenuis* from the Gourits River system in having relatively longer fins and a narrower caudal peduncle. In the light of the mtDNA differentiation found in the present study, the taxonomic status of these two lineages should be re-evaluated. According to Moritz's (1994) definition (historically isolated and divergent) and one of the criteria (monophyly of mtDNA), the Gourits and Keurbooms-Bitou lineages of *P. tenuis* can be considered to be two separate Evolutionarily Significant Units (ESU's). In addition it is likely that at least the Bitou versus Keurbooms populations of *P. tenuis* and Gourits versus Gamtoos populations of *P. asper* may be different Management Units (Moritz, 1994). The evolutionary processes discovered thus far will be relatively simple to manage. This will only require that the Gourits lineage and the Keurbooms-Bitou lineage of *P. tenuis* remain isolated. Other possibly more recent processes of isolation that should be allowed to continue is between the Keurbooms and Bitou River systems for *P. tenuis* and between the Gourits and Gamtoos River systems for *P. asper*. Evolutionary processes within populations from the Gourits River system may be complex and further samples will have to be analysed to identify these.

Pseudobarbus asper is currently listed as Vulnerable and *P. tenuis* as Endangered (IUCN, 2003), mainly due to predation from alien fish species and excessive water extraction. During a survey in 2000 (E. R. Swartz, unpublished), the alien sharptooth catfish *Clarias gariepinus* (Burchell, 1822) were recorded far upstream in the Gamtoos River system (33° 13' 30" S 24° 15' 30" E). Only a single *P. asper* individual was recorded from three localities during the survey. It is therefore possible that *P. asper* may soon be exterminated from that system due to predation from *C. gariepinus*. Furthermore, low numbers of *P. asper* were collected during

recent surveys in the Gourits River system. Indigenous fish have not been excluded from mainstream areas in the Gourits River system as much as other river systems in the Cape Floristic Region (Bills, 1999; Swartz, 2000; Swartz *et al.*, 2004; E. R. Swartz, unpublished; Chapter 5), but deterioration of mainstream habitats is nonetheless of concern.

The potential rapid and extensive loss in distribution range of *P. asper* in the Gamtoos River system should urgently be confirmed and their conservation status should be re-evaluated. Thus far only two to four populations of the Keurbooms-Bitou lineage of *P. tenuis* are known to survive and additional surveys are needed to assess this lineage's conservation status as well. During the surveys for the present study new populations of *P. tenuis* were discovered in the Gourits River system (E. R. Swartz, unpublished). Several of these populations occur in headwater streams where impacts are low. This lineage can be effectively protected if these populations can be secured from invasion by alien fish species and if population sizes remain large enough.

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Chapter 5

Historical lineages and evolutionary processes in *Pseudobarbus burchelli* (Teleostei, Cyprinidae) within and among different riversystems of the south coast of South Africa

Abstract

The primary freshwater species, *Pseudobarbus burchelli*, occurs in four river systems that would have formed part of only two palaeoriver systems during the last glacial maximum about 18 000 years ago. Unexpectedly, divergence in mitochondrial control region and cytochrome *b* sequences showed that three historically isolated lineages occur within only the western palaeoriver system. A Breede lineage was widespread across the currently isolated Breede, Duiwenhoks and Goukou River systems. Two geographically restricted lineages were recorded in the Tradou catchment within the Breede River system and in the currently isolated Heuningnes River system. The Heuningnes lineage would have been able to colonise the Breede and Duiwenhoks River systems through a common confluence during the last glacial maximum. Similarly, the Tradou lineage would have been able to migrate downstream in the Tradou catchment before the introduction of alien fishes, but no evidence of introgression between the any of these lineages were found. This suggests that some level of ecological speciation may have occurred, probably aided by waterfalls and sea level transgressions. Despite probably not having a common confluence during the last glacial maximum, there was a lack of differentiation between the Goukou River system and sites of the western palaeoriver system (Breede and Duiwenhoks River systems). The only explanation for this is recent river capture or translocation. Mostly isolation type evolutionary processes were inferred with nested clade analysis, with some migration type processes within the Breede and Tradou lineages.

Introduction

The redfins (genus *Pseudobarbus*) is represented by seven described species that occur in the Cape Floristic Region (CFR) of South Africa, with one species in the highlands of Lesotho. Most of these cyprinid minnow species have a single pair of oral barbels. However, two species have two distinct pairs of barbels. They form a monophyletic lineage (Chapter 6) and are restricted to the south-western river systems of the CFR. The earliest described redfin, *Barbus (Pseudobarbus) burchelli* Smith 1841, had two pairs of barbels. It is unclear, however, from where this species was described. It remained to be determined whether the redfins from the Breede River system region (Fig. 5.1) or the ones from the neighbouring Berg River system was conspecific to with the species that Smith (1841) described (Skelton, 1988). Apart from Smith's name, the oldest name available for the Breede species was *Gnathendalia vulnerata* Castelnau 1861 and for the Berg species it was *Barbus burgi* Boulenger 1911.

Barnard (1943) placed Boulenger's *Barbus burgi* in synonymy with Smith's species. Without providing a justification, Jubb (1965) reversed this decision, placing *Gnathendalia vulnerata* in synonymy with Smith's (1841) species. When Skelton (1988) defined a monophyletic redfin genus, he raised Smith's (1841) subgenus name to a full generic name and accepted Jubb's (1965) nomenclatural changes to maintain taxonomic stability. Skelton (1988) assigned a specimen (AMG 7223) from the Tradou catchment as neotype for *P. burchelli*. Bloomer & Impson (2000) found two divergent genetic lineages within *Pseudobarbus burgi*, which may be different species. If differentiation has occurred within *P. burchelli* to a similar extent to the differentiation within *P. burgi*, then it may be necessary to make further taxonomic changes.

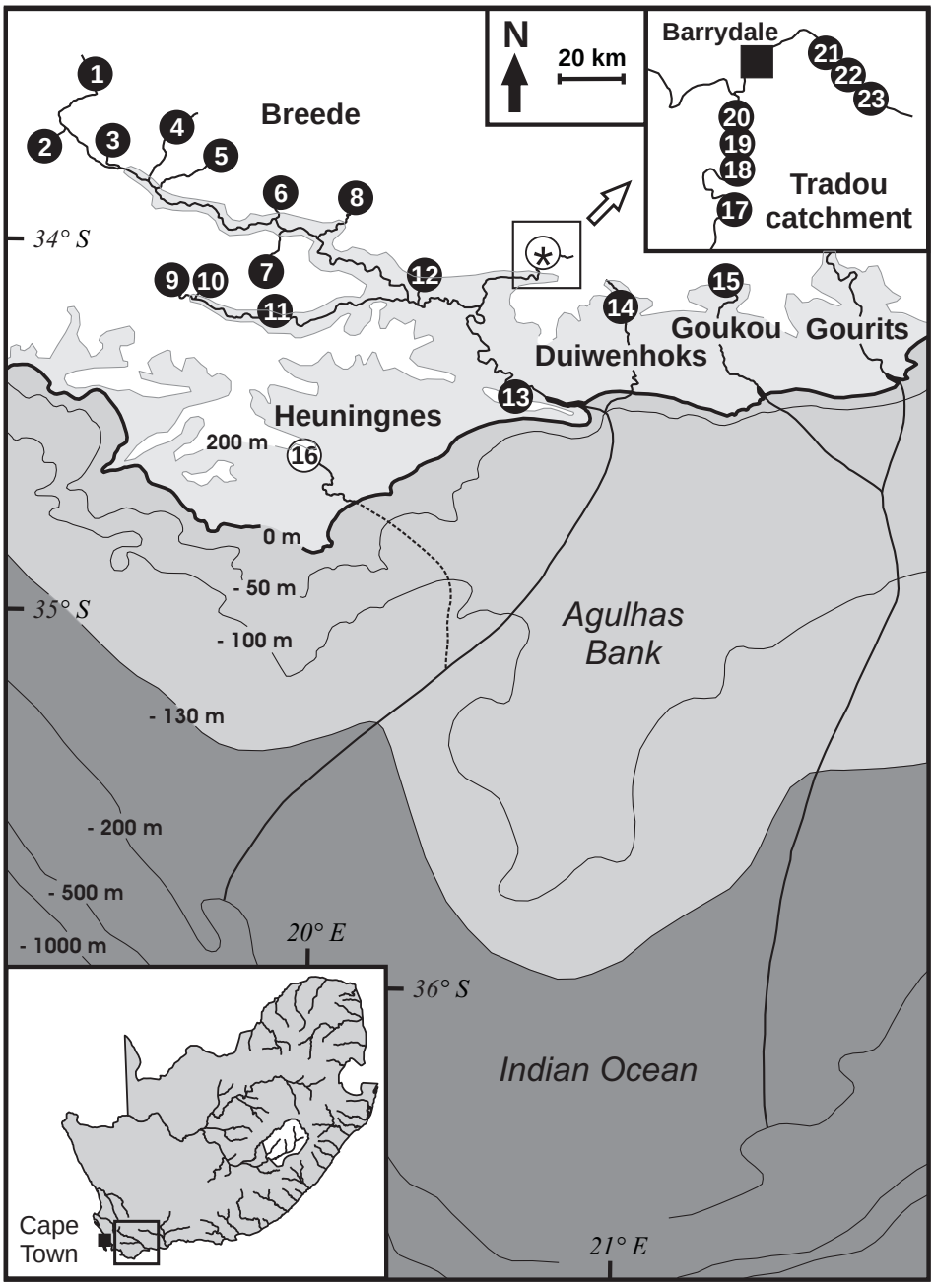

Fig. 5.1. Map of the Agulhas Bank region of the southern coast of South Africa where *P. burchelli* samples were collected. In the main map, solid circles show where the Breede lineage was collected. The open numbered circle (locality 16) shows where the Heuningnes lineage was collected and the open circle with the asterisk indicates the Tradou catchment where the Tradou lineage was collected. Insert maps show the Tradou catchment and localities from where the Tradou lineage was collected and the position of the study area in relation to South Africa. Currently isolated river systems (solid lines in un-shaded or light shaded area) and possible LGM palaeoriver courses based on the geological literature (solid

lines according to Dingle & Rogers (1972) or dashed lines based on the available bathymetry in the medium and dark shaded area).

In terms of distribution across different river systems, *P. burchelli* is the second most widely distributed redfin species. The only other redfin species that occurs in more river systems is *P. afer* in which four major lineages were found (Chapter 3). *Pseudobarbus burchelli* have been recorded in the Breede, Duiwenhoks, Goukou and Heuningnes River systems (Skelton, 1988) (Fig. 5.1). The Breede is the largest of these river systems and drains inland areas beyond the coastal ranges of the southern Cape Fold Mountains. In the Breede River system, several *P. burchelli* populations are currently isolated in clear and oligotrophic mountain tributary streams, which is typical habitat for most of the redfin species. Particularly alien bass species from North-America (*Micropterus salmoides* and *M. dolomieu*) dominate the mainstream areas. Very little is known about the range of *P. burchelli* before the introduction of alien fishes, but it is likely that this has, like other redfins, become very restricted (Skelton, 1987; 1988).

The Duiwenhoks and Goukou River systems are much smaller and do not penetrate the southern coastal ranges of the Cape Fold Mountains. The habitat in these river systems is also oligotrophic. In the Breede, Duiwenhoks and Goukou River systems, *P. burchelli* occurs in rivers that originate almost entirely in Table Mountain sandstones, but also flow over Bokkeveld marine sediments (Keyser, 1998) at altitudes that range from 50 m to 535 m above sea level. These rivers have low mineral and suspension loads (Day *et al.*, 1998). In contrast, the Heuningnes River system drains over Bokkeveld marine sediments and Sandveld sands. It is eutrophic and carries high mineral and suspension loads. The Heuningnes River system also flows along a low gradient and the altitude where *P. burchelli* was recorded for the present study was only about 50 m above sea level. *Pseudobarbus burchelli* is listed as Endangered (IUCN, 2003), mainly because of predation from the alien fishes. Excessive water extraction

and bulldozing of riverbeds are further threats to their survival. Local *P. burchelli* populations are therefore at risk of extinction, which could result in unknown unique lineages going extinct.

Fish from different river systems often share a close relationship because of river capture events (Brito *et al.*, 1997; Mesquita *et al.*, 2001; Waters & Wallis, 2000; Chapter 4). However, the confluence of rivers during low sea levels also played an important role in the distribution of freshwater fishes of the Cape Floristic Region (see Ketmaier *et al.*, 2004; Chapter 3). Several major sea level regressions occurred during the late Pleistocene. The most recent was a –130 m regression during the last glacial maximum (LGM) about 18 000 years BP (Ramsay & Cooper, 2002; Rogers, 1985; Tankard, 1976). These regressions would have caused several neighbouring river systems to have a common confluence before reaching the sea.

A major offshore feature of the area where *P. burchelli* occurs is the Agulhas Bank. This is a shallow area of the continental shelf that would have been exposed during lower sea levels (Fig. 5.1). The major river systems, the Breede and Gourits, were never linked during low sea levels with their courses having followed different directions across the Agulhas Bank (Dingle & Rogers, 1972; Dingle *et al.*, 1983). Confluences would, however, have occurred between these major river systems and some of the smaller river systems as a result of exposure of the Agulhas Bank. The last major transgression occurred during the early Pliocene (about 3.4 - 5.2 myr BP) and reached levels of around + 200 m (Butzer & Helgren, 1972) to over + 300 m (Siesser & Dingle, 1981) along the south coast of South Africa. During this time, the lowland areas and smaller river systems would have been drowned. Later

transgressions apparently never reached more than + 30m above present sea levels (Butzer & Helgren, 1972; Rogers, 1985).

The evolutionary processes (migration and isolation type processes) that played an important role in the evolution of populations of *P. burchelli*, may be inferred by assessing geographic genetic structuring and differentiation. An attempt can then be made to associate these evolutionary processes with the geological and climatic processes of river capture and confluence of river systems during low sea levels. A basic step in conservation management should be to allow evolutionary processes that have shaped current intraspecific diversity, to continue into the future (Crandall *et al.* 2000; Moritz 1999; 2002).

Materials and Methods

Sampling

Pseudobarbus burchelli specimens were collected by snorkelling with a handnet or with a 3m seine net. Either whole fish samples were stored in liquid nitrogen in the field and transferred to a –70 °C freezer upon returning to the laboratory, or muscle, fin-clips or whole fish samples were placed in EtOH (Department of Genetics, University of Pretoria). The remaining carcasses and/or additional samples were fixed in formalin and deposited in the South African Institute for Aquatic Biodiversity (SAIAB) collection (Grahamstown) as voucher specimens.

Map reconstructions and geographic distance measurement

Geographic distance among sampled localities were measured along current river courses from a GIS layer (South African Department of Environmental Affairs and Tourism) and if necessary, along possible palaeoriver courses on the Agulhas Bank (Fig. 5.1). The palaeoriver courses were constructed from the bathymetry of the South African Navy Charts, seismic profiling of offshore sediments by Birch (1980) and reviews published on offshore stratigraphical, sedimentological and bathymetric studies (Dingle *et al.*, 1987; Dingle & Rogers, 1972). If it was presumed that certain river systems did not connect before reaching the continental shelf, then the geographic distance measurement followed the – 200 m contour line between the proposed palaeoriver systems. This contour is relatively close to the – 130 m contour that is used as a surrogate for the LGM's coastline and the edge of the continental shelf on the Agulhas Bank. Geographic distances among sampled localities were also measured along current river courses and then along the current coastline. The + 200 m contour (see Fig. 5.1) was used as an indication of which areas would have been available for occupation by redfins during the early Pliocene transgression (Butzer & Helgren, 1972).

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from frozen or EtOH preserved tissue using standard protocols of chemical digestion and phenol/chloroform extraction (Sambrook *et al.*, 1989). The 5' end of the mitochondrial DNA control region was amplified (PCR) with primers specially designed for cyprinids (Chapter 2), namely L16560 (5' CCAAAGCCAGAATTCTAAC 3') in the tRNA (Thr) on the 5' side of control region and H677 (5' GTCGCGCAAAAACCAAAG 3') within the 3' side of control region. These primer names are according to sequence positions of the 3' base of each primer in the complete mtDNA genome sequence of *Cyprinus carpio* (Chang *et al.* 1994). The primers

GluF (5' AACCACCGTTGTATTCAACTACAA 3') and ThrR (5' ACCTCCGATCTTCGGATTACAAGACCG 3') from Machordom & Doadrio (2001a) were used to amplify most of the mitochondrial cytochrome *b* gene. Reagents (apart from the primers) and conditions for amplification, purification and cycle sequencing were the same for control region and cytochrome *b*.

Amplification was performed in 50 µl volumes containing 1 x buffer, 2 mM $MgCl_2$, 0.2 mM of each of the four nucleotides (Promega), 25 pmol of each primer, 1.5 U of Super-Therm DNA polymerase (Southern Cross Biotechnology) and 100-200 ng template DNA. Conditions for amplification involved an initial denaturation of 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 54°C and 45 seconds at 72°C and a final extension of 5 minutes at 72°C. PCR products were purified using High Pure™ PCR Product Purification Kit (Boehringer Mannheim), followed by elution in ddH_2O. Cycle sequencing was performed in 10 µl volumes, with 2 µl of ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), 1.6 pmol of a single primer (L16560 or H677 for control region or GluF or ThrR for cytochrome *b*) and 100 ng of purified DNA as template. PCR cycling and cycle sequencing was performed in a Geneamp® PCR System 9700 (Applied Biosystems).

Nucleotide sequences were determined through ABI 377 or ABI 3100 automated sequencers. Consensus sequences were obtained from the forward and reverse sequences and by comparing these to sequences from other individuals through alignment and inspection in Sequence Navigator 1.01 (Applied Biosystems). The consensus sequences were aligned using Clustal X (Thompson *et al.*, 1997) and checked manually.

Genetic analysis

The nucleotide substitution model that best fits the data was selected from 56 such models with the Akaike test in MODELTEST version 3.06 (Posada & Crandall, 1998). The proportion of invariable sites (I) and the α value of the gamma distribution (rate variation among sites) was also estimated. Genetic distances among the alleles were based on these parameters. Control region and cytochrome *b* sequences were combined for neighbour-joining estimation of phylogenetic relationships, which was done in the program PAUP* (Swofford, 2002).

Gene (δ) and nucleotide diversities (π) and their standard errors were calculated for each lineage with ARLEQUIN version 2.000 (Schneider *et al.*, 2000). In the case of gene diversity, the standard errors only take sampling variance into account, whereas in the case of nucleotide diversity, both sampling and stochastic variance is taken into account. An AMOVA was also performed in ARLEQUIN (Excoffier *et al*., 1992), which was tested for significance with permutation tests (10 000 replicates) for control region, cytochrome *b* and combined sequences. Groups for the AMOVA analysis were defined as *P. burchelli* from first the Breede (excluding samples from the Tradou catchment), Duiwenhoks and Goukou River systems, secondly the Heuningnes River system and lastly those from the Tradou catchment. Six (localities 1; 2-8; 9-15; 16; 17-20; 21-23) or eight (localities 1; 2-5; 6-8; 9-12; 13-15; 16; 17-20; 21-23) regions within groups were defined. The Tamura-Nei substitution model with the gamma correction found in MODELTEST 3.06 was used to calculate distances on which ϕ_{ST} were based.

Genealogies were determined with the program TCS (Clement *et al.*, 2000) based on 95% confidence of connections among alleles (Templeton *et al.*, 1992). Alleles in these genealogies were nested hierarchically from the tips to the interior without nesting interiors until they could be nested with tip clades (Cunningham, 2002). Exact contingency tests were performed on each nested clade to test whether a lack of association between alleles or clades and their geographic location could be rejected (Templeton & Sing, 1993). This was done by comparing observed χ^2 values to distributions of χ^2 generated from 10000 random permutations of the original data in GEODIS version 2.0 (Posada *et al.*, 2000). Clade distances (D_c), nested clade distances (D_n), average interior versus tip clade distances (IT_c) and average interior versus tip nested clade distances (IT_n) were calculated in the same program based on the nested design and the geographic distances.

According to Templeton *et al.* (1995), D_c is a measure of how geographically widespread individuals in a clade are, and D_n is a measure of the geographical distribution of individuals in a clade compared to all individuals in the nested clade. Different historical processes influence these geographic distance measures (D_c, D_n, IT_c and IT_n) differently. This may indicate which type of process has occurred (Templeton *et al.*, 1992). Templeton's (2004) inference key was used to assist in interpreting these distance patterns and to identify evolutionary processes as either migration-type or isolation-type processes. Nested clade analysis was done for control region, cytochrome *b* and combined sequences.

Results

Survey

A total of 46 individuals from 22 localities was analysed for control region from the Breede (38 individuals; 19 localities), Duiwenhoks (1 individual; 1 locality), Goukou (1 individual; 1 locality) and Heuningnes (6 individuals; 1 locality) River systems. For cytochrome *b*, 41 individuals were analysed from 22 localities from the Breede (31 individuals; 19 localities), Duiwenhoks (1 individual; 1 locality), Goukou (1 individual; 1 locality) and Heuningnes (8 individuals; 1 locality) River systems. Only the individuals from the Baviaans and Bok Rivers were not analysed for both control region and cytochrome *b* (Table 5.1; Fig. 5.1).

Map reconstructions

Two major historical river systems were inferred from the reconstructions of a – 130 m sea level on the Agulhas Bank (light shaded area in Fig. 5.1). The Breede and Duiwenhoks River systems would have formed a western palaeoriver system, whereas according to Birch (1980) and Dingle & Rogers (1972) the Goukou River system would have had a common confluence with the Gourits River system to form an eastern palaeoriver system. The Heuningnes River system would have had a common confluence with the Breede and Duiwenhoks River systems before reaching the – 130 m sea level, unless it flowed in a much further westerly position compared to the position indicated in Fig. 5.1. Geographic distances among localities along the current river courses, the proposed palaeoriver courses and along the – 200 m contour, ranged from 1 – 1106 km. When the current coastline was used to connect the different river systems, geographic distances ranged from 1 – 450 km (Table 5.2).

Table 5.1. Localities where *P. burchelli* specimens were collected. Locality 18 was collected by N. D. Impson. All other collections were made by E. R. Swartz and fellow collectors (see acknowledgements).

Code	Locality	Latitude	Longitude	Date
Breede River system				
1	Koekedou	33º 21’ 30” S	19º 17’ 00” E	24/03/2001
2	Wit	33º 34’ 30” S	19º 08’ 30” E	21/03/1998 & 23/03/2001
3	Jan Dutoits	33º 35’ 30” S	19º 19’ 45” E	23/03/2001
4	Hex	33º 31’ 50” S	19º 32’ 25” E	23/03/2001
5	Nuy	33º 37’ 45” S	19º 41’ 00” E	19/03/2001
6	Willem Nels	33º 45’ 25” S	19º 52’ 05” E	19/03/2001
7	Hoeks	34º 01’ 30” S	19º 50’ 30” E	21/03/2001
8	Kogmanskloof	33º 46’ 20” S	20º 07’ 10” E	22/03/2001
9	Baviaans	34º 02’ 10” S	19º 33’ 30” E	12/03/2001
10	Gobos	34° 02’ 20” S	19° 37’ 10” E	12/03/2001
11	Bok	34° 07’ 10” S	19° 51’ 10” E	11/03/2001
12	Leeu	34° 00’ 00” S	20° 20’ 00” E	09/03/2001
13	Melkhout	34º 22’ 20” S	20º 38’ 20” E	17/03/2001
Duiwenhoks River system				
14	Duiwenhoks	34° 05’ 30” S	20° 57’ 40” E	07/03/2001
Goukou River system				
15	Kruis	34° 00’ 52” S	21° 17’ 24” E	26/04/2000
Heuningnes River system				
16	Grashoek	34º 34’ 15” S	19º 56’ 45” E	15/03/2001

Table 5.1. Continued.

Code	Locality	Latitude	Longitude	Date
Tradou catchment (Breede River system)				
17	Lower Tradou	33° 57’ 24” S	20° 42’ 28” E	17/10/2002
18	Tradou tributary	33° 56’ 51” S	20° 42’ 32” E	1997/1998
19	Middle Tradou	33° 56’ 30” S	20° 42’ 27” E	15-16/10/2002
20	Upper Tradou	33° 56’ 07” S	20° 42’ 39” E	18/10/2002
21	Lower Huis	33° 54’ 35” S	20° 44’ 24” E	17/10/2002
22	Middle Huis	33° 54’ 56” S	20° 44’ 46” E	17/10/2002
23	Upper Huis	33° 55’ 09” S	20° 45’ 04” E	17/10/2002

Table 5.2. Geographic distances (km) among sampled localities of *P. burchelli* along inland river courses, proposed palaeoriver courses and the –200 m contour line (below diagonal) or along inland river courses and the current coastline (above diagonal).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1) Koekedou		45	68	91	97	132	151	164	278	279	247	208	303	375	439	450	265	268	269	270	278	279	280
2) Wit	45		43	66	72	107	126	139	253	254	222	183	278	350	414	425	240	243	244	245	253	254	255
3) Jan Dutoits	68	43		41	47	82	101	114	228	229	197	158	253	325	389	400	215	218	219	220	228	229	230
4) Hex	91	66	41		48	83	102	115	229	230	198	159	254	326	390	401	216	219	220	221	229	230	231
5) Nuy	97	72	47	48		83	102	115	229	230	198	159	254	326	390	401	216	219	220	221	229	230	231
6) Willem Nels	132	107	82	83	83		35	48	162	163	131	92	187	259	323	334	149	152	153	154	162	163	164
7) Hoeks	151	126	101	102	102	35		57	171	172	140	101	196	268	332	343	158	161	162	163	171	172	173
8) Kogmanskloof	164	139	114	115	115	48	57		158	159	127	88	183	255	319	330	145	148	149	150	158	159	160
9) Baviaans	278	253	228	229	229	162	171	158		7	33	100	195	267	331	342	157	160	161	162	170	171	172
10) Gobos	279	254	229	230	230	163	172	159	7		34	101	196	268	332	343	158	161	162	163	171	172	173
11) Bok	247	222	197	198	198	131	140	127	33	34		69	164	236	300	311	126	129	130	131	139	140	141
12) Leeu	208	183	158	159	159	92	101	88	100	101	69		115	187	251	262	77	80	81	82	90	91	92
13) Melkhout	303	278	253	254	254	187	196	183	195	196	164	115		78	142	153	110	113	114	115	123	124	125
14) Duiwenhoks	380	355	330	331	331	264	273	260	272	273	241	192	83		138	181	182	185	186	187	195	196	197
15) Kruis	1106	1081	1056	1057	1057	990	999	986	998	999	967	918	809	824		245	246	249	250	251	259	260	261
16) Grashoek	533	508	483	484	484	417	426	413	425	426	394	345	236	251	779		257	260	261	262	270	271	272
17) Lower Tradou	265	240	215	216	216	149	158	145	157	158	126	77	110	187	913	340		3	4	5	13	14	15
18) Tradou tributary	268	243	218	219	219	152	161	148	160	161	129	80	113	190	916	343	3		1	2	10	11	12
19) Middle Tradou	269	244	219	220	220	153	162	149	161	162	130	81	114	191	917	344	4	1		1	9	10	11
20) Upper Tradou	270	245	220	221	221	154	163	150	162	163	131	82	115	192	918	345	5	2	1		8	9	10
21) Lower Huis	278	253	228	229	229	162	171	158	170	171	139	90	123	200	926	353	13	10	9	8		1	2
22) Middle Huis	279	254	229	230	230	163	172	159	171	172	140	91	124	201	927	354	14	11	10	9	1		1
23) Upper Huis	280	255	230	231	231	164	173	160	172	173	141	92	125	202	928	355	15	12	11	10	2	1	

Sequence variation and diversity

The region of control region analysed corresponds with positions 17 – 615 in *Cyprinus carpio* (Chang *et al.*, 1994). It yielded 48 variable sites, with 36 parsimony informative (4 with indels) and 12 autapomorphic sites (1 with indels), which defined 26 alleles in the 46 individuals that were analysed. The substitution model of Tamura & Nei (1993) with I = 0 and α = 0.013 was selected with the MODELTEST analysis. For cytochrome *b*, the region that corresponds to positions 15350 – 16049 in *Cyprinus carpio* yielded 51 variable sites, with 40 parsimony informative and 11 autapomorphic sites. Twenty alleles were detected in the 41 individuals that were analysed. The substitution model of Tajima & Nei (1984) with I = 0.846 and α being equal among sites best fitted the cytochrome *b* data. The neighbour-joining tree (Fig. 5.2) was based on combined control region and cytochrome *b* sequences and the general time-reversible substitution model (see Lanave *et al.*, 1984) with I = 0.759 and α being equal among sites. Three lineages are evident from this tree (Breede, Heuningnes and Tradou).

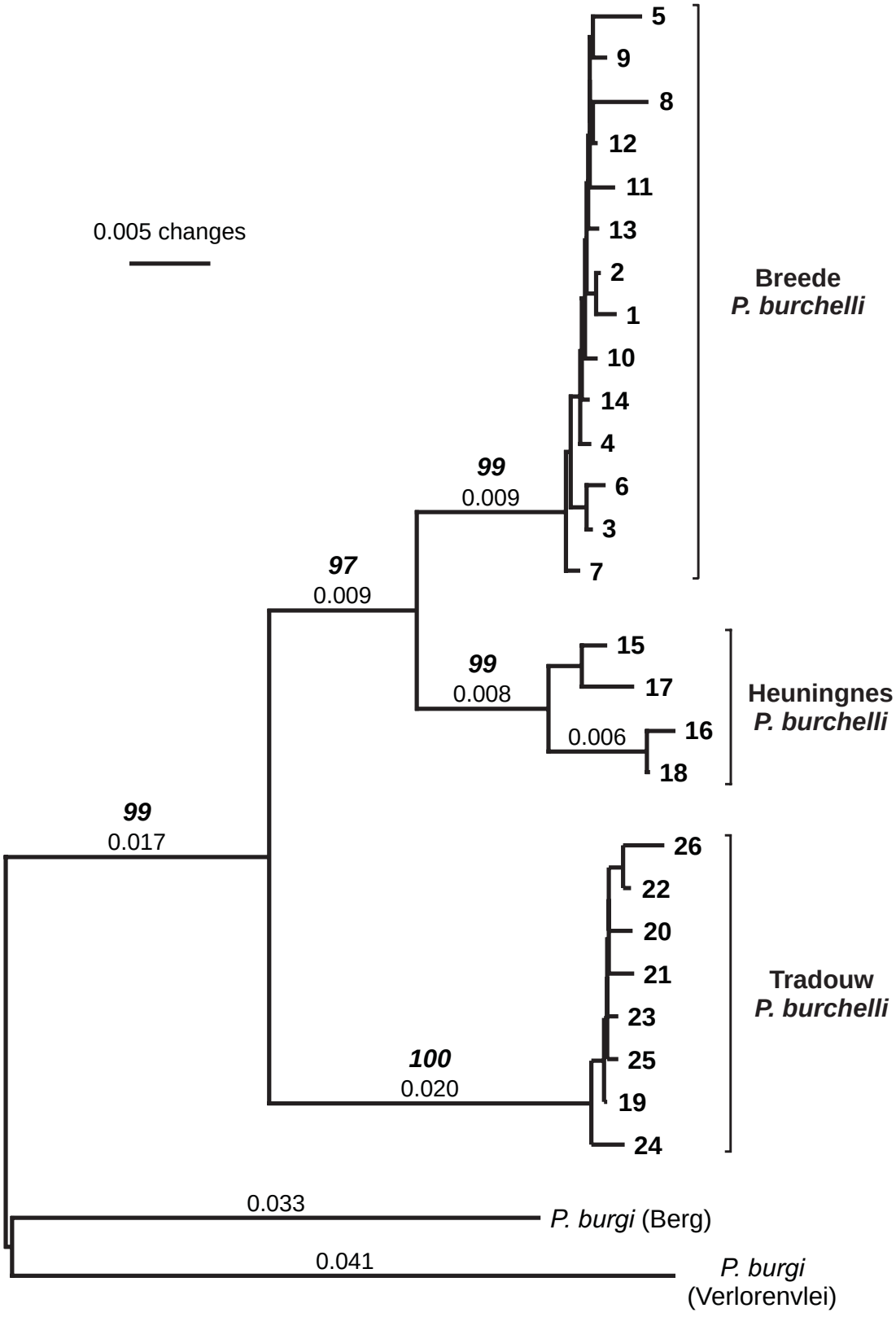

Fig. 5.2. Neighbor-joining phylogram based on combined control region and cytochrome *b* sequences, showing genetic distances among lineages of *P. burchelli*. Allele numbers (regular text), allele sample size (in brackets) and bootstrap support (italic text) are also shown.

The range of genetic distances among control region (CR) and cytochrome *b* (CYT) alleles within these identified lineages (D_{CR} = 0-0.015; D_{CYT} = 0-0.014) did not overlap with the genetic distances between lineages (D_{CR} = 0.021-0.064; D_{CYT} = 0.022-0.068). The largest genetic distances were between alleles of the Tradou lineage and those of the Breede and Heuningnes lineages (D_{CR} = 0.033-0.064; D_{CYT} = 0.038-0.068). Genetic distances were lower between the Breede and Heuningnes lineages (D_{CR} = 0.021-0.033; D_{CYT} = 0.022-0.036). The genetic distances within the Heuningnes lineage (D_{CR} = 0-0.015; D_{CYT} = 0-0.014) was much larger compared to the genetic distances within the Breede lineage (D_{CR} = 0-0.007; D_{CYT} = 0-0.009) and the Tradou lineage (D_{CR} = 0-0.009; D_{CYT} = 0-0.006). This was surprising since the Heuningnes lineage was only recorded and analysed from a single locality.

Gene diversity was significantly lower in the Tradou lineage compared to the Breede lineage for control region, cytochrome *b* and the combined sequences. The Tradou also had lower gene diversity compared to the Heuningnes lineage, but this difference was only significant for cytochrome *b*. There were no cases where gene diversity was significantly different between the Breede and Heuningnes lineages (Table 5.3). Nucleotide diversity in the Heuningnes lineage was higher than the Tradou and Breede lineages for control region, cytochrome *b* and the combined sequences. All the comparisons between the Heuningnes lineage and the Tradou lineage were significant. Only the nucleotide diversity based on control region was significantly different between Heuningnes and Breede lineages. The nucleotide diversities of the Breede and Tradou lineages were not significantly different (Table 5.3).

Table 5.3. Gene (δ) and nucleotide (π) diversity of the Breede, Heuningnes and Tradou lineages of *P. burchelli* for control region, cytochrome *b* and combined control region and cytochrome *b* sequences. Standard errors are shown in brackets.

	Control region	Cytochrome *b*	Combined
Gene diversity			
Breede	0.958 (0.033)	0.850 (0.077)	0.991 (0.028)
Heuningnes	0.867 (0.129)	0.893 (0.086)	0.867 (0.129)
Tradou	0.690 (0.105)	0.588 (0.135)	0.779 (0.099)
Nucleotide diversity			
Breede	0.009 (0.005)	0.003 (0.002)	0.003 (0.002)
Heuningnes	0.211 (0.123)	0.007 (0.004)	0.009 (0.005)
Tradou	0.006 (0.004)	0.001 (0.001)	0.002 (0.001)

Genetic structuring

For control region, only two alleles were shared among sites (alleles 20 and 21), both within the Tradou catchment and the Tradou lineage (Table 5.4 – 5.5; Fig. 5.1). Three alleles (1, 15 and 16) were shared among sites for cytochrome *b*. The cytochrome *b* alleles 15 and 16 showed similar distributions to the control region alleles 20 and 21. However, the cytochrome *b* allele 1 was the most widespread allele found in the present study. It was also the only allele that was shared among different river systems, occurring in six localities across the Breede, Duiwenhoks and Goukou River systems. Twenty-four control region alleles and seventeen cytochrome *b* alleles were only recorded at a single locality.

Table 5.4. Frequency of control region (D), cytochrome *b* (C) and combined control region and cytochrome *b* (B) alleles among sampled localities of the Breede and Heuningnes lineages of *P. burchelli*.

Control region allele number	N	Breede, Duiwenhoks, Goukou and Heuningnes River system localities															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
D1	4	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D2	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D3	2	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D4	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
D5	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
D6	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
D7	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
D8	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
D9	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
D10	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
D11	1	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
D12	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
D13	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
D14	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
D15	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
D16	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
D17	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
D18	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
D19	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Total	**26**	**6**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**6**

Locality names (See Table 5.1 for full locality descriptions): 1) Koekedou; 2) Wit; 3) Jan Dutoits; 4) Hex; 5) Nuy; 6) Willem Nels; 7) Hoeks; 8) Kogmanskloof; 9) Baviaans; 10) Gobos; 11) Bok; 12) Leeu; 13) Melkhout; 14) Duiwenhoks; 15) Kruis; 16) Grashoek

Table 5.4. Continued…

Cytochrome *b* allele number	N	Breede, Duiwenhoks, Goukou and Heuningnes River system localities															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
C1	6	-	-	1	-	-	-	-	1	-	1	-	-	1	1	1	-
C2	3	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C3	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C4	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
C5	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
C6	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
C7	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
C8	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
C9	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
C10	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
C11	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
C12	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
C13	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
C14	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Total	**24**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**8**

Locality names (See Table 5.1 for full locality descriptions): 1) Koekedou; 2) Wit; 3) Jan Dutoits; 4) Hex; 5) Nuy; 6) Willem Nels; 7) Hoeks; 8) Kogmanskloof; 9) Baviaans; 10) Gobos; 11) Bok; 12) Leeu; 13) Melkhout; 14) Duiwenhoks; 15) Kruis; 16) Grashoek

Table 5.4. Continued…

Combined allele number	N	Breede, Duiwenhoks, Goukou and Heuningnes River system localities															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
B1	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B2	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B3	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B4	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
B5	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
B6	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
B7	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
B8	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
B9	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
B10	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
B11	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-
B12	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
B13	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
B14	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
B15	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
B16	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
B17	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
B18	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Total	**21**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**1**	**0**	**1**	**1**	**1**	**1**	**6**

Locality names (See Table 5.1 for full locality descriptions): 1) Koekedou; 2) Wit; 3) Jan Dutoits; 4) Hex; 5) Nuy; 6) Willem Nels; 7) Hoeks; 8) Kogmanskloof; 9) Baviaans; 10) Gobos; 11) Bok; 12) Leeu; 13) Melkhout; 14) Duiwenhoks; 15) Goukou; 16) Heuningnes

Table 5.5. Frequency of control region (D), cytochrome *b* (C) and combined control region and cytochrome *b* (B) alleles among sampled localities of the Tradou lineage of *P. burchelli*.

Allele number	N	Localities for *P. burchelli* from the Tradou						
		17	18	19	20	21	22	23
Control region								
D20	11	1	7	1	2	-	-	-
D21	3	-	-	2	-	1	-	-
D22	2	-	-	-	-	-	-	2
D23	1	1	-	-	-	-	-	-
D24	1	-	-	-	-	1	-	-
D25	1	-	-	-	-	-	1	-
D26	1	-	-	-	-	-	1	-
Total	**20**	**2**	**7**	**3**	**2**	**2**	**2**	**2**
Cytochrome *b*								
C15	11	1	4	1	2	-	1	2
C16	2	-	-	1	-	1	-	-
C17	1	1	-	-	-	-	-	-
C18	1	-	-	1	-	-	-	-
C19	1	-	-	-	-	1	-	-
C20	1	-	-	-	-	-	1	-
Total	**17**	**2**	**4**	**3**	**2**	**2**	**2**	**2**
Combined sequences								
B19	8	1	4	1	2	-	-	-
B20	2	-	-	1	-	1	-	-
B21	2	-	-	-	-	-	-	2
B22	1	1	-	-	-	-	-	-
B23	1	-	-	1	-	-	-	-
B24	1	-	-	-	-	1	-	-
B25	1	-	-	-	-	-	1	-
B26	1	-	-	-	-	-	1	-
Total	**17**	**2**	**4**	**3**	**2**	**2**	**2**	**2**

Locality names (See Table 5.1 for full locality descriptions): 17) Lower Tradou; 18) Tradou tributary; 19) Middle Tradou; 20) Upper Tradou; 21) Lower Huis; 22) Middle Huis; 23) Upper Huis

With six or eight regions that were specified within the three lineages (latter specified as groups), differentiation among the groups accounted for all the variation in the case of control region, 92.9 % of the variation in the case of cytochrome *b* and 90.3-90.4 % of the variation when sequences were combined (Table 5.6). Overall ϕ_{ST} was also high (0.911 - 0.999). Only 0-0.8 % and 0-8.9 % of the variation was explained by differentiation among regions within groups and within regions respectively. When different regions within the Breede lineage were defined as groups, variation within regions explained most of the variation (62.4 - 78.5 %), with very little of the variation explained by differentiation among the specified groups (11.4 - 14.1 %) or among regions within groups (7.4-26.2 %). This *a priori* structure also yielded much lower ϕ_{ST} (0.215 - 0.375).

Table 5.6. AMOVA results for the *a priori* structures among *P. burchelli* populations. Asterisks indicate significant results ($p < 0.05$).

	Among groups	Among populations within groups	Within populations	Overall ϕ_{ST}
Control region				
All lineages (8 regions)	98728.075 (100%)*	-0.957 (0%)*	9.692 (0%)*	0.999*
All lineages (6 regions)	98727.916 (100%)*	-0.553 (0%)*	9.436 (0%)*	0.999*
Breede lineage (5 regions)	0.343 (11.4%)	0.789 (26.2%)	1.883 (62.4%)*	0.375*
Cytochrome *b*				
All lineages (8 regions)	14.183 (92.9%)*	0.055 (0.4%)*	1.023 (6.7%)*	0.933*
All lineages (6 regions)	14.182 (92.9%)*	0.050 (0.3%)*	1.029 (6.8%)*	0.933*
Breede lineage (5 regions)	0.139 (13.1%)	0.091 (8.6%)	0.835 (78.4%)*	0.216*
Combined				
All lineages (8 regions)	20.717 (90.4%)*	0.162 (0.7%)*	2.037 (8.9%)*	0.911*
All lineages (6 regions)	20.696 (90.3%)*	0.181 (0.8%)*	2.039 (8.9%)*	0.911*
Breede lineage (5 regions)	0.291 (14.1%)	0.154 (7.4%)	1.620 (78.5%)*	0.215*

Nested clade analysis

No ambiguous branches were found in the cladograms among alleles for control region (Fig. 5.3 A), cytochrome *b* (Fig. 5.3 B) or combined control region and cytochrome *b* alleles (Fig. 5.3 C). The three lineages could not be connected in any of the cladograms with the program TCS, since there were more than ten mutational steps between them. All the alleles within clades 3-2 (Fig. 5.3 A), 3-1 (Fig. 5.3 B) and 2-6 (Fig. 5.3 C) were from the single locality in the Heuningnes River system (Heuningnes lineage) and therefore exact contingency tests for geographic association could not be done. The Breede lineage was restricted to clades 3-1, 2-1 and 4-1 and the Tradou lineage was restricted to clades 3-3, 2-4 and 3-4 in Fig. 5.3 A, B and C respectively.

Using the –200 m contour for geographic distances, a significant association between the clades or alleles within them and geographic position was detected in clades 1-10, 2-2, 3-1, 3-3, 4-1 and 5-1 for control region ($19.000 < \chi^2 < 48.000$; $0.000 < p < 0.038$), clades 4-1 and 5-1 for cytochrome *b* ($0.000 < \chi^2 < 24.000$; $p = 0.000$) and clades 2-7, 4-1, 5-1 and 6-1 for the combined sequences ($21.000 < \chi^2 < 45.000$; $0.000 < p < 0.034$). The remaining clades did not show a significant association between their clades or alleles and geographic position in the control region ($2.000 < \chi^2 < 19.000$; $0.168 < p < 1.000$), cytochrome *b* ($2.000 < \chi^2 < 48.000$; $0.117 < p < 1.000$) and combined sequence ($2.000 < \chi^2 < 18.000$; $0.100 < p < 1.000$) cladograms.

A

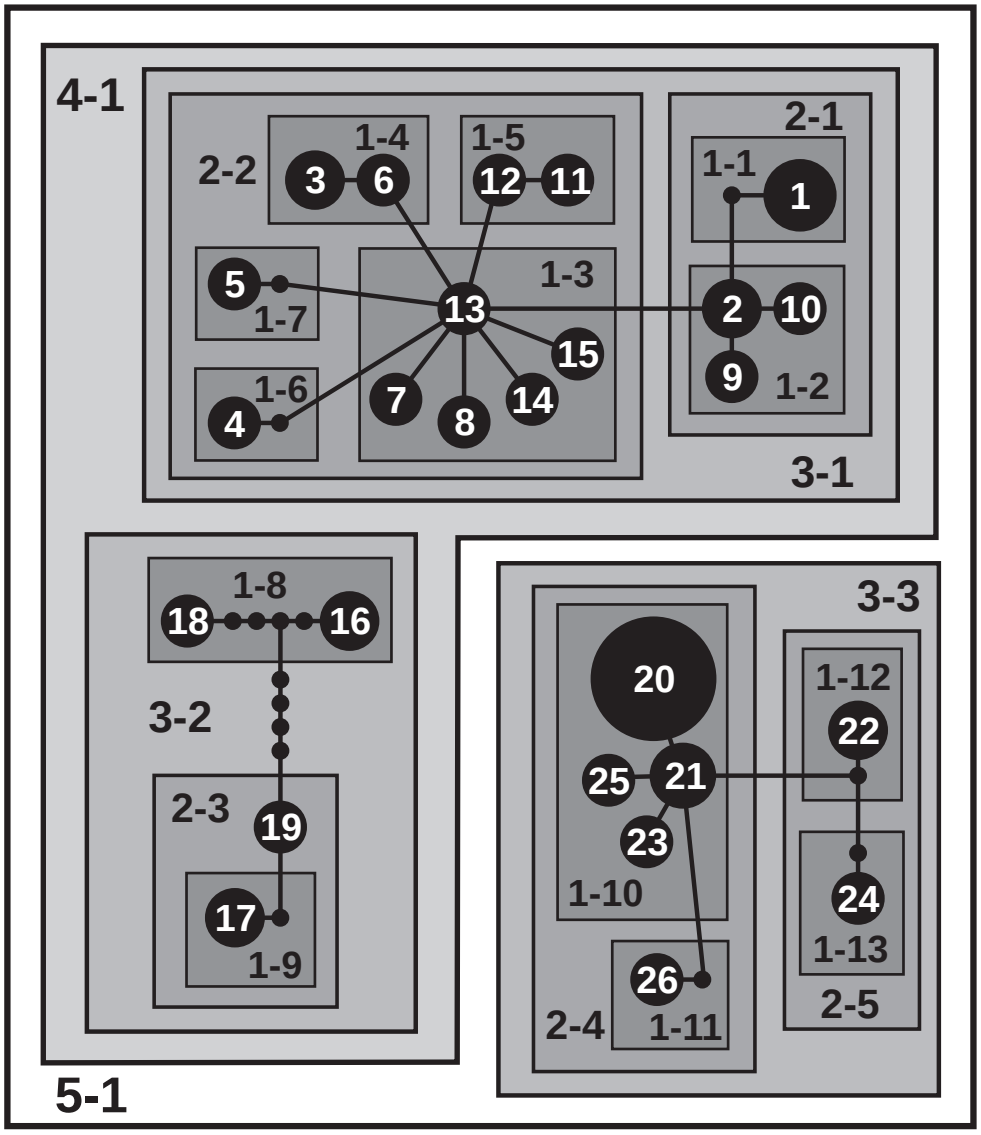

Figure legend below.

B

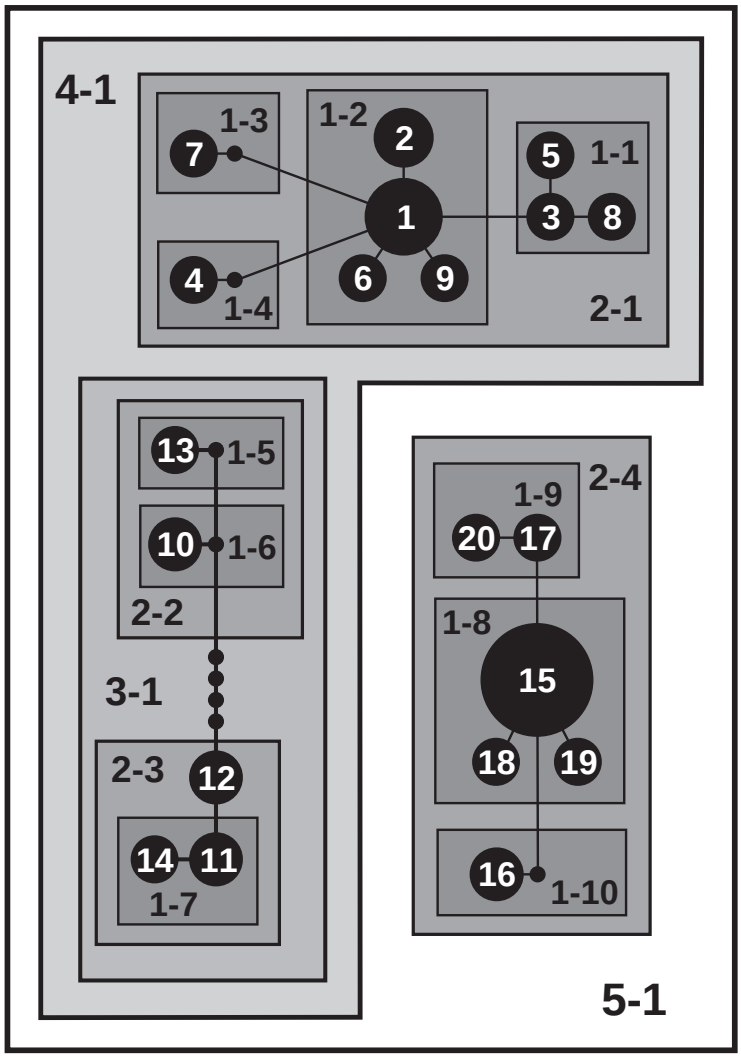

Figure legend below.

C

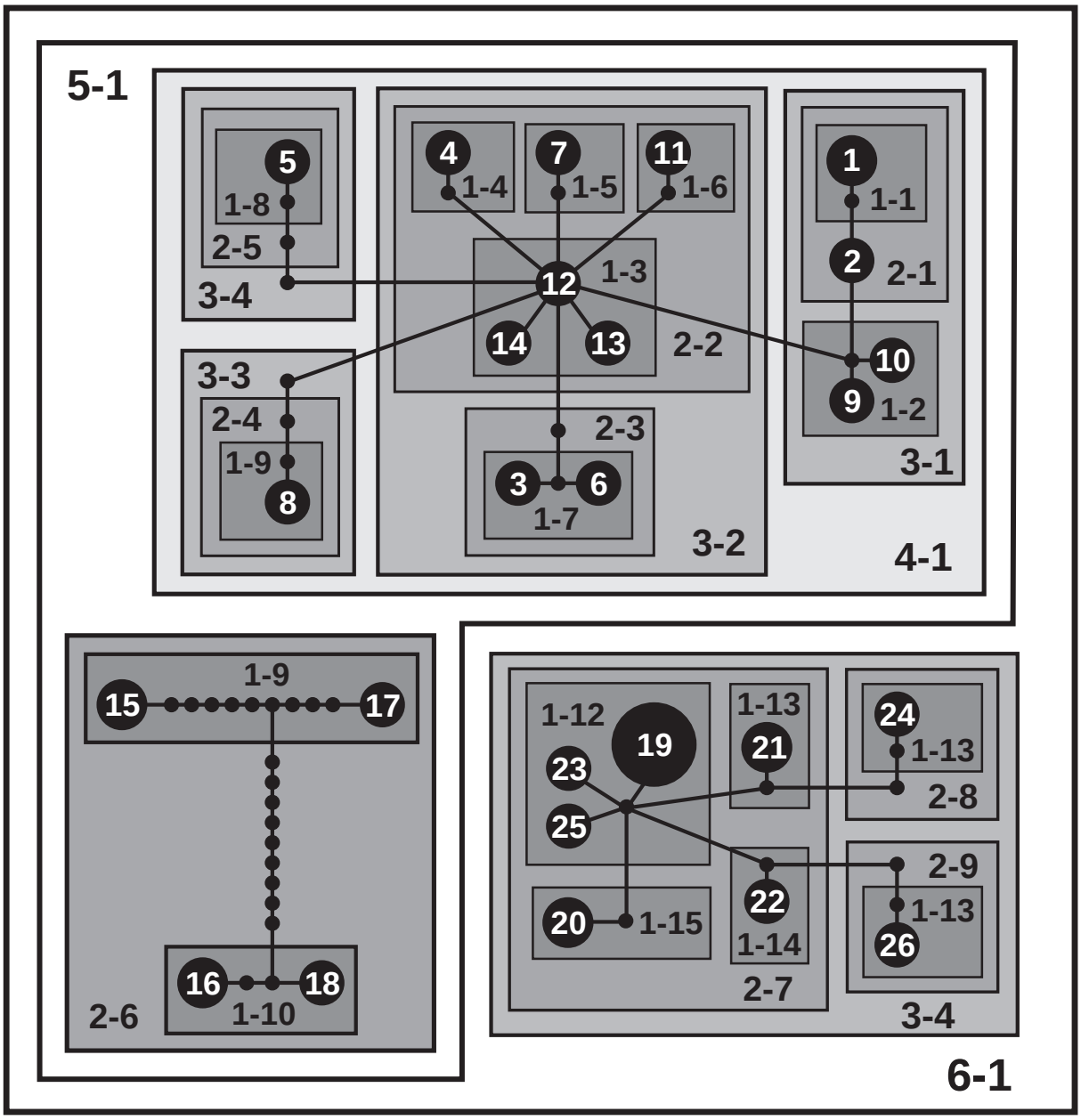

Fig. 5.3. Nested clade design of the control region (A), cytochrome *b* (B) and combined (C) alleles of *P. burchelli*. The Breede, Heuningnes and Tradou lineages are restricted to clades 3-1, 3-2 and 3-3 for control region, 2-1, 3-1 and 2.4 for cytochrome *b* and 4-1, 2-6 and 3-4 in the combined analysis respectively.

Only isolation type processes were inferred for higher-level clades based on the key of Templeton (2004), because of the geographic isolation and divergence between the Breede, Heuningnes and Tradou lineages (Table 5.7). For cytochrome *b*, there were no lower level clades that showed a significant association between the clades or alleles within them and geographic position. For control region and the combined control region and cytochrome *b* analysis, however, both isolation and migration type processes were inferred within the Breede and Tradou lineages. There were no changes in the type of processes that were inferred for control region, cytochrome *b* or the combined analysis when the current coastline was used to calculate geographic distances instead of using the –200 m contour.

Table 5.7. Evolutionary processes within *P. burchelli* identified through nested clade analysis and with the inference key of Templeton (2004). None of the inferences differed between analyses where the –200 m contour or the current coastline were used to calculate river distances.

Clade	Localities in clade	Inference chain	Migration or isolation	Inferred evolutionary process
Control region:				
1-10	All Tradou localities, except 23	1, 2, 3, 4, No	Migration/ Isolation	Restricted gene flow with isolation by distance
2-2	All Breede localities, except 1, 7 and 10	1, 2, 3, 4, No	Migration/ Isolation	Restricted gene flow with isolation by distance
3-1	Breede	1, 2, 11, 17, No	Inconclusive	Inconclusive
3-3	Tradou	1, 2, 11, 12, No	Migration	Contiguous range expansion
4-1	Breede and Heuningnes	1, 19, No	Isolation	Historical isolation
5-1	Breede, Heuningnes and Tradou	1, 19, No	Isolation	Historical isolation
Cytochrome *b*:				
4-1	Breede and Heuningnes	1, 19, No	Isolation	Historical isolation
5-1	Breede, Heuningnes and Tradou	1, 19, No	Isolation	Historical isolation
Combined control region and cytochrome *b*:				
2-7	All Tradou localities, except two individuals	1, 2, 11, 12, No	Migration	Contiguous range expansion
4-1	Breede	1, 2, 11, 17, No	Inconclusive	Inconclusive
5-1	Breede and Heuningnes	1, 19, No	Isolation	Historical isolation
6-1	Breede, Heuningnes and Tradou	1, 19, No	Isolation	Historical isolation

Discussion

Lineage distribution and evolutionary processes

Historical isolation and a relatively long period of divergence were evident between the three lineages found in *P. burchelli*. Surprisingly, the largest divergence was found within the Breede River system between the Breede and Tradou lineages. The Tradou lineage was only recorded in the Tradou catchment where it occurs in two fragmented populations one within Tradou's Pass and the other above the town of Barrydale (Fig. 5.1). In contrast, the Breede lineage is one of the most widespread redfin lineages recorded thus far, occurring across the Breede (excluding the Tradou catchment), Duiwenhoks and Goukou River systems. At least 20 populations of the Breede lineage were recorded during the present study and subsequent surveys in the Breede River system. The Heuningnes lineage seems to have the most restricted range of the *P. burchelli* lineages as it was only recorded from a single pool in the Grashoek River of the Heuningnes River system, despite surveys at six localities in the Heuningnes and neighbouring Uilkraal River systems.

Differentiation within the Breede lineage of *P.burchelli* was low, despite its wide distribution across multiple river systems. The low level of differentiation is reflected in the AMOVA analysis and the relatively low genetic distances among its alleles. With the NCA analysis of control region alleles, restricted gene flow with isolation by distance was inferred as an evolutionary process within the Breede lineage. This suggests that the Breede lineage must have been able to maintain at least low levels of migration across its range in the Breede River system and between the latter and the Duiwenhoks and Goukou River systems.

Pseudobarbus burchelli is the largest redfin species, based on specimens from the Breede River system (Skelton, 1988). This might be an adaptation to mainstream environments.

Previously there would have been very little competition from other fishes in the mainstream areas of the Breede River system. Only the cyprinid *Barbus andrewi* and anabantid *Sandelia capensis* would have been of similar size or larger than *P. burchelli* in mainstream areas of the Breede River system. *Sandelia capensis* occur in sympatry with *P. burchelli* at most localities and would therefore not have excluded the latter species from mainstream environments. *Barbus andrewi* is a larger mainstream cyprinid species, but is probably mostly omnivorous. However, since the introduction of alien fishes, mainstream populations of *P. burchelli* have been eliminated and adaptation to mainstream areas cannot be investigated.

Genetic differentiation was low within the Tradou lineage. Mostly migration type processes were identified within this lineage. High levels of gene flow can be expected within the Tradou lineage, because of the close proximity of the localities and because this lineage would have had a continuous distribution across the Tradou catchment before anthropogenic impacts. However, alleles were not randomly distributed. Restricted gene flow with isolation by distance and contiguous range expansion were potential evolutionary processes within the Tradou lineage. No evolutionary processes were inferred for the Heuningnes lineage, because it was only recorded from a single locality. Apart from the Grashoek River, *P. burchelli* has also been recorded in the two other major rivers of the Heuningnes River system, namely the Kars and Nuwejaars Rivers (Barnard, 1943; Skelton, 1988). The Heuningnes lineage displayed the highest gene and nucleotide diversity and divergence among its alleles, despite being analysed from a single locality. This, together with the historical records, suggests a much larger historical population size.

Role of sea level regressions and differentiation among river systems

If only confluence during lower sea levels played a role in connecting populations of *P. burchelli* that are currently isolated in different river systems, then one would have expected the Breede, Duiwenhoks and Heuningnes River systems to share a recent history of migration and that the population in the Goukou River system has been isolated. This was not the case. As expected, the close relationship between populations of the Breede River system and the one from the Duiwenhoks River system can be explained through the common confluence these river systems had during the lower sea levels of the LGM (Fig. 5.1). However, the close relationship between the latter two systems and the Goukou River system cannot be explained through connection during lower sea levels. The Goukou River system would have had a common confluence with the Gourits River system and this eastern palaeoriver system never had a common confluence with the historical Breede-Duiwenhoks-Heuningnes River system. The historical Gourits-Goukou River system would have flowed in a southerly direction across the Agulhas Bank, whereas the historical Breede-Duiwenhoks-Heuningnes River system flowed in a south-westerly direction.

It is possible that a river capture occurred between the Duiwenhoks and Goukou River systems. This capture would have had to be relatively recent to explain the lack of differentiation between the Goukou River system and the rest of the Breede lineage. The similar habitat of the Breede, Duiwenhoks and Goukou River systems would have allowed the Breede lineage to survive with minimal adaptation after river capture. Alternatively, the occurrence of the Breede lineage in the Goukou River system is as a result of recent translocation by humans. However, a unique blue sheen was observed in large specimens from the latter river system. It will be necessary to investigate whether this colour pattern is

heritable and whether it is restricted to this river system. Based on the present analysis a prediction of whether a potential translocation occurred from the Breede or the Duiwenhoks River systems cannot be made.

It is surprising that *P. tenuis* and *P. asper* from the Gourits River system do not also occur in the Goukou River system. If *P. burchelli* occurred in the Goukou River system during the LGM, then it is also surprising that the species does not occur in the Gourits River system. It is possible that the Goukou River system was isolated from the mainstream Gourits River system through waterfalls or unsuitable habitat and/or extinctions may have occurred. It is also possible that a river capture event occurred after the LGM and therefore after the Gourits and the Goukou river systems were isolated from each other.

Most of the southern coastal lowland areas that are now drained by the Heuningnes, Duiwenhoks and Goukou River systems, would have been drowned during the early Pliocene transgression (Fig. 5.1). Only when very slow substitution rates of 0.5 – 1 % are used for control region and cytochrome *b*, does the age of differentiation between the Heuningnes and Breede lineages resemble the approximate 3.4 - 5.2 myr BP of the early Pliocene. These substitution rates are slower than the 3 % that Bernatchez & Danzmann (1993) used for control region in salmonids, the 8-10 % that Brown *et al.* (1993) used for control region in sturgeon and the 2.8 % that Ortí *et al.* (1994) used for cytochrome *b* in sticklebacks. The colonisation of the Heuningnes River system by *P. burchelli* is therefore more recent than the early Pliocene transgression. This colonization could have occurred as a result of the confluence of the historical Breede-Duiwenhoks River system and the Heuningnes River. However, isolation since the LGM regression represents insufficient time to allow for the divergence that has occurred between the Breede and the Heuningnes lineages. A substitution

rate of at least ten times that used by Bernatchez & Danzmann (1993), Brown *et al.* (1993) and Ortí *et al.* (1994) for mitochondrial control region and cytochrome *b* in fish is required. It suggests that earlier isolation occurred.

One possibility is that the Heuningnes and the historical Breede-Duiwenhoks River system never had a common confluence before reaching the –130 m LGM sea level and that *P. burchelli* reached the Heuningnes River system through river capture rather than confluence during low sea levels. But that requires the Heuningnes to have flowed in a much further westerly position than what has been indicated in Fig. 5.1. Even then, a common confluence with the Breede-Duiwenhoks River system during the LGM seems likely. It is possible that ecological differentiation occurred between the Heuningnes and Breede lineages. Their current distribution would then be the result of their unique adaptations to the different environments in which they now occur. Habitat preference could explain why both lineages do not occur in both river systems, considering that the LGM connection would only have been about 18 000 years ago.

Ecological differentiation could have occurred without other isolating mechanisms. Alternatively, the presence of waterfalls in the offshore reaches between the Heuningnes River and the historical Breede-Duiwenhoks River system may have enhanced differentiation by preventing upstream migration into the Heuningnes River. Bloomer & Impson (2000) reported a similar situation to the Heuningnes lineage of the present study. They found eutrophic adapted (Verlorenvlei River system) and oligotrophic adapted (Berg River system) lineages within *P. burgi*. The divergence between these two *P.burgi* lineages are much older, however, than between the Heuningnes and Breede lineages of *P. burchelli*.

Parapatric differentiation of the Tradou lineage

Compared to the other *P. burchelli* lineages the divergence of the Tradou lineage is older than the divergence between the Breede and Heuningnes lineages. Based on the current distributions, the differentiation between the Tradou lineage and the other lineages probably occurred within the Breede River system. A waterfall marks the lower limit of distribution of the Tradou lineage, which would have prevented upstream gene flow. Downstream migration might have been possible. However, there is no evidence from mtDNA alleles that relatively recent interbreeding between these two lineages occurred. This suggests that the Tradou lineage was either never in contact with the Breede lineage since the initial divergence of mtDNA alleles into different monophyletic lineages, or that they were not able to breed with the Breede lineage in areas where their distributions overlapped. Alien fishes now dominate the river below the waterfall where overlap between these two lineages would have been most likely. Therefore a direct assessment of whether these two lineages can interbreed cannot now be done in their natural environment.

Similar lineage divergence events within a single river system have occurred in cyprinid species elsewhere in the Cape Floristic Region. For example, speciation between *Barbus erubescens* and *B. calidus* was probably aided by waterfalls in the Twee River catchment (Olifants River system), which mark the lower distributional limit for *B. erubescens* (Skelton, 1974a; Swartz *et al.*, 2004). These waterfalls would have prevented upstream gene flow, but it is surprising that *B. erubescens* did not spread to the rest of the Olifants River system. Similary, it is surprising that the Tradou lineage of *P. burchelli* did not extend its range in the Breede River system. It may suggest that the Tradou lineage is in some way adapted to the Tradou catchment.

Another reason may be competition avoidance. Apart from one eel (*Anguilla mossambica*), no other indigenous fishes were recorded with the Tradou lineage of *P. burchelli*. The Breede lineage of *P. burchelli* may be better adapted to compete with mainstream fish. A similar argument was proposed as a reason why *B. erubescens* did not extend its range in the Olifants River system, since historically it only occured with the much smaller *Galaxias zebratus* (Skelton, 1974a; Swartz *et al.*, 2004). This raises the possibility that parapatric speciation occurred between the Tradou lineage and a Breede-Heuningnes ancestor. Because of the possibility of downstream gene flow, some level of ecological speciation reinforced by breeding segregation would have had to occur.

Taxonomic and conservation implications

The results of this study require that the taxonomic status of the Breede, Heuningnes and Tradou lineages be re-investigated, because of the large mtDNA divergence between them. These lineages have unique colour patterns and probably have unique adaptations to their respective habitats. The lack of reciprocal monophyly of these lineages despite current or historical connections that would have allowed it, is further support that breeding segregation may have occurred. Several taxonomic changes will have to be made if these lineages prove to be different species and if previous taxonomic decisions are accepted.

If the neotype from the Tradou catchment that Skelton (1988) assigned the name *Pseudobarbus burchelli* proves to be of the Tradou lineage, then the name *P. burchelli* will have to be assigned to this lineage. In this case the name *Gnathendalia vulnerata* Castelnau 1861 under the combination *Pseudobarbus vulneratus*, will then become available for the widespread Breede lineage. There are no designated names available for the Heuningnes lineage (Skelton, 1988). It is unlikely that Smith's (1841) *Barbus (Pseudobarbus) burchelli* was originally described from the Tradou catchment, but the above mentioned change will cause the least taxonomic instability.

The Breede, Heuningnes and Tradou lineages can be considered to be Evolutionarily Significant Units (ESU's) according the definition of Moritz (1994). The historical isolation process that have led to the divergence of these lineages must be allowed to continue, by keeping these lineages isolated and by conserving the different habitats in which they occur. Other evolutionary processes that will have to be conserved are possible historical restricted gene flow within the Breede and Tradou lineages. In the case of the Breede lineage, it may be difficult to restore corridors between the currently isolated populations. However, at least 20 populations of this lineage were recorded during the present study and subsequent surveys. If the size of the populations remains large enough to prevent inbreeding, most of the genetic diversity can be effectively conserved.

Urgent conservation actions are needed to secure the survival of both the Heuningnes and Tradou lineages. More surveys are needed to establish the full range of the Heuningnes lineage. The range of the Tradou lineage has been established and is restricted to the Tradou catchment (E. R. Swartz, unpublished). The river reach area between Tradou's Pass and above the town of Barrydale can be rehabilitated. Restoring a corridor between these two

fragmented populations will allow the Tradou lineage to maintain a larger effective population size and will also allow the evolutionary process of restricted gene flow to continue.

Acknowledgements

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Chapter 6

Phylogeny and biogeography of the genus *Pseudobarbus* (Cyprinidae) in southern Africa

Abstract

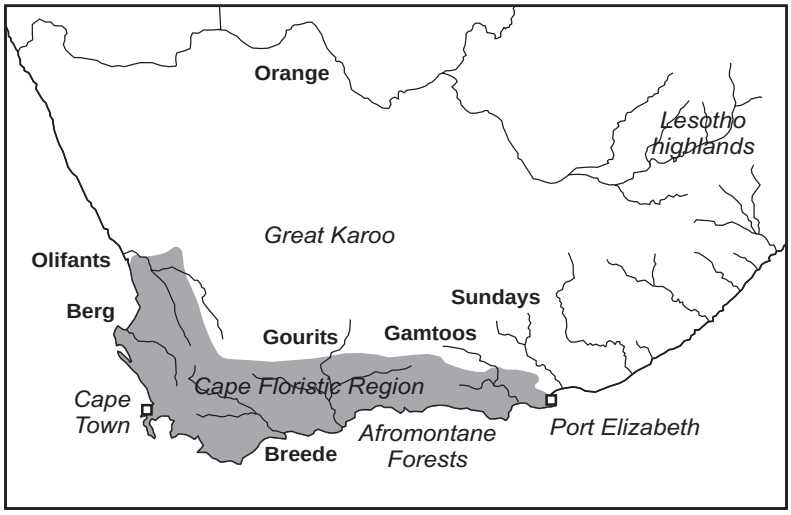
Biogeographic hypotheses regarding the evolution of the genus *Pseudobarbus* have been, to date, formulated on the basis of morphological data only. However, several phylogeographic studies based on mitochondrial DNA have shown that 15 historically isolated lineages exist within the seven currently described species. In the present study, the relationships among the historically isolated lineages of *Pseudobarbus* were reconstructed using both molecular and morphological data. For the molecular analyses, the mitochondrial control region, cytochrome *b* and 16S and a combined dataset of all these were used for comparison to a morphological dataset. There were conflicts between the molecular and morphological phylogenies suggesting convergent evolution and homoplasy in some of the morphological characters. When a combined molecular and morphological analysis was done, however, the morphological characters improved resolution of the deeper relationships. The combined molecular and morphological analysis suggests that the earliest divergence in *Pseudobarbus* was between *P. quathlambae* in Lesotho and the other species that are currently restricted to the Cape Foristic Region in South Africa. The molecular analyses also suggest a close relationship between *P. phlegethon* in the Olifants River system on the west coast of South Africa and a lineage of *P. afer* that occur in small river systems in Afrotemperate Forests on the south coast. The latter relationship can only be explained through previous occurrence and subsequent extinction of ancestral populations in the Gourits River system and also suggests that the polytypic *P. afer* is polyphyletic. Several species descriptions may be necessary to adequately reflect the taxonomic diversity within *Pseudobarbus*.

Introduction

The cyprinid genus *Pseudobarbus* comprises seven currently recognised species (Skelton, 1988). Six of the species occur in river systems associated with the Cape Floristic Region (CFR) and the Cape Fold Mountains in south-western South Africa, while one species is restricted to the highlands of Lesotho (Fig. 6.1A). *Pseudobarbus* is the largest genus of the Cape ichthyofauna, which can be distinguished from the larger Karoo ichthyofauna (Skelton, 1986; 1994b) mainly on the bases of regional ecological differences. Together they comprise the temperate ichthyofauna (Skelton, 1986; 1994b), which is dominated by cyprinids in terms of species diversity (Skelton *et al.*, 1991).

An interesting feature of the temperate cyprinids is that several of the species are of polyploid origin. *Pseudobarbus* species are all tetraploid (Naran, 1997). There are also diploid and tetraploid '*Barbus*' and hexaploid *Labeobarbus* species (Naran, 1997; Oellermann & Skelton, 1990). The tetraploid '*Barbus*' species are endemic to southern Africa and not closely related to the North-African and European tetraploid *Barbus*. It has been established that the sister group to *Pseudobarbus* is the southern African tetraploid '*Barbus*' species, whilst the sister group to all the southern African tetraploid *Pseudobarbus* and '*Barbus*' species are the pan-African diploid '*Barbus*' (Machordom & Doadrio, 2001b; Tsigenopoulos *et al.*, 2002; E. R. Swartz *et al.*, unpublished).

A



B

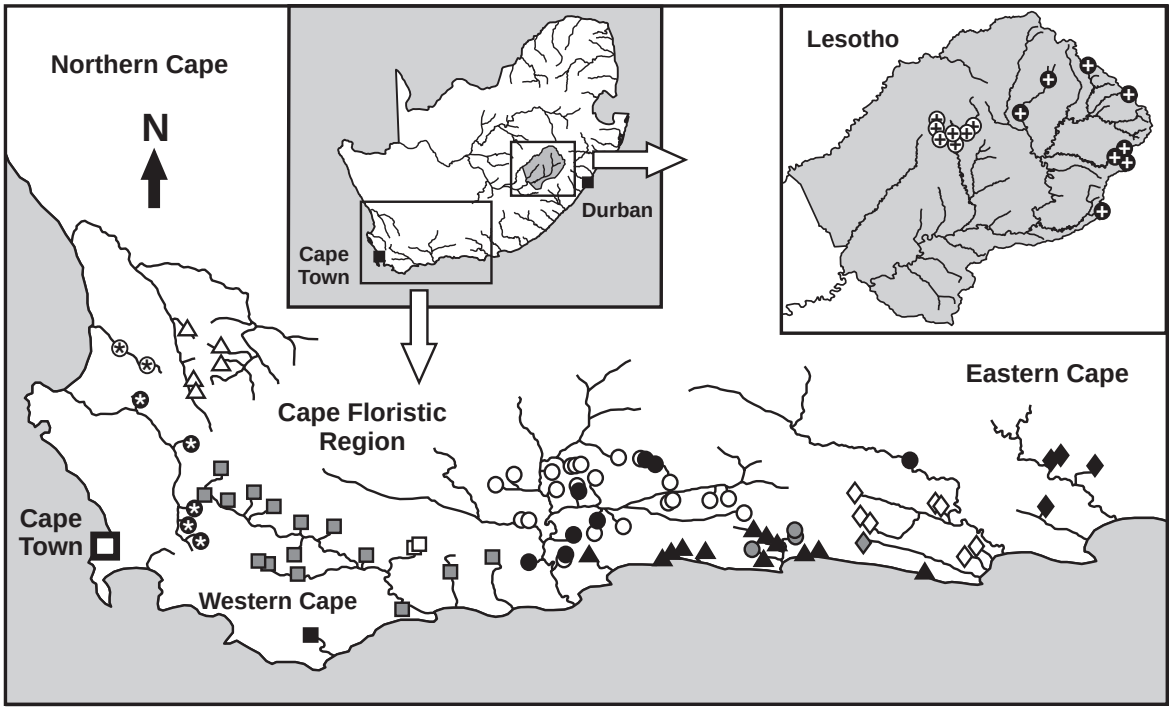


Fig. 6.1. Regions and river systems referred to in the text (A) and the sampling sites for the present study which indicate distribution of historically isolated lineages of *Pseudobarbus* in the Cape Floristic Region and Lesotho (B). All these sites were analysed using control region, whilst selected sites from each of the lineages were analysed using cytochrome *b* and 16S (see Table 6.2-6.3). Lineages on main map: *P. phlegethon* (white triangle); Verlorenvlei (white circle with black asterisk) and Berg (black circle with white asterisk) *P. burgi*; Breede, Heuningnes and Tradou *P. burchelli* (grey, black and blue squares respectively); *P. asper* (black circle); Gourits (white circle) and Keurbooms (grey circle) *P. tenuis*; Forest, Krom, St. Francis and Algoa *P. afer* (black triangle and grey, white and black diamonds respectively). Lineages on insert: Mohale (white circle with black cross) and Eastern *P. quathlambae* (black circle with white cross).

Boulenger (1911) classified African barbs into four broad categories based on scale radii and the shape of the last unbranched dorsal ray. All the southern African tetraploid barbs have radiating scales, which distinguishes them from the large hexaploid barbs, which have scales with parrallel striae, but not from the diploid '*Barbus*' species (Oellermann & Skelton, 1990; Skelton, 1976). Barbs with radiating scale striae were further divided into three categories, namely those with bony and smooth, bony and serrated or soft and not serrated primary dorsal fin-rays (Boulenger, 1911). *Pseudobarbus* species fall within the latter category, having soft and flexible dorsal rays (Skelton, 1988). All the other southern African tetraploid barbs fall in the second category with their serrated primary dorsal spine. It was on the basis of their serrated dorsal spine that the tetraploid southern African barbs, *Barbus calidus* and *B. erubescens*, were not included in the genus *Pseudobarbus*, despite having red fins which is a striking characteristic of the latter genus (Skelton, 1988).

A further important step in defining the genus *Pseudobarbus*, was the recognition that the Maloti minnow in Lesotho (*P. quathlambae*) belonged to it. The Maloti minnow was originally described as *Labeo quathlambae* from a Drakensberg tributary in South Africa by Barnard (1943), mainly because of its small scales and subterminal mouth. After it was feared extinct (Jubb, 1966), Greenwood & Jubb (1967) did a detailed osteological study on the specimens collected from the type locality. They concluded that it was not a *Labeo* species and could not clearly relate it to any other cyprinid genus. They therefore placed it in a monotypic genus *Oreodiamon*, which means "spirit of the mountain", referring to its extinct status. Following its rediscovery in Lesotho (Pike & Tedder, 1973), Skelton (1974b) collected live specimens of the Maloti Minnow and recognised, amongst other characteristics, that they have red fins.

When Skelton (1980; 1988) reviewed the taxonomy and established relationships within *Pseudobarbus*, he found that several morphological characters are shared between the Maloti Minnow (*P. quathlambae*) and *P. tenuis* (southern parts of the Western Cape), which suggested a sister species relationship. The latter was surprising given the large geographic distance between these two species. Based on the morphological characters, *P. phlegethon* from the Olifants River system in the western parts of the CFR was inferred as the sister species to *P. quathlambae* and *P. tenuis*. *Pseudobarbus afer*, which is the most widespread redfin species, and the Karoo adapted *P. asper* were inferred as sister species. Skelton (1980; 1988) suggested that *P. afer* is a polytypic species because of large variation in several morphological characters.

There has also been taxonomic uncertainties regarding the two species with two pairs of barbels (*P. burgi* and *P. burchelli*), because these species are closely related (Jubb, 1965; Skelton, 1980; 1988) and because it is not clear from where *Barbus (Pseudobarbus) burchelli* Smith 1841 was described. No original type material exists and no type locality was specified. A decision had to be made whether a species from the Breede River system region or one from the neighbouring Berg River system was to be assigned as the species that Smith described. Apart from Smith's name, the oldest name available for the Breede species was *Gnathendalia vulnerata* Castelnau 1861 and for the Berg species it was *Barbus burgi* Boulenger 1911.

Both Barnard (1943) and Jubb (1965) suggested that it was not possible to decide whether *Barbus (Pseudobarbus) burchelli* was the Berg or Breede species. Barnard (1943) decided to place Boulenger's *Barbus burgi* in synonymy with Smith's species. However, Jubb (1965) placed *Gnathendalia vulnerata* in synonymy with Smith's species after P. H. Greenwood

examined the type skins of Castelnau's *Gnathendalia vulnerata* and concluded that they agreed with Smith's description of *Barbus (Pseudobarbus) burchelli*. No specific reasons were provided by Jubb (1965) why he did not accept Barnard's (1943) decision, but presumably *Barbus burgi* was in general usage.

When Skelton (1988) defined a monophyletic redfin genus, he raised Smith's subgenus name to a full generic name and accepted Jubb's (1965) nomenclatural changes to maintain taxonomic stability. Skelton (1980) suggested that the earliest divergence in *Pseudobarbus* was between *P. burgi* and all the other *Pseudobarbus* species. A phylogenetic re-analysis of the parsimony informative characters discussed by Skelton (1980) is presented in the present study in conjunction with a molecular phylogeny of the group.

The morphological relationships were easily explained through differentiation between adjacent river systems, apart from the large geographic distance between *P. quathlambae* and *P. tenuis*. Skelton (1980) suggested that an ancestor of the latter species must have occurred in Karoo tributaries of the Orange River system (Fig. 6.1A). These populations would have given rise to *P. tenuis* in the Gourits River system where they now occur in sympatry with *P. asper*. Sympatry between *P. tenuis* and *P. asper* was therefore likely the result of secondary contact. Redfins do not currently occur in the area of the Orange River system where Skelton (1980) proposed an exchange between the Orange and Gourits River systems. Extinction of the proposed ancestral populations in Karoo tributaries of the Orange River system therefore had to be assumed.

The Olifants and Orange River systems shared a common confluence (Dingle & Hendey, 1984) not later than the early Tertiary (De Wit, 1993). There are also several fish species linked to the Karoo ichthyofauna that confirm such a connection (Skelton, 1986). Of the three species of *Austroglanis*, one occurs in the Orange River system and two occur in the Olifants River system. *Labeobarbus capensis* from the Olifants River system seem to be closely related to *L. aeneus* and *L. kimberleyensis* from the Orange River system. Two other cyprinid species that occur in the Olifants River system have Orange River system and Karoo connections. *Labeo seeberi* from the Olifants River system is closely related to *L. umbratus* in the Orange, Gourits, Gamtoos and other Karoo type river systems (Reid, 1985). *Barbus anoplus* from the Olifants River system is closely related to several other populations of this species across the Orange River system and rivers that drain the Great and Little Karoo. A close relationship between *P. phlegethon* and an ancestor of *P. quathlambae* and *P. tenuis* was therefore consistent with geological and biogeographic evidence.

Skelton (1980; 1988) noted morphological variation between populations within species, but the differences was not clear or consistent enough to warrant full species status for these unique populations. However, mtDNA evidence suggests that 15 historically isolated lineages can be identified within the seven *Pseudobarbus* species (Fig. 6.1B): Mohale and Eastern lineages were identified within *P. quathlambae* (Chapter 2), four historically isolated lineages (Algoa, St. Francis, Krom and Forest) were found within *P. afer* (Chapter 3), Gourits and Keurbooms lineages were found within *P. tenuis* (Chapter 4), *P. burchelli* consists of the Breede, Heuningnes and Tradou lineages (Chapter 5) and Bloomer & Impson (2000) identified the Berg and Verlorenvlei lineages within *P. burgi*. Swartz *et al.* (2004) found fixed allelic differences at seven allozyme loci between the Olifants and Doring populations of *P. phlegethon*, but these differences were not as clearly reflected in the mtDNA sequences

(Chapter 3). This discrepancy may reflect interesting population histories within the Olifants River system, but for the purpose of the present study *P. phlegethon* will be referred to as a single lineage.

Minor differentiation was also found within the Forest lineage of *P. afer* (Chapter 3) that could have interesting phylogeographic implications, but these lineages were not divergent enough to influence phylogeny reconstruction. Only one mutational step distinguished the Gamtoos populations of *P. asper* from some Gourits alleles of the same species, and therefore this species was also considered as a single lineage (Chapter 4). These lineages are more informative with regards to phylogeny reconstruction than the currently described species, since they represent more of the diversity within *Pseudobarbus*. The historically isolated lineages are also more appropriate units for understanding the biogeography of the genus.

To investigate their phylogenetic relationships, representatives of all 15 historically isolated lineages were included in the present study. Representatives from all the southern African serrated tetraploid '*Barbus*' species were included as outgroups (*B. calidus*, *B. erubescens*, *B. serra*, *B. andrewi*, *B. trevelyani* and *B. hospes*). The aim of the present paper was to establish the phylogenetic relationships based on mtDNA between the 15 historically isolated lineages, to compare these relationships to inferences based on morphology and to test previous hypotheses regarding the taxonomy, biogeography and evolution of the genus *Pseudobarbus*.

Materials and Methods

Sampling

Sequences and specimens were available from Chapters 2-5, Bloomer & Impson (2000) and Swartz *et al.* (2004). Additional *P. burgi*, *P. phlegethon* and outgroup specimens were collected by snorkelling with a handnet or with a 3m seine net. Whole fish samples were stored in liquid nitrogen in the field and transferred to a –70 °C freezer upon returning to the laboratory or muscle, fin-clips or whole fish samples were placed in EtOH (Department of Genetics, University of Pretoria). The remaining carcasses and/or additional samples were fixed in formalin and deposited in the South African Institute for Aquatic Biodiversity collection (Grahamstown) as voucher specimens.

DNA extraction, amplification and sequencing

Standard protocols of chemical digestion and phenol/chloroform extraction were used to isolate total genomic DNA from the frozen or EtOH preserved tissue (Sambrook *et al.*, 1989). Most of the 5’ end and part of the 3’ end of the mitochondrial DNA control region was amplified (PCR) with the primers L16560 (5’ CCAAAGCCAGAATTCTAAC 3’) and H677 (5’ GTCGCGCAAAAACCAAAG 3’) (Chapter 2). From Machordom & Doadrio (2001a), the primers GluF (5’ AACCACCGTTGTATTCAACTACAA 3’) and ThrR (5’ ACCTCCGATCTTCGGATTACAAGACCG 3’) were used to amplify almost the entire mitochondrial cytochrome *b* gene. The widely used vertebrate primers 16Sar and 16Sbr (Palumbi *et al.*, 1991) were used to amplify part of the 3’ end of the 16S mtDNA ribosomal gene.

Reagents (apart from the primers) and conditions for amplification, purification and cycle sequencing were the same for control region, cytochrome *b* and 16S. Amplification was performed in 50 µl volumes containing 1 x buffer, 2 mM $MgCl_2$, 0.2 mM of each of the four nucleotides (Promega), 25 pmol of each primer, 1.5 U of Super-Therm DNA polymerase (Southern Cross Biotechnology) and 100-200 ng template DNA. Conditions for amplification involved an initial denaturation of 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 54°C and 45 seconds at 72°C and a final extension of 5 minutes at 72°C.

PCR products were purified using the High Pure™ PCR Product Purification Kit (Boehringer Mannheim), followed by elution in ddH_2O. Cycle sequencing was performed in 10 µl volumes, with 2 µl ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems), 1.6 pmol of a single primer (L16560 or H677 for control region, GluF or ThrR for cytochrome *b* or 16Sar or 16Sbr for 16S) and 100 ng of purified DNA as template. PCR cycling and cycle sequencing were performed in a Geneamp® PCR System 9700 (Applied Biosystems). Nucleotide sequences were determined through ABI 377 or ABI 3100 automated sequencers.

Consensus sequences were obtained from the forward and reverse sequences of each specimen and by comparing these to sequences from other individuals through alignment and inspection in Sequence Navigator 1.01 (Applied Biosystems). The consensus sequences were aligned using Clustal X (Thompson *et al.*, 1997) and checked manually. Several control region sequences were available from Chapters 2-5. Some *Pseudobarbus* cytochrome *b* sequences were available from Machordom & Doadrio (2001b) and Tsigenopoulos *et al.* (2002) under the GenBank accession numbers AF180848 - AF180851 and AF287449 - AF287454 respectively.

Morphology

External and internal morphological characters were coded for comparison to the molecular analysis from Skelton (1980). Morphological data were not available for three of the historically isolated lineages, namely the Heuningnes and Tradou lineages of *P. burchelli* and the Mohale lineage of *P. quathlambae*. There were also no clear morphological characters investigated by Skelton (1980) that distinguish between the *P. afer* or *P. tenuis* lineages. Characters were ordered according to character states in the outgroups *B. calidus* and *B. erubescens*.

Phylogenetic analysis

Control region, cytochrome *b* and 16S sequence datasets were first analysed separately and then were combined to assess the robustness of relationships across datasets. As expected, congruence between these datasets were not rejected with a partition homogeneity test (p = 0.410) as implemented in PAUP* (Swofford, 2002), since they are linked in the mitochondrial genome. The morphological dataset was also analysed separately and finally, combined with the combined molecular dataset. Only representatives of the historically isolated *Pseudobarbus* lineages were used for more computationally intensive phylogenetic analyses. To assess the phylogenetic relationships among the historically isolated lineages of *Pseudobarbus*, neighbour joining (NJ; Saitou & Nei, 1987), maximum likelihood (ML; Felsenstein, 1981) and maximum parsimony (MP; Hennig, 1966) analyses were performed in PAUP*. In addition, Bayesian analyses were performed in MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001).

The nucleotide substitution model that best fits the molecular data was selected from 56 such models with the Akaike test in MODELTEST version 3.06 (Posada & Crandall, 1998). Ti:Tv ratio, proportion of invariable sites (I) and the α value of the gamma distribution (rate variation among sites) were also estimated. NJ, ML and Bayesian analyses were based on these models. Starting trees for the ML analyses were obtained through the NJ method. The optimal tree was obtained through a heuristic search with ten random sequence additions. Branch support was assessed with 1000 bootstrap replicates (Felsenstein, 1985) each with ten replicates of random taxon addition. Gaps were treated as missing data for the ML and Bayesian analyses.

Bayesian posterior probabilities were estimated following the same model that was used in the ML analysis with invariable sites (I) and rate variation among sites (α or gamma distribution). After experimental runs, the "revmat with multiplier", "gamma shape with multiplier" and "prop. invariants with beta proposal" parameters were made more stringent. To allow for finer scale sampling of rate variation among different sequence regions, ten gamma shape categories were used. The temperature between the chains was lowered compared to the default settings, to allow for better sampling among the different chains. One cold and three heated Monte Carlo Markov chains (MCMC) were run simultaneously for one million generations.

The log-likelihood scores were plotted against the generation number to establish when the runs became stable. This occurred between 3 000 to 10 000 generations. Based on this, the first 100 000 generations were discarded as "burnin" to be confident that the MCMC chains were only sampling optimal trees. The remaining trees were sampled every 100 generations, yielding 9 000 trees for each of the analyses from which the posterior probabilities were

estimated. This process was repeated four times to assess whether the different analyses gave consistent results. Once the stability was confirmed, a run of five million generations was done that yielded 49 000 trees after again discarding the first 100 000 generations as "burnin".

The MP analyses for the molecular and morphological datasets were performed through heuristic searches with TBR branch swapping and 1000 random additions of taxa. Branch support was assessed with 10 000 non-parametric bootstrap replicates (Felsenstein, 1985), each with 10 replicates of random taxon addition. For control region and 16S, single gaps were treated as a 5th character state. The sequence regions where adjacent gaps occurred were coded as different character states of a single character.

Results

Sequence analysis

The different datasets and the analyses to which they were subjected are summarised in Table 6.1. From Chapters 2-5, analysis of 259 individuals from 25 river systems and 102 localities yielded 115 control region alleles. The locality information, number of individuals and number of alleles analysed for cytochrome *b* (63 individuals, 51 alleles, 45 localities, 19 river systems), 16S (49 individuals, 31 alleles, 39 localities, 19 river systems) and the additional control region sequences (17 individuals, 17 alleles, 12 localities, 5 river systems) are given in Tables 6.2 – 6.3. The same mtDNA regions were consistently used, which were positions 17-615 for control region, 15350-16429 for cytochrome *b* and 2959-3521 for 16S with reference to the mtDNA genome sequence of *Cyprinus carpio* (Chang *et al.* 1994). This yielded 609-

614, 1080 and 568 base pairs for control region, cytochrome *b* and 16S respectively and a combined analysis of 2257 base pairs.

Parsimony informative characters for the molecular datasets are given in Table 6.1. Forty-eight of the cytochrome *b* amino acids varied within *Pseudobarbus*. Amino acid differences were 0-8 within *Pseudobarbus* lineages, 1-25 between *Pseudobarbus* lineages, 17-31 between *Pseudobarbus* lineages and the southern African tetraploid '*Barbus*' outgroups and 1-27 among the latter outgroups. Genetic distances based on the HKY substitution model with I = 0 and equal rates among sites varied between 1 – 10.3% for control region, between 1.6 - 15.8% for cytochrome *b* and between 0 – 4% for 16S between lineages, compared to 0 – 2% for control region, 0 – 2.5% for cytochrome *b* and 0 – 0.7% for 16S within lineages.

Table 6.1. Genetic and morphological datasets and the phylogenetic analyses that were employed in the present study.

Data	N ingroup taxa	N outgroup taxa	Analysis	Gap treatment	N characters or bases	N trees	Score/Ntrees
Control region	127	4	NJ	Missing data	614	-	
Control region	15	2	MP	5th state & coded	101		
Control region	24	5	MP	5th state & coded	139		398(3)
Control region	15	2	NJ & ML	Missing data	609	-	
Cytochrome *b*	44	6	MP	-	348		967(24)
Cytochrome *b*	44	6	NJ	-	1080	-	
Cytochrome *b*	15	2	MP	-	269		
Cytochrome *b*	15	2	NJ & ML	-	1080	-	
16S	24	7	MP	5th state & coded	50		112 (836)
16S	15	2	MP	5th state & coded	21		
16S	15	2	NJ & ML	Missing data	568	-	
Combined DNA	24	5	MP	5th state & coded	512		1338 (3)
Combined DNA	24	5	MP	5th state & non-coded	515		
Combined DNA	24	5	MP	Missing data	502		
Combined DNA	24	5	NJ, BA	Missing data	2257	-	
Combined DNA	15	2	MP	5th state & coded	409		
Combined DNA	15	2	MP	5th state & non-coded	412		
Combined DNA	15	2	MP	Missing data	402		
Combined DNA	15	2	NJ, ML & BA	Missing data	2257	-	
Morphology	8	2	MP (ordered)	-	39		65 (2)
Morphology	8	2	MP (unordered)	-	36		
Morphology & DNA	12	2	MP	5th state & coded	384		850 (2)

Table 6.2. Localities of additional samples that were analysed for the present study. Other samples are from Chapters 2-5, Bloomer & Impson (2000) and Swartz *et al*. (2004). See acknowledgements for collectors and field assistants. The plus signs refers to sites where different species occurred in sympatry.

Code	Species	River system	Locality	Latitude	Longitude	Date
1	*P. afer*	Kaaimans	Kaaimans	33° 58’ 14” S	22° 33’ 30” E	16/04/2000
2		Klein Brak	Klein Brak	33° 57’ 10” S	21° 58’ 45” E	22/04/2000
3		Tsitsikamma	Tsitsikamma	34° 05’ 49” S	24° 26’ 52” E	02/04/2000
4		Swartkops	Blindekloof	33° 40’ 20” S	25° 19’ 00” E	03/03/2001
5		Sundays	Kabouga	33° 15’ 43” S	25° 22’ 45” E	21/03/2003
6			Kabouga tributary	33° 15’ 58” S	25° 25’ 19” E	21/03/2003
7			Wit (Otto’s Pool)	33° 20’ 08” S	25° 41’ 33” E	10/05/2000
8		Krom	Krom	33° 51’ 48” S	23° 58’ 46” E	06/05/2000
9		Kabeljous	Gheis	33° 56’ 26” S	24° 48’ 17” E	01/04/2000
10		Gamtoos	Ys	33° 39’ 58” S	24° 34’ 25” E	08/05/2000
11^{+}	*P. asper*	Gourits	Langtou	33° 58’ 30” S	21° 47’ 20” E	23/04/2000
12		Gamtoos	Groot	33° 19’ 05” S	24° 20’ 50” E	23/09/1998 & 06/02/2001

Table 6.2. Continued.

Code	Species	River system	Locality	Latitude	Longitude	Date
13	*P. tenuis*	Gourits	Bos	33° 43’ 50” S	21° 30’ 22” E	07/05/2000
14			Aaps	33° 19’ 39” S	22° 27’ 44” E	03/02/2000
15			Kruis	33° 28’ 45” S	21° 54’ 10” E	04/02/2000
11[+]			Langtou	33° 58’ 30” S	21° 47’ 20” E	23/04/2000
16		Bitou	Kransbos	33° 55’ 20” S	23° 13’ 15” E	11/04/2000
17		Keurbooms	Diep	33° 52’ 07” S	23° 29’ 52” E	27/02/2001
18			Langbos	33° 51’ 15” S	23° 29’ 26” E	27/02/2001
19	*P. burchelli*	Breede	Koekedou	33º 21’ 30” S	19º 17’ 00” E	24/03/2001
20			Hoeks	34º 01’ 30” S	19º 50’ 30” E	21/03/2001
21			Kogmanskloof	33º 46’ 20” S	20º 07’ 10” E	22/03/2001
22			Leeu	34° 00’ 00” S	20° 20’ 00” E	09/03/2001
23	*P. burchelli*	Breede	Tradou	33° 56’ 51” S	20° 42’ 32” E	Date!
24		Duiwenhoks	Duiwenhoks	34° 05’ 30” S	20° 57’ 40” E	07/03/2001
25		Goukou	Goukou	34° 00’ 52” S	21° 17’ 24” E	26/04/2000
26		Heuningnes	Grashoek	34º 34’ 15” S	19º 56’ 45” E	15/03/2001

Table 6.2. Continued.

Code	Species	River system	Locality	Latitude	Longitude	Date
27	*P. burgi*	Berg	Olifants	33° 50’ 28” S	19° 07’ 03” E	19/02/2000
28			Krom	33º 37’ 10” S	19º 10’ 15” E	29/03/2001
29			Platkloof	32º 52’ S	18º 41’ E	2000*
30			Hugo	33º 44’ S	19º 02’ E	2000*
31			Leeu	33º 09’ S	19º 03’ E	2000*
32		Verlorenvlei	Redelinghuis	32º 28’ 30” S	18º 32’ 30” E	2000* & 02/04/2001
33			Het Kruis	32º 35’ 50” S	18º 45’ 00” E	30/03/2001
34	*P. phlegethon*	Olifants	Upper Breekkrans	32° 33’ 43” S	19° 16’ 41” E	25/03/1998
35			Lower Breekrans	32° 33’ 34” S	19° 17’ 57” E	23/03/2002
36			Driehoeks	32° 28’ 59” S	19° 16’ 42” E	27/03/1998
37[+]			Noordhoeks	32° 43’ 19” S	19° 04’ 14” E	18/02/1998
38			Rondegat	32° 21’ 05” S	19° 02’ 00” E	16/02/1998
39[+]			Thee	32° 47’ 40” S	19° 05’ 50” E	24/03/1998

Table 6.2. Continued.

Code	Species	River system	Locality	Latitude	Longitude	Date
40	*P. quathlambae*	Orange	Lower Matsoku	29° 15’ 35” S	28° 33’ 29” E	27/09/2000-06/10/2000
41			Senqu	28° 55’ 32” S	29° 01’ 27” E	27/09/2000-06/10/2000
42			Moremoholo	29° 07’ 29” S	29° 19’ 38” E	27/09/2000-06/10/2000
43			Tsoelikane	29° 53’ 51” S	29° 07’ 14” E	27/09/2000-06/10/2000
44	*P. quathlambae*	Orange	Senqunyani	29° 25’ 25” S	28° 06’ 24” E	27/09/2000-06/10/2000

Table 6.2. Continued.

Code	Species	River system	Locality	Latitude	Longitude	Date
Outgroups						
45	*B. calidus*	Olifants	Tra Tra	32° 17’ 00” S	19° 12’ 40” E	31/03/1998
46			Boskloof	32° 33’ 29” S	19° 03’ 32” E	23/02/1998
37+			Noordhoeks	32° 43’ 19” S	19° 04’ 14” E	18/02/1998
39+			Thee	32° 47’ 40” S	19° 05’ 50” E	24/03/1998
47			Oudste	32° 49’ 33” S	19° 05’ 48” E	24/03/1998
48	*B. erubescens*	Olifants	Twee			
49			Heks			April/May 2002
50	*B. serra*	Olifants	Driehoeks	32° 29’ 27” S	19° 17’ 20” E	20/11/1998
51			Upper Olifants	32° 58’ 19” S	19° 10’ 54” E	11/03/1998
52			Tra Tra	32° 16’ 00” S	19° 12’ 38” E	14/11/1998
53			Oorlogskloof			1999
54	*B. andrewi*	Breede	Brandvlei			1999
55	*B. trevelyani*	Buffalo	Cwengcwe			1993
56	*B. hospes*	Orange	Vioolsdrif			1999

Table 6.3. Sequences contributed towards *Pseudobarbus* phylogenetics by the present study from different river systems in South Africa.

Pseudobarbus lineages	River system	Cytochrome *b*		16S		Control region	
		$N_{alleles}$	N_{total}	$N_{alleles}$	N_{total}	$N_{alleles}$	N_{total}
Forest *P. afer*	Kaaimans	1	1	1	1	-	-
	Klein Brak	2	2	1	2	-	-
	Tsitsikamma	1	1	1	1	-	-
Algoa *P. afer*	Swartkops	1	1	1	1	-	-
	Sundays	2	3	2	3	-	-
Krom *P. afer*	Krom	1	2	1	1	-	-
St. Francis *P. afer*	Kabeljous	2	2	1	1	-	-
P. asper	Gourits	2	2	1	2	-	-
	Gamtoos	1	1	1	1	-	-
Breede *P. burchelli*	Breede	2	2	2	4	-	-
	Duiwenhoks	1	1	-	-	-	-
	Goukou	1	1	1	1	-	-
Heuningnes *P. burchelli*	Heuningnes	2	2	1	1	-	-
Tradou *P. burchelli*	Breede	1	2	1	2	-	-
Berg *P. burgi*	Berg	2	2	1	1	8	8
Verlorenvlei *P. burgi*	Verlorenvlei	1	1	1	2	4	4

Table 6.3. Continued.

Pseudobarbus lineages	River system	Cytochrome *b*		16S		Control region	
		$N_{alleles}$	N_{total}	$N_{alleles}$	N_{total}	$N_{alleles}$	N_{total}
P. phlegethon	Olifants	4	6	3	7	-	-
Eastern *P. quathlambae*	Orange	3	5	2	3	-	-
Mohale *P. quathlambae*	Orange	2	2	1	1	-	-
Gourits *P. tenuis*	Gourits	4	4	2	2	-	-
Keurbooms *P. tenuis*	Bitou	-	-	1	1	-	-
	Keurbooms	1	2	1	1	-	-
Outgroups							
B. calidus	Olifants	4	5	1	2	1	1
B. erubescens	Olifants	2	3	1	1	1	1
B. serra	Olifants	2	3	1	2	1	1
B. andrewi	Breede	1	2	1	1	1	1
B. trevelyani	Buffalo	2	2	2	2	-	-
B. hospes	Orange	3	3	1	2	1	1

Model based molecular analysis

The fifteen historically isolated *Pseudobarbus* lineages that has been identified in Chapters 2-5 and by Bloomer & Impson (2000) are shown in the neighbour joining phylogram in Fig. 6.2. The neighbour joining phylogram was based on the HKY85 substitution model (Hasegawa *et al.*, 1985) with a Ti:Tv ratio of 4.222, I = 0.681 and α = 0.844 found in MODELTEST. Apart from all the localities from Chapters 2-5 the NJ analysis in Fig. 6.2 also included re-analysed samples from all the *P. burgi* populations that Bloomer & Impson (2000) investigated, an additional *P. burgi* population and five of the seven *P. phlegethon* populations that were investigated by Swartz *et al.* (2004). Only representatives from the 15 historically isolated lineages presented in Fig. 6.2 were included in the ML, Bayesian and ML phylogenetic analyses.

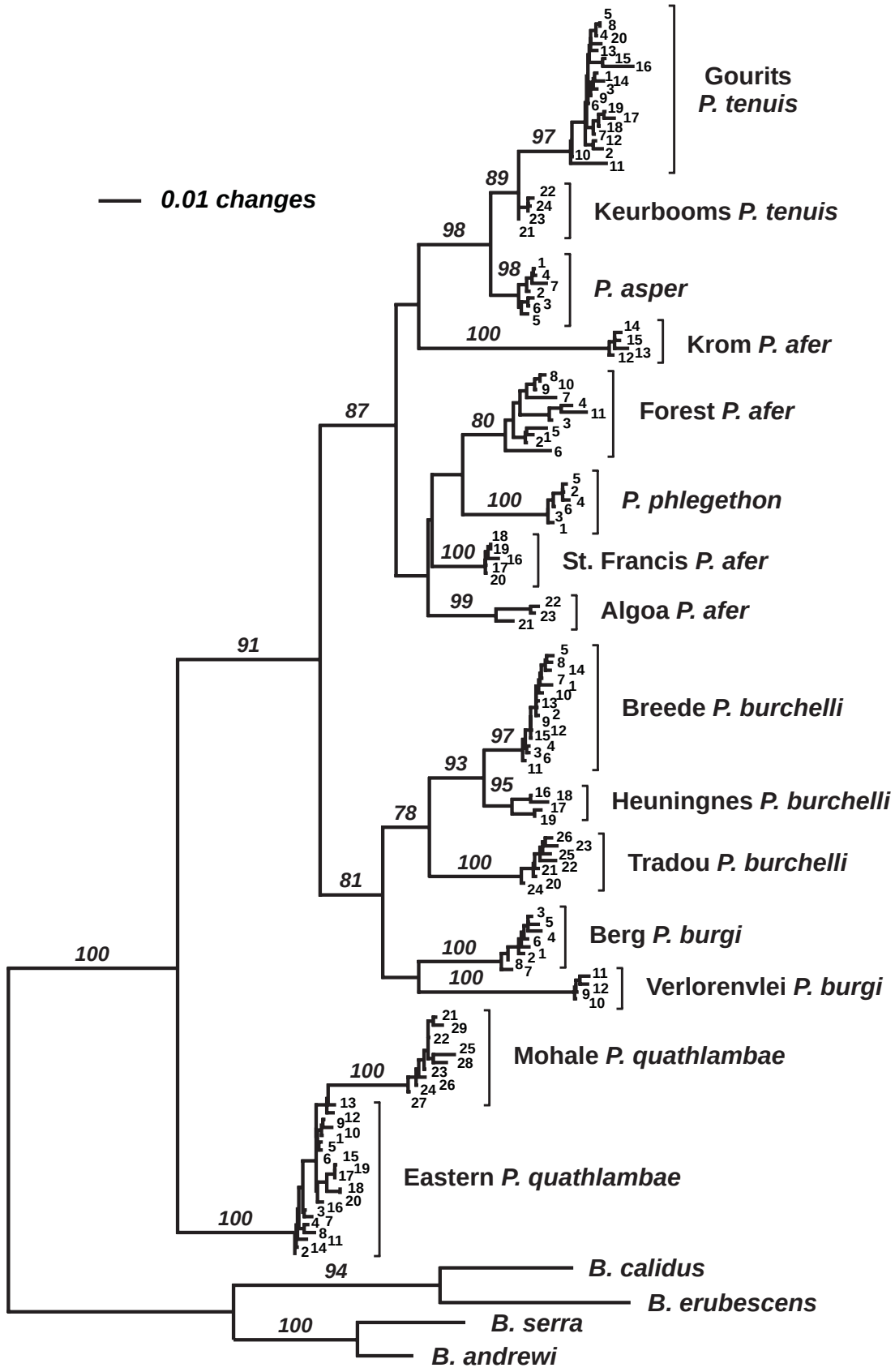


Fig. 6.2. Neighbour joining phylogram showing the 15 historically isolated lineages that were identified in Chapters 2-5 and by Bloomer & Impson (2000). Numbers above branches refer to bootstrap support (1000 replicates) and the smaller numbers refer to control region alleles.

The ML analysis on the combined genetic dataset was based on the GTR substitution model as found in MODELTEST with I = 0.631, α = 0.998 and with base frequencies being A = 0.314, C = 0.221, G = 0.162 and T = 0.303. From the ML phylogram in Fig. 6.3 with only single representatives of each of the 15 *Pseudobarbus* lineages, it is evident that the lineages of *P. burchelli* and *P. quathlambae* form two monophyletic groups with 100% bootstrap and Bayesian posterior probability support in both cases. However, *P. phlegethon* groups with the Forest lineage of *P. afer* with moderate bootstrap and significant Bayesian posterior probability support, suggesting that *P. afer* as a species may not be monophyletic. In addition, the two *P. burgi* lineages are also not monophyletic. Instead, the Berg lineage of *P. burgi* groups with the *P. burchelli* lineages with low bootstrap support, but with significant Bayesian posterior probability support. There was also a lack of bootstrap or Bayesian posterior probability support for the two *P. tenuis* lineages being monophyletic.

Deeper relationships were well resolved, but with mostly little support. There was moderate bootstrap support for the deepest split within *Pseudobarbus* being between the two *P. quathlambae* lineages and all the other lineages of *Pseudobarbus*. However, there was strong support for all the *Pseudobarbus* lineages with two pairs of barbels being monophyletic and for all the *Pseudobarbus* lineages being monophyletic compared to *B. calidus* and *B. erubescens*. When all the taxa for which the combined dataset was available were subjected to ML (not shown) and Bayesian analyses (posterior probability values shown in Fig. 6.4), the inferences regarding relationships did not change.

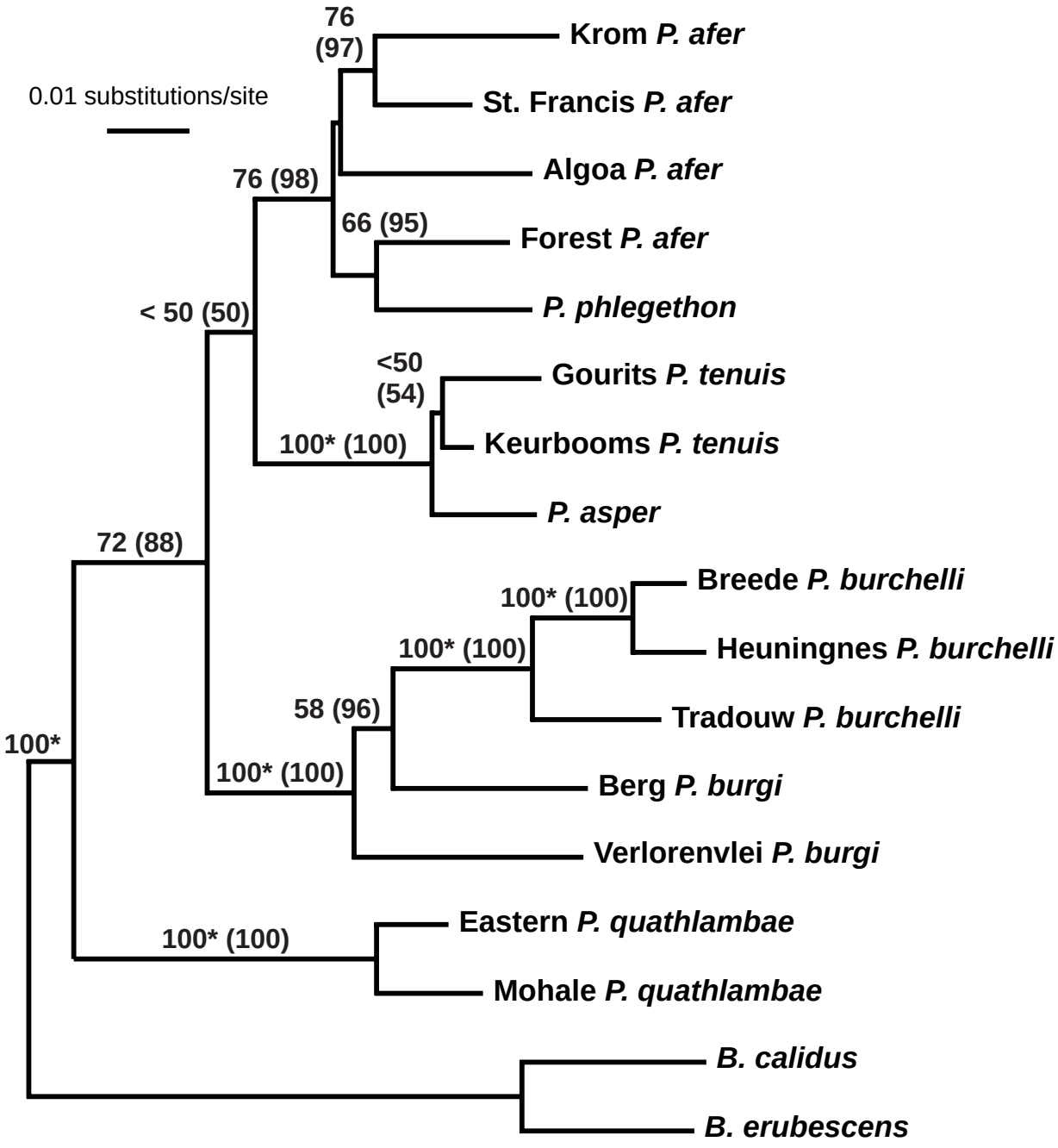


Fig. 6.3. Maximum likelihood phylogram based on the combined genetic dataset, showing relationships among the 15 historically isolated lineages of *Pseudobarbus*. Values above the branches are bootstrap support based on 1000 likelihood bootstrap replicates done with a heuristic search, with Bayesian posterior probabilities (in brackets) based on a run of 5 million generations. Bootstrap support values that were also > 50 in ML analyses done separately on control region, cytochrome *b* and 16S are indicated with asterisks.

Control region and 16S datasets of the 15 lineages (same taxa as in Fig. 6.3) showed much less resolution when they were analysed separately using ML (trees not shown). Topology differences between the ML trees based on the combined dataset (Fig. 6.3), cytochrome *b* and 16S (not shown) were only evident in relationships where there was lack of bootstrap support. Only the ML tree based on control region (not shown) showed weak bootstrap support for the two *P. burgi* lineages being monophyletic, compared to a relationship that groups the Berg lineage with the *P. burchelli* lineages in the three other datasets. Bootstrap values in the combined ML analysis that were also > 50 in the separate ML analysis on the same taxa for control region, cytochrome *b* and 16S, are indicated with asterisks in Fig. 6.3.

Parsimony based molecular analysis

MP analysis was based on all the taxa for which the combined dataset was available (Fig. 6.4) and on only representatives of the 15 *Pseudobarbus* lineages (same taxa as Fig. 6.3). The latter was done in order to make direct comparisons to the model based analyses (same taxa as in Fig. 6.3). The two datasets (24 ingroup taxa versus 15 ingroup taxa) did not differ from each other in terms of relationships that were inferred from the MP analyses. The strict consensus tree of three most parsimonious trees with all the taxa for which the combined dataset was available is shown in Fig. 6.4. The tree scores were 1338 steps with CI = 0.504, RI = 0.734 and RC = 0.370. From this consensus tree, it is evident that the three lineages of *P. burchelli*, the two lineages of *P. burgi* and the two lineages *P. quathlambae* form three monophyletic groups with 100%, 63% and 100% bootstrap support respectively.

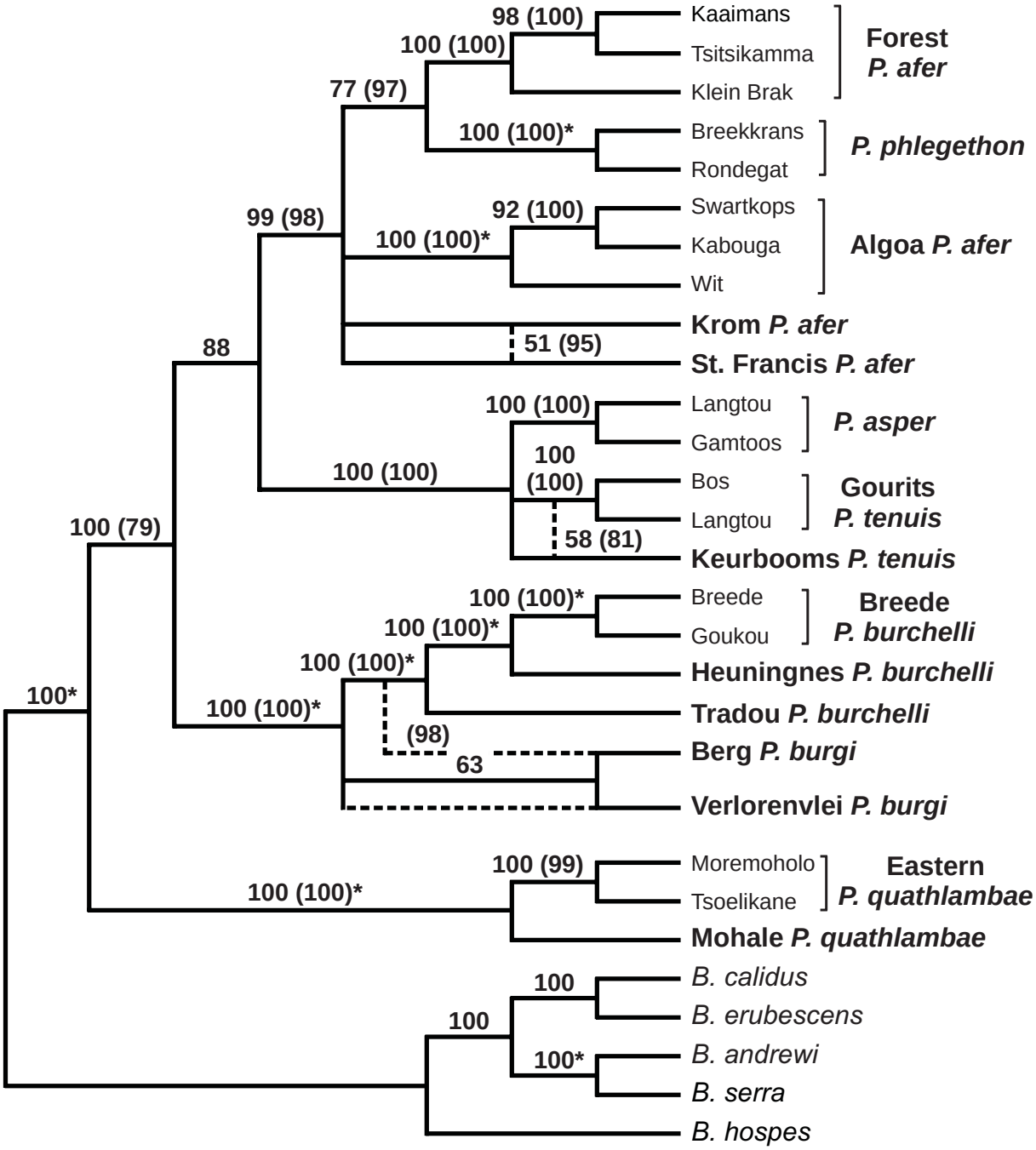


Fig. 6.4. Maximum parsimony strict consensus tree based on the combined genetic dataset, showing relationships among the 15 historically isolated lineages of *Pseudobarbus*. Values above the branches are bootstrap support based on 1000 parsimony bootstrap replicates done with a heuristic search, and Bayesian posterior probabilities (in brackets) based on a run of five million generations. Broken lines indicate alternative relationships that were supported by the Bayesian analysis, which in two cases also had weak bootstrap support from the MP analysis. Bootstrap support values that were also > 50 in MP analyses done separately on control region, cytochrome *b* and 16S are indicated with asterisks.

The *P. afer* lineages were not monophyletic in the MP analysis, due to support for *P. phlegethon* and the Forest lineage of *P. afer* being sister groups with moderate bootstrap support. From the MP analysis it is unsure whether the two *P. tenuis* lineages are monophyletic, since the relationships between them and *P. asper* are unresolved. There was strong support for the clade with *P. asper* and the two *P. tenuis* lineages as the sister group to the *P. afer* and *P. phlegethon* complex, for the deepest split within *Pseudobarbus* being between the two lineages of *P. quathlambae* and all the other lineages of *Pseudobarbus* and that all the *Pseudobarbus* lineages were monophyletic compared to five of the six species of southern African serrated tetraploid barbs.

The control region dataset analysed separately for only the taxa for which combined analysis sequences were available, showed less resolution compared to the combined analysis. Two of the sequence regions (two base pairs each) had to be coded because of adjacent gaps. In addition, 16 characters had single gaps as a 5th character state. The strict consensus tree of the control region MP analysis is shown in Fig. 6.5. The three most parsimonious trees had a CI = 0.500, RI = 0.716 and RC = 0.358 and was 398 steps. Only the two *P. burgi*, two of the three *P. burchelli* and two *P. quathlambae* lineages were clearly monophyletic in terms of the currently described species. The deeper relationships were the same as the combined analysis.

The cytochrome *b* dataset analysed separately for all taxa for which sequences were available showed very similar relationships to the combined analysis. Twenty-four equally parsimonious trees were found which were 967 steps with CI = 0.454, RI = 0.810 and RC = 0.368. The strict consensus of these trees is shown in Fig. 6.6. The resolution was slightly lower due to failure to resolve the relationships between the two lineages of *P. burgi* and the *P. burchelli* lineages. Deeper relationships also differed from the combined analysis in that

the double barbed lineages of *Pseudobarbus* was the sister group of the *P. afer* and *P. phlegethon* complex, but with weak bootstrap support.

The 16S dataset with all available sequences was the most unresolved in terms of the MP analyses. A sequence region of four base pairs had to be coded because of adjacent gaps. The consensus of 836 equally most parsimonious trees based on the 16S dataset is shown in Fig. 6.7. The most parsimonious trees had a CI = 0.634, RI = 0.839 and RC = 0.532 and had a length of 112 steps. Only the three *P. burchelli* lineages and the two *P. quathlambae* lineages were clearly monophyletic in terms of the currently described species. Apart from relationships among the three *P. burchelli* lineages, support for the double barbed *Pseudobarbus* lineages and *Pseudobarbus* itself being monophyletic, no other relationships were resolved with the 16S dataset. The bootstrap values of the clades that were also supported in the combined analysis and in separate analyses of cytochrome *b*, control region and 16S (same taxa) are indicated with asterisks in Fig. 6.4.

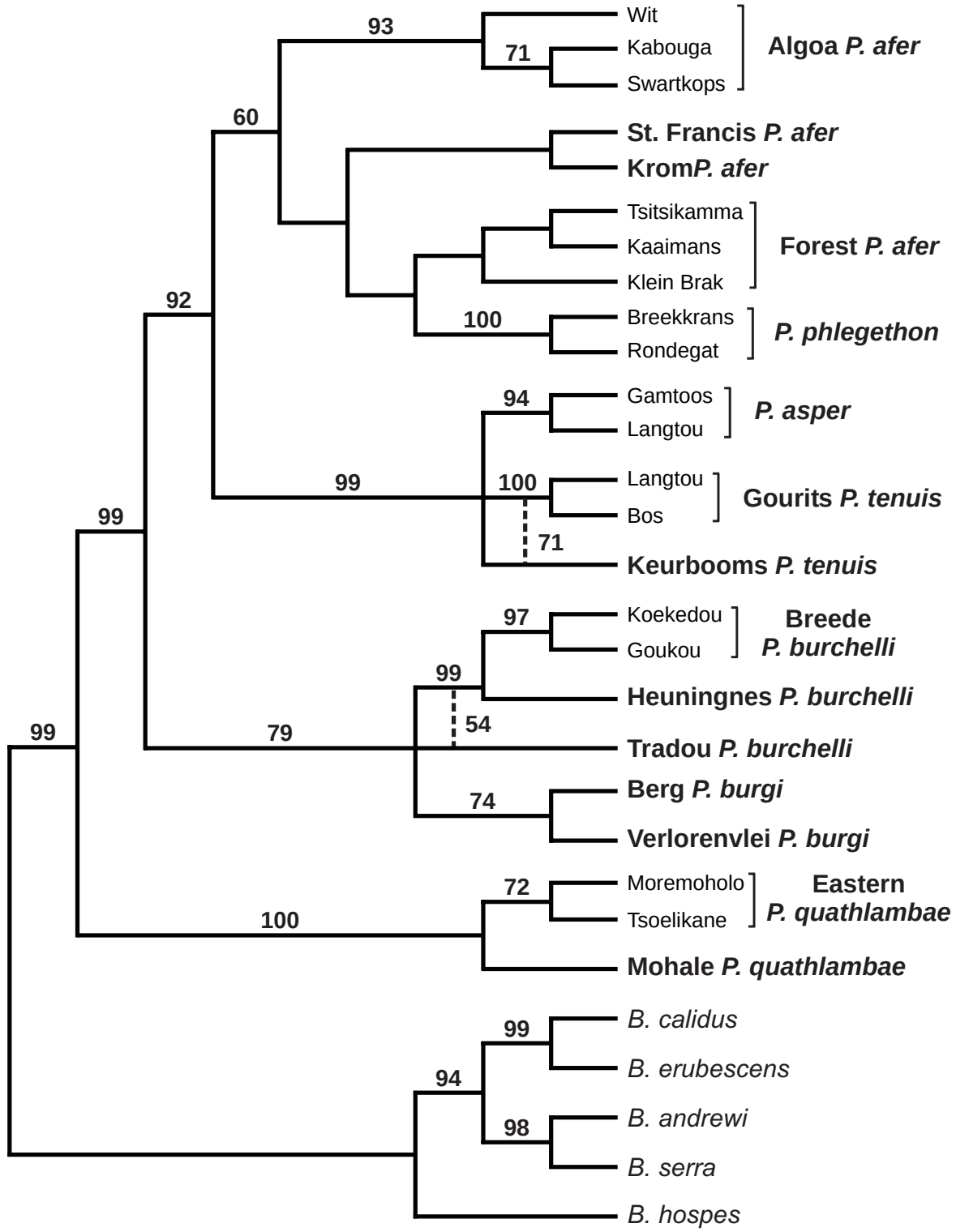


Fig. 6.5. Maximum parsimony strict consensus tree based on control region, showing the relationships among lineages of *Pseudobarbus*. Only control region sequences that were included in the combined analysis are shown here. Bootstrap support values based are shown above branches. Broken lines indicate relationships for which there was weak bootstrap support.

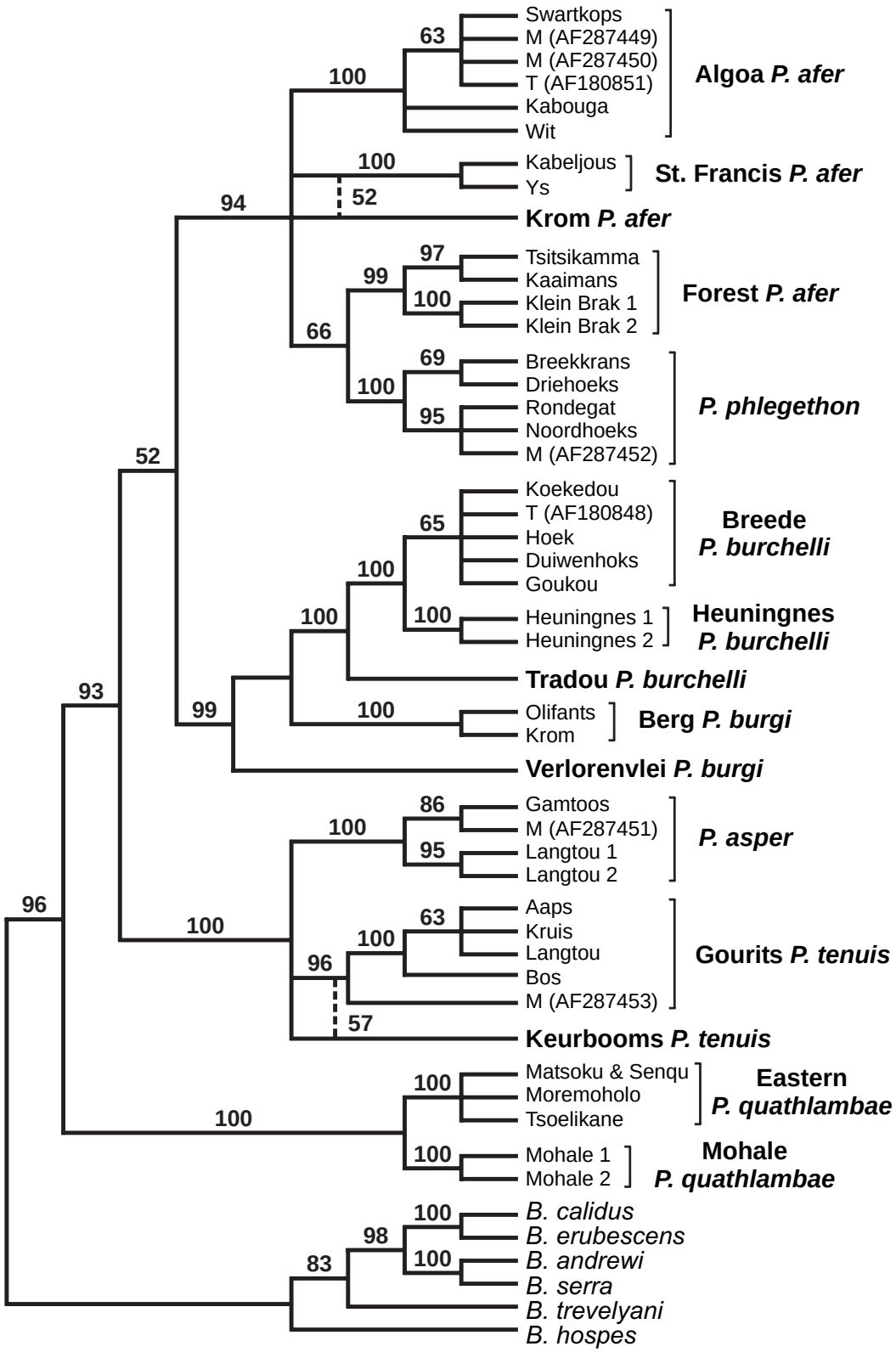


Fig. 6.6. Maximum parsimony strict consensus tree based on cytochrome *b*, showing the relationships among lineages of *Pseudobarbus*. Bootstrap support values are shown above branches. Broken lines show relationships for which there was weak bootstrap support.

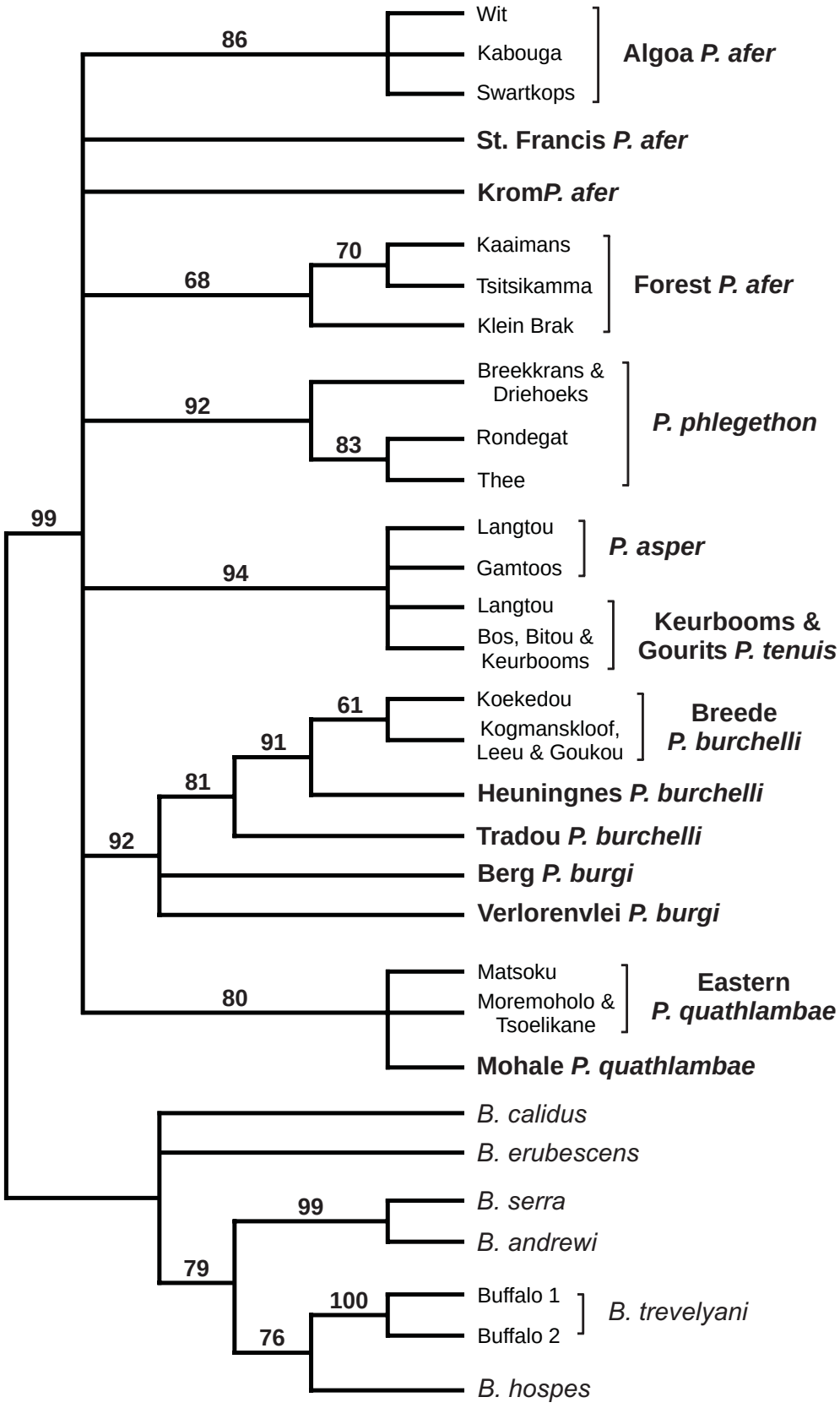


Fig. 6.7. Maximum parsimony strict consensus tree based on 16S, showing the relationships among lineages of *Pseudobarbus*. Bootstrap support values are shown above branches.

Parsimony based morphological analysis

When characters were ordered according to characters states in *B. calidus* and *B. erubescens*, there were 39 parsimony informative characters in the morphological dataset. Three of these characters were left unordered due to uncertain transformation between character states. Twenty-three characters were synapomorphic for *Pseudobarbus* (Table 6.4) and only 16 characters were parsimony informative within *Pseudobarbus* (Table 6.5). Two most parsimonious trees were found with CI = 0.857, RI = 0.868 and RC = 0.744 with a length of 63 steps. The strict consensus of these two trees is shown in Fig. 6.8. There was support strong support for monophyly of *Pseudobarbus*. In addition, there was support for a group that included *P. afer*, *P. phlegethon*, *P. asper* and *P. tenuis*, which was also supported in the molecular analyses (Fig. 6.3 and 6.4). However, this group also included *P. quathlambae*.

The strong association between *P. quathlambae* and *P. tenuis* (80% bootstrap support) is the major difference between the morphological and molecular analyses. When all the characters were left unordered, only 36 characters were parsimony informative. Since 16 equally most parsimonious trees were found with CI = 0.925, RI = 0.918 and RC = 0.849 with a length of 53 steps, the consensus tree has much less resolution than the dataset that was ordered. Apart from this, there was moderate bootstrap support for the two *P. burgi* lineages being monophyletic which was not the case in the analysis where the characters were ordered. Bootstrap support values for the unordered morphological dataset are indicated in brackets above branches of the consensus tree in Fig. 6.8.

Table 6.4. Synapomorphic morphological characters from Skelton (1980) for *Pseudobarbus* compared to the serrated redfin outgroups (*B. calidus* and *B. erubescens*).

Character	Character state	
	Serrated redfins	Pseudobarbus
Primary dorsal spine serrations	Strong or weak *	Soft (absent)
Branched anal fin rays	Six or seven *	Five
Pelvic axillary scale	Present	Reduced or absent
Breast scales	Normal	Reduced
Mouth shape	U-shaped	Sickle shaped
Placement of cusps	Symmetrical	Asymmetrical
Tubercles type	Erupted	Conical
Tubercle pattern	Scattered	"*Pseudobarbus* pattern"
Supraethmoid frontal gap	Absent	Present
Supraethmoid groove	Deep groove	Shallow groove
Condyles	Not concave	Concave
Pterosphenoids	Joined	Divided
Concavity on lateral ethmoids	Absent	Present
Lachrymal shape	High peak	Low peak
Basioccipital process	50-60º	30-40º
Supraopercular	Present	Absent
Quadrate	Deep excavation	Shallow excavation
Premaxillae & maxillae	Elongate	Truncate
Urohyal	Abrupt flanges	Tapered flanges
Pharyngeals	Slender	Broad
Pectoral girdle	Monomorphic	Dimorphic
Weberian apparatus neural crest	Bi-lateral flanges	No bi-lateral flanges
Supraneurals	Well developed	Vestigial or absent
Intramuscular bones	Well developed	Reduced

Asterisks indicate where the character state differed between *B. calidus* and *B. erubescens*. The first state is that of *B. calidus* and the second is that of *B. erubescens*.

Tabele 6.5. Morphological characters and character states from Skelton (1980) that were parsimony informative within *Pseudobarbus*. Only lineages that were investigated by Skelton (1980) and that showed differences within the currently described species were included in the analysis. Characters that were unordered are indicated with asterisks.

Character state	**1 Barbels**	**2 Anterior barbel**	**3 Scale size**	**4 Outer row teeth**	**5 Gut**	**6 Tubercle size**	**7 Neurocranium shape**
0	Two	Long	Large	Two	Short	Very small	Triangular
1	One	Short	Medium	One	Moderate	Small	Rectangular
2	-	Absent	Small	Absent	Long	Moderate	-
3	-	-	-	-	-	Large	-
P. afer	1	2	0	0	1	3	1
P. phlegethon	1	2	0	1	1	2	1
P. asper	1	2	1	0	2	3	1
P. tenuis	1	2	0	2	0	3	1
P. burchelli	0	0	0	0	1	3	1
Berg	0	1	0	0	1	3	1
Verlorenvlei	0	1	0	0	2	3	1
P. quathlambae	1	2	2	2	0	1	0
B. calidus	0	0	0	0	0	0	0
B. erubescens	0	0	0	0	0	0	0

Tabele 6.5. Continued.

Character state	**8 Ethmoid**	**9* Supraorbital**	**10* Dermosphenotic**	**11* Exoccipital process**	**12 Opercle**
0	Ossified	Elongate	Moderate	Absent	Normal
1	Reduced	Rounded	Well developed	Wide	Slender
2	-	Absent	Fused	Pointed	
P. afer	0	2	0	2	0
P. phlegethon	0	1	0	2	0
P. asper	0	1	0	2	0
P. tenuis	1	1	0	1	1
P. burchelli	0	1	0	2	0
Berg	0	1	1	2	0
Verlorenvlei	0	1	1	2	0
P. quathlambae	1	2	2	1	1
B. calidus	0	0	0	0	0
B. erubescens	0	0	0	0	0

Tabele 6.5. Continued.

Character state	**13 Metapterygoid**	**14 Gill rakers**	**15 Sensory lateral line canal**
0	Concave	Large, 9-12	Extensive
1	Convex	Small, 9-12	Angulo-articular & posterior dentary
2	Deep notch	Small, 7-9	Only posterior dentary
3	-	-	Absent
P. afer	1	1	3
P. phlegethon	1	1	3
P. asper	1	1	3
P. tenuis	2	1	3
P. burchelli	1	1	1
Berg	1	1	2
Verlorenvlei	1	1	1
P. quathlambae	2	2	3
B. calidus	0	0	0
B. erubescens	0	0	0

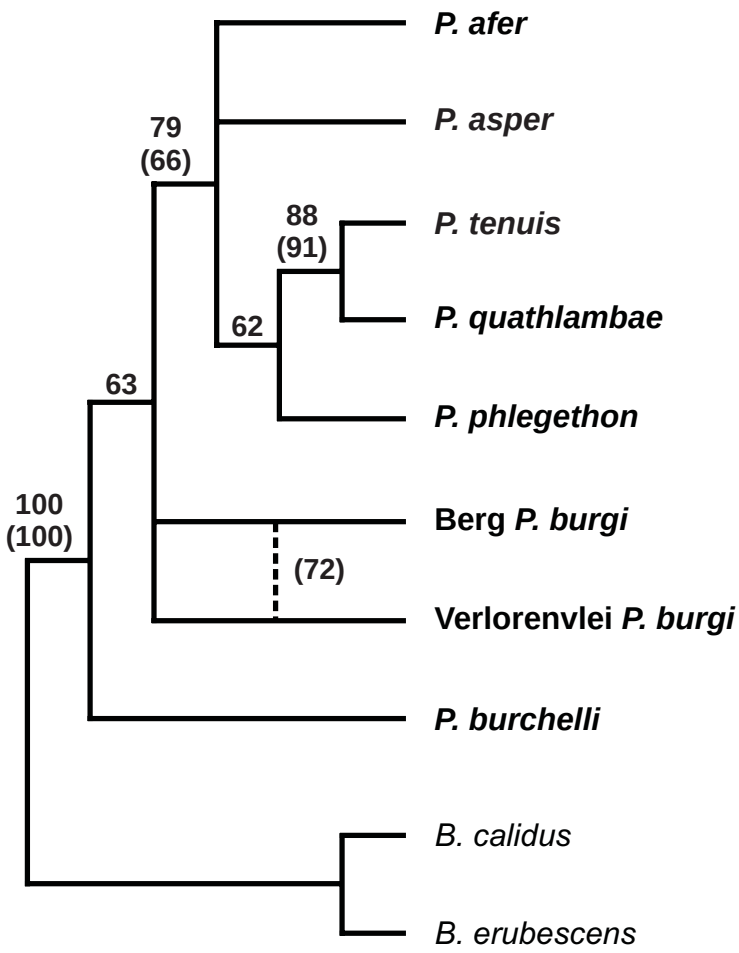


Fig. 6.8. Strict consensus of two equally most parsimonious trees based n 40 parsimony informative morphological characters that were investigated by Skelton (1980). Characters were ordered according to character states in *B. calidus* and *B. erubescens*. The broken line indicates a relationship that was recovered from the MP analysis based on the unordered dataset. Bootstrap support values based on the ordered and unordered datasets (latter in brackets) are shown above branches.

Parsimony based combined molecular and morphological analysis

The combined molecular and morphological analysis was based on the same taxa as the morphological analysis, except that the *P. afer* and *P. tenuis* lineages were not collapsed into just two taxa in the former, because of mtDNA divergence. The combined molecular and morphological analysis produced 384 parsimony informative characters (40 morphological and 344 molecular). Four of the morphological characters were unordered, whist the other morphological characters were ordered according to character states in the outgroups (*B. calidus* and *B. erubescens*).

The MP analysis produced two most parsimonious trees with CI = 0.559, RI = 0.592 and RC = 0.331 with a length of 849 steps. The consensus of these trees is shown in Fig. 6.9. A clade with *P. afer* lineages associated with *P. phlegethon*, one with the two *P. tenuis* lineages grouping with *P. asper* and a clade with all the lineages with two pairs of barbels were strongly supported. A clade with all the lineages with a single pair of barbels (excluding *P. quathlambae*) had moderate bootstrap support. There was also moderate bootstrap support for *P. phlegethon* grouping with the Forest lineage of *P. afer* and high bootstrap support for the two *P. tenuis* lineages being monophyletic.

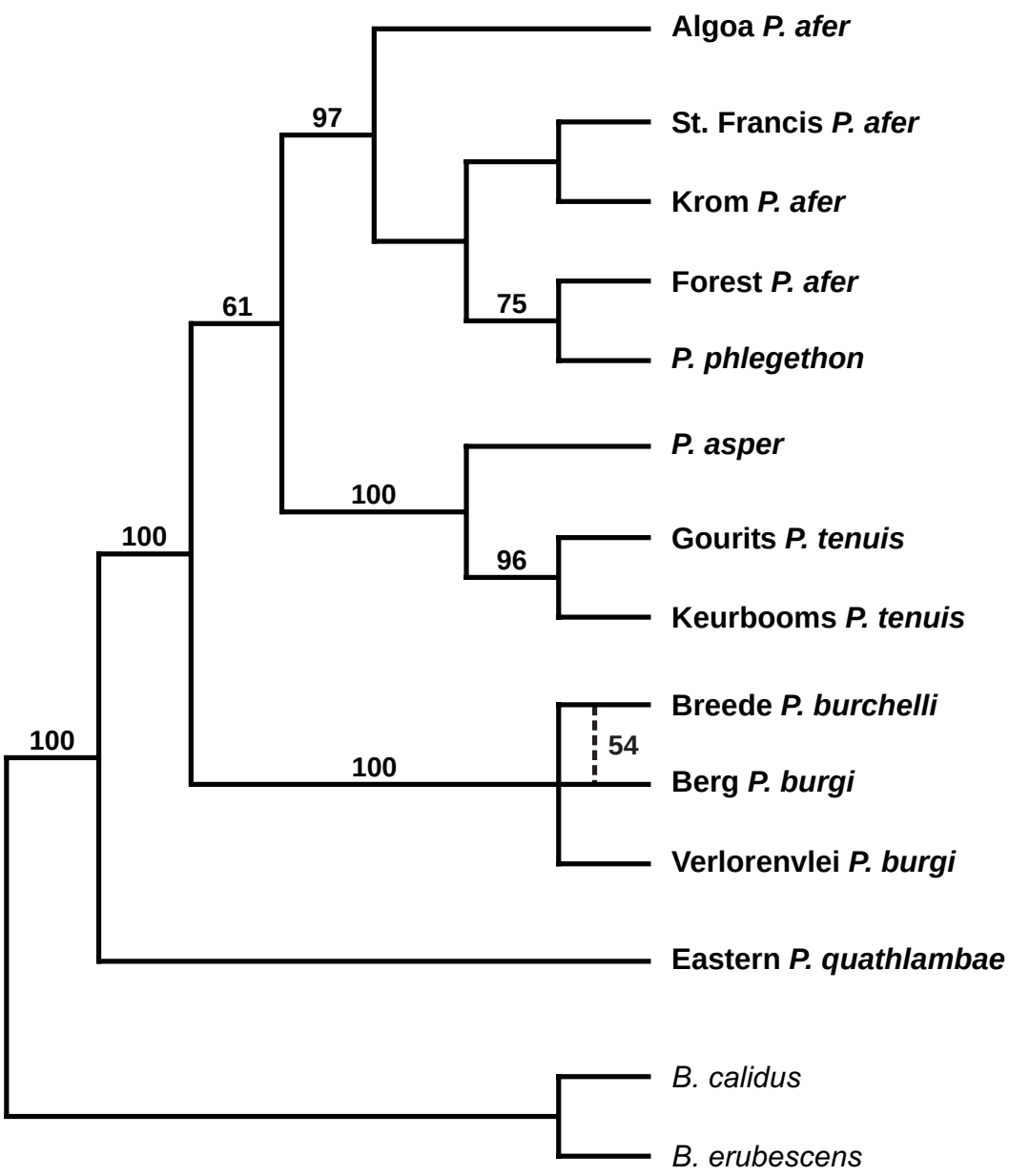


Fig. 6.9. Maximum parsimony strict consensus tree based on a combined dataset of control region, cytochrome *b*, 16S and morphological characters, showing the relationships among lineages of *Pseudobarbus* for which both genetic and morphological datasets were available. Bootstrap support values are shown above branches.

Summary cladograms

Simple phylograms and cladograms that summarise the results of the present phylogenetic study for comparative purposes are presented in Fig. 6.10. All the branches with bootstrap support < 60% and Bayesian posterior probability support < 95% were collapsed. There was only one conflict in relationships in the molecular analyses (Fig. 6.10A-G). It concerned the position of the Berg lineage of *P. burgi*. In the Bayesian analysis (Fig. 6.10B) it groups with the *P. burchelli* lineages, but in the MP analysis (Fig. 6.10E) the two *P. burgi* lineages group together.

There were no conflicts between the combined morphological and DNA analysis (Fig. 6.10I) and the model based or MP molecular analyses, since the position of the Berg lineage of *P. burgi* were unresolved in relation to the two other lineages with two pairs of barbels that were analysed. However, the MP analysis based only on the morphological characters (Fig. 6.10H) differed substantially from the molecular analysis, mainly because of the close relationship that was inferred between *P. quathlambae* and *P. tenuis*. Further important differences were that the *Pseudobarbus* lineages with two pairs of barbels were not recovered as a monophyletic lineage and *P. phlegethon* did not group with *P. afer* as in the molecular analyses. Cladograms that summarise results across the model based molecular analyses (Fig. 6.10C-D) and across all the molecular analyses (Fig. 6.10F-G) are presented to show consistency of relationships across methods.

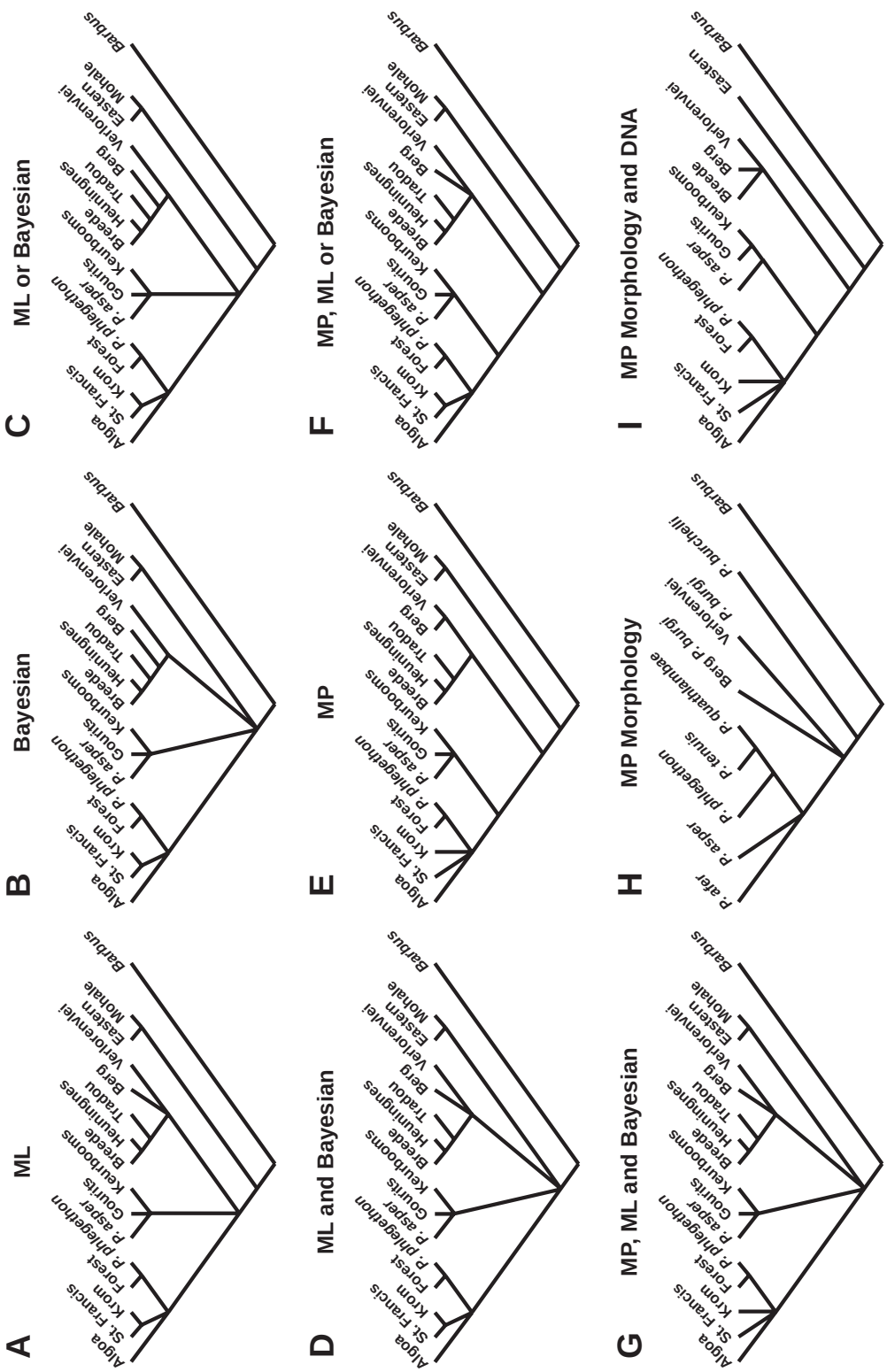


Fig. 6.10. Cladograms summarising genetic (A-G), morphological (H) and combined morphological and genetic (I) support for relationships in *Pseudobarbus*. Only relationships with bootstrap support greater than or equal to 60% and significant Bayesian posterior probabilities greater than or equal to 95% are shown.

Discussion

Phylogenetic relationships

The most important difference between the morphological and molecular analyses is the phylogenetic position of *P. quathlambae*. The morphological data set places this species as the sister species to *P. tenuis*. However, the molecular data strongly support a close relationship between *P. tenuis* and *P. asper*. Skelton (1980) suggested that *P. asper* was the sister species to *P. afer*. There has even been considerable confusion between the latter two species. Western populations of *P. afer* that mainly fall within the Forest lineage in the present study, were initially placed with *P. asper* (Barnard, 1943; Jubb, 1965). Surprisingly, however, the molecular analysis suggests a close relationship between the Forest lineage of *P. afer* and *P. phlegethon*. In the model based molecular analyses there were support for the St. Francis and Krom lineages being sister groups. The relationship between the Algoa lineage compared to the other *P. afer* lineages and *P. phlegethon* was unresolved in all of the analyses.

The molecular data and several morphological characters support a monophyletic group of all the lineages with two pairs of barbels. Within this group, the three *P. burchelli* lineages were clearly monophyletic. However, the relationship of the two *P. burgi* lineages was either monophyletic or unresolved although their sister relationship to *P. burchelli* was well supported. More work will have to be done to resolve the phylogenetic position of the Berg lineage of *P. burgi*. Apart from the single lineages of *P. asper* and *P. phlegethon*, only the three lineages of *P. burchelli* and the two lineages of *P. quathlambae* were clearly monophyletic in terms of the currently described species.

Deeper relationships within *Pseudobarbus* were not well resolved by the Bayesian analysis of the combined molecular dataset or the MP analysis of the morphological characters. However, both the ML and MP analyses based on the combined molecular dataset suggested that the lineages with a single pair of barbels are not monophyletic. This is because the earliest divergence within *Pseudobarbus* is between the two *P. quathlambae* lineages and all the other *Pseudobarbus* lineages, which include the lineages with two pairs of barbels. The next divergence was between the lineages with a single pair of barbels (excluding *P. quathlambae*) and the lineages with two pairs of barbels. The ML and MP analyses therefore suggest that the *P. afer* - *P. phlegethon* complex and the lineage with *P. asper* and *P. tenuis* are sister groups.

The combined morphological and molecular MP analysis showed the best resolution of deeper relationships compared to the morphological or molecular analyses on their own. It is also not in conflict with the cladograms that were constructed from comparisons across methods (Fig. 6.10F-G). Including the morphological characters in the molecular dataset therefore did not reduce the resolution of the tree where there was conflict between the morphological and molecular characters. Instead, it seems to have contributed towards resolving relationships where there was not strong support across all the molecular analyses.

Morphological character evolution within the framework provided by the molecular data

Of the parsimony informative morphological characters investigated by Skelton (1980), 24 were synapomorphic for *Pseudobarbus* and therefore consistent with all the phylogenies that were recovered in the present study. Apart from these, only neurocranium shape was completely consistent with the relationships recovered from the molecular analyses. Of all the

Pseudobarbus lineages, the neurocranium shape of *P. quathlambae* is most similar to the tetraploid barb outgroups. The neurocranium proportion shows a close relationship between the two *P. tenuis* lineages (shallow and broad). The well-developed dermosphenotic in *P. burgi* is an important character to distinguish the two lineages of this species from *P. burchelli*. This character is consistent with the results of the MP molecular analysis, but would not have evolved in a parsimonious manner if the Berg lineage of *P. burgi* groups with the *P. burchelli* lineages as suggested by the Bayesian molecular analyses.

The relationships that were recovered by the molecular analysis suggest that several of the morphological characters investigated by Skelton (1980) may have evolved twice in the evolution of *Pseudobarbus*. The anterior barbels may have been lost once in *P. quathlambae* and a second time in all the other *Pseudobarbus* species that have a single pair of barbels. All the southern African tetraploid barbs have two pairs of barbels, which suggest that this state was retained in *P. burchelli* and *P. burgi*. The reduction in length of the anterior barbels in *P. burgi* may therefore be an unrelated process to the disappearance of this character in the *Pseudobarbus* species with only a single pair of barbels. The reduction of scale size also seems to have evolved once in *P. quathlambae* and a second time in *P. asper*, with larger scales being the norm in all the other *Pseudobarbus* species and in all the southern African tetraploid barbs.

The increase in tubercle size also appears homoplasic. The small tubercle size of *P. quathlambae* is most similar to the southern African serrated tetraploid barbs. All the other *Pseudobarbus* lineages would have undergone an increase in tubercle size, apart from *P. phlegethon* where a secondary decrease in tubercle size occurred. The loss of the supraorbital bone must also have occurred twice, once in *P. quathlambae* and a second time in *P. afer*.

Gill rakers and the dermosphenotic may also have evolved in a non-parsimonious manner if one assumes the reduction in number of the former and absence of the latter to be derived characteristics as was only the case in *P. quathlambae*. A reduction of ossification of the ethmoid, widening of the exoccipital process, elongation of the opercle and deepening of the concave edge of the metapterygoid must also have occurred twice in the evolution of *Pseudobarbus*, because of similarities in these features between *P. quathlambae* and *P. tenuis*. The elongation of the opercle can probably be grouped with other characteristics of these two species in which the head and body form has become more slender.

Several other characters investigated by Skelton (1980) may have even more complex evolutionary histories when compared to the relationships recovered from the molecular analyses. The reduction in number of outer row pharyngeal teeth may have occurred three times, once in *P. quathlambae*, a second time in *P. tenuis* and to a lesser extent a third time in *P. phlegethon*. An increase in gut length must have occurred in the double barbed lineages and in the *P. afer – P. phlegethon* complex. *Pseudobarbus asper* and the Verlorenvlei lineage of *P. burgi* would have independently undergone further increase in gut length. The sensory lateral line canal has a complex evolutionary history. It has been completely lost from the angulo-articular and posterior dentary in the single barbed *Pseudobarbus* lineages (therefore twice in the evolution of *Pseudobarbus*). This canal has been retained in the angulo-articular and posterior dentary in all the double barbed *Pseudobarbus* lineages, except for the Berg lineage of *P. burgi* where it has been lost in the angulo-articular (apparently independently to all the *Pseudobarbus* lineages with a single pair of barbels).

If the molecular data are to be followed, it seems as if most of the synapomorphic morphological characters that place *P. tenuis* and *P. quathlambae* as sister groups can be attributed to convergent evolution. Convergence in morphological characters such as slenderness for fast flowing tributary streams and easy movement between cobbles and under boulders, could have contributed to convergence of morphological characters between these two species. Whereas most of the *Pseudobarbus* lineages are adapted to oligotrophic mountain tributary streams *P. asper* and the Verlorenvlei lineage of *P. burgi* occur in mainstream, intermittently flowing habitats. Adaptation to these habitats seems to have caused an increase of gut length associated with their more eutrophic environment. The other lineage that may have similar adaptations is the Heuningnes lineage of *P. burchelli* that occur in the eutrophic Heuningnes River system. It therefore seems as if several morphological characters may have undergone convergence in the evolution of *Pseudobarbus*. However, apart from improving the resolution of the deeper relationships, several morphological characters have the potential to resolve species delineations within *Pseudobarbus*.

Biogeography and evolution

The cladogram in Fig. 6.10F that summarises the results across the molecular analyses, presents a good working hypothesis of the relationships within *Pseudobarbus*, for comparison to existing biogeographic hypotheses. It presents all the clades for which there was support in any of the molecular analyses, as long as there were no conflicts, in which case the relationships were unresolved. It also presents all the lineages that were investigated in the present study and does not conflict with the combined morphological and molecular analysis (Fig. 6.10I).

From the cladogram in Fig. 6.10F, relationships can be mapped onto the regions currently occupied by the *Pseudobarbus* lineages (Fig. 6.11). It is reasonable to assume that the divergence between the ancestor of *Pseudobarbus* and the southern African serrated tetraploid barbs (Machordom & Doadrio, 2001b; Tsigenopoulos *et al.*, 2002; E. R. Swartz *et al.*, unpublished) occurred within the region now occupied by the temperate ichthyofauna, since all sister species occur within this region. The current phylogenetic investigation challenges the biogeographic conclusions made by Skelton (1986; 1994b) in one major respect. Because the morphological characters showed that *P. quathlambae* and *P. tenuis* were sister species, Skelton (1980; 1988) had to suggest a link through the Great Karoo and a link between the Gourits and Orange River systems with subsequent extinction of redfins in the Karoo tributaries of the Orange River system. Based on the molecular analyses, such an explanation is unnecessary.

The molecular evidence suggests that the earliest divergence within *Pseudobarbus* was between *P. quathlambae* and the common ancestor of all the other lineages of *Pseudobarbus*. It is unclear how or between which river systems this initial divergence occurred. The most likely connection from the geological data that would account for the current distribution of *Pseudobarbus* species in both the Orange River system and river systems of the CFR, is through connection between the current Orange River system and the Olifants River system (see De Wit, 1993; Dingle & Hendey, 1984). The two lineages of *P. quathlambae* would have diverged at a later stage within Lesotho between the Mohale catchment and eastern rivers within Lesotho.

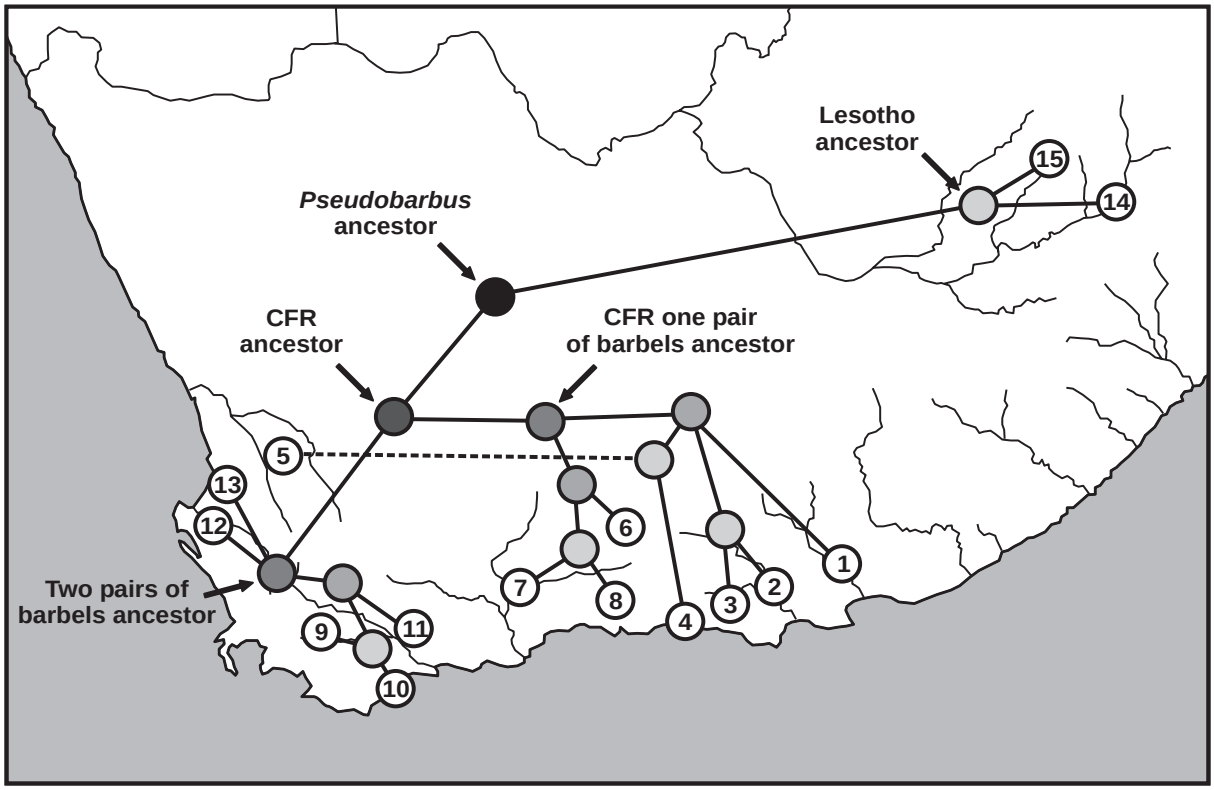


Fig. 6.11. Hypothesised biogeography of *Pseudobarbus* based on the relationships recovered across the different phylogenetic analyses of the present study. Lineages: Algoa, St. Francis, Krom, Forest *P. afer* (1-4 respectively); *P. phlegethon* (5); *P. asper* (6); Gourits and Keurbooms *P. tenuis* (7-8 respectively); Breede, Heuningnes and Tradou lineages of *P. burchelli* (9-11 respectively); Berg and Verlorenvlei *P. burgi* (12-13 respectively; Eastern and Mohale *P. quathlambae* (14-15 respectively).

The next divergence within *Pseudobarbus* would have occurred between lineages with two pairs of barbels that currently occur in the south-western river systems of the CFR (excluding the Olifants River system) and the lineages with a single pair of barbels that are associated with most of the other river systems of the CFR (including the Olifants River system). Differentiation then occurred within these lineages. Within the lineage with two pairs of barbels, differentiation occurred between river systems associated with the current Verlorenvlei, Berg and Breede River systems, to give rise to three lineages (Verlorenvlei, Berg and the currently described *P. burchelli*). The next step was differentiation within the Breede River system between a Breede lineage and one that is currently restricted to the Tradou catchment (a tributary of the Breede River system). Finally, differentiation occurred between the Breede and Heuningnes River systems to give rise to the currently recognised Breede and Heuningnes lineages.

Within the lineages that have a single pair of barbels in the CFR, differentiation occurred between a group that currently occurs in the Gourits, Gamtoos, Keurbooms and Bitou River systems (*P. asper* and *P. tenuis*) and a complex group that occur in eastern river systems of the CFR and in the Olifants River system. From the molecular evidence it is unclear what the earliest divergence was between *P. asper* and the two lineages of *P. tenuis*. However, the morphological evidence strongly suggests that the two *P. tenuis* lineages are monophyletic. Therefore the most likely scenario is that a *P. tenuis* ancestor first diverged from *P. asper*, followed soon by differentiation between a *P. tenuis* lineage from the Keurbooms and Bitou River systems and a *P. tenuis* lineage from the Gourits River system. *Pseudobarbus asper* and *P. tenuis* occur in sympatry in the Gourits River system. Their close genetic relationship raises the possibility that ecological speciation occurred or if secondary invasion occurred, at least ecological displacement and selection for different habitat preference. The divergence

between *P. asper* and *P. tenuis* seems to be relatively recent. Therefore, rapid morphological differentiation occurred between them, compared to for example the *P. afer* lineages that seem to be much older, but with less morphological differentiation.

The more complex group of lineages with a single pair of barbels, involve *P. phlegethon* and the four lineages of *P. afer*. The earliest divergences in this group gave rise to a lineage associated with Algoa Bay, one associated with St. Francis Bay and a puzzling lineage associated with several coastal river systems in the southern Afrotemperate Forest area of the CFR and the Olifants River system on the west coast. The final steps in the evolution of the *P. afer* and *P. phlegethon* complex, would have been differentiation between the populations of *P. afer* from the Afrotemperate Forests (Forest lineage) and *P. phlegethon* and between the Krom River system (Krom lineage) and the Gamtoos and associated river systems (St. Francis lineage).

Whereas the relationships suggested by the present molecular analysis provides biogeographic scenario's that are much simpler in terms of the occurrence of *P. quathlambae* and *P. tenuis* to those proposed by Skelton (1980; 1988), it suggests novel phylogenetic relationships that are difficult to explain. All of the relationships based on the molecular analyses can be explained through proximity of river systems and through processes of river capture or confluence of different river systems during lower sea levels, except for the close relationship between *P. phlegethon* and the Forest lineage of *P. afer*. The only logical link between these lineages is through the Gourits River system. An ancestor of *P. phlegethon* and the Forest lineage must have occurred in the Gourits River system, but most likely has subsequently been extirpated. Apart from possible sympatric speciation between *P. asper* and *P. tenuis* (Chapter 4) and possible parapatric differentiation between the Breede and Tradou lineages of

P. burchelli (Chapter 5), all other speciation and differentiation events within *Pseudobarbus* seem to be the result of isolation and allopatric divergence between different river systems.

Taxonomic implications

The support for the Forest lineage of *P. afer* being the sister group to *P. phlegethon* in ML, Bayesian and MP molecular analyses suggests that *P. afer* is not monophyletic. Unless it can be justified that *P. phlegethon* should be synonymised with *P. afer*, the latter species should be redefined. The type material for *P. afer* was probably collected in the region of the Sundays or Swartkops River systems (Skelton, 1988), therefore the Algoa lineage. There is already a name available for the Krom lineage of *P. afer*. Smith (1936) described *Barbus senticeps* from the Krom River system, which was later synonymised with *P. afer* by Jubb (1965). The St. Francis and Forest lineages of *P. afer* are, therefore, the only unnamed taxa.

The Berg and Verlorenvlei lineages of *P. burgi* also seems to be distinct taxa, because of clear differences in at least two morphological characters (gut length and presence or absence of the sensory lateral line canal in the angulo-articular) (Skelton, 1980), a large genetic divergence between them and weak or contradictory support for them being monophyletic in the molecular analysis. There was also no molecular support to suggest that the two lineages of *P. tenuis* are monophyletic compared to *P. asper*, but in the combined molecular and morphological analysis *P. tenuis* was clearly monophyletic. Pending further investigation, the two lineages of *P. tenuis* may also represent different taxa, since Skelton (1988) noted differences in fin lengths and caudal peduncle proportions.

A revision of the taxonomic status of the three lineages of *P. burchelli* will not only be of importance for species delineation, but can have important consequences for the taxonomic history of the genus *Pseudobarbus*. In his review, Skelton (1988) resolved the confusion regarding the name *Barbus (Pseudobarbus) burchelli* Smith 1841, by assigning samples from the Tradou catchment as neotype specimens for the name *Pseudobarbus burchelli*. However, it appears as if the Tradou and Breede lineages of this species may be different species. Therefore the name *Gnathendalia vulnerata* Castelnau 1861 may have to be resurrected for the widespread Breede lineage.

Apart from possible species descriptions, a revision of *Pseudobarbus* would help to resolve many of the taxonomic and species delineation issues. The historically isolated lineage diversity and number of variable morphological characters between these lineages of *Pseudobarbus* seems to be disproportionate to the number of species that has been recognised. There fore the data presented in the present study as well as previous work (Chapters 2-5; Bloomer & Impson, 2000; Skelton, 1980; 1988; Swartz *et al.*, 2004), should lead to taxonomic changes that would significantly increase the recognised taxonomic diversity of this genus.

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Chapter 7

Thesis conclusion

Diversity

In the present thesis, following up on the work done by Bloomer & Impson (2000) and Swartz *et al.* (2004), 15 historically isolated lineages have been identified within *Pseudobarbus*. Most of these lineages appear to be different taxa (species or subspecies). When described, it would significantly increase the species diversity of the genus *Pseudobarbus* and therefore the Cape and Temperate Ichthyofaunas. Based on mitochondrial DNA, the four lineages of *P. afer* are not monophyletic, since *P. phlegethon* groups with the Forest lineage of *P. afer*. Placing *P. phlegethon* in synonymy with *P. afer* or one of its lineages will be unjustified, because the taxonomic status of the former species is firmly established (Skelton, 1988). The taxonomic status of all four lineages of *P. afer*, must therefore be revised. The Gourits and Keurbooms lineages of *P. tenuis* identified in the present thesis and the Verlorenvlei and Berg lineages of *P. burgi* identified by Bloomer & Impson (2000), did show morphological variation or differences within currently recognised species according to Skelton (1980; 1988).

Taxonomic re-assessments are also justified for the three lineages of *P. burchelli*, because of potential allopatric and parapatric speciation events. Swartz *et al.* (2004) recorded seven fixed allozyme allelic differences between Olifants and Doring populations of *P. phlegethon*, that are, however, not reflected in the mitochondrial DNA analysis. Skelton (1988) nonetheless noted variation in morphological characters between populations of these catchments, and there is a colour pattern difference that warrants further taxonomic investigation. There is an urgent need to assess the conservation status of the 15 historically isolated lineages of

Pseudobarbus as well as the Olifants and Doring populations of *P. phlegethon*, and to put conservation management actions in place to ensure their continued survival.

Phylogenetic relationships

Relationships recovered between species and historically isolated lineages of *Pseudobarbus* from the molecular analyses in the present thesis differ from those recovered from the morphological investigation by Skelton (1980). The main difference is the phylogenetic position of *P. quathlambae*. Several morphological characters suggest a close relationship between the latter species and *P. tenuis*. However, the molecular analyses suggest that the earliest divergence in *Pseudobarbus* is between *P. quathlambae* and all the other lineages of *Pseudobarbus*. The molecular analyses also indicate a close relationship between *P. tenuis* and *P. asper*, despite several morphological characters suggesting otherwise.

Another surprising result was that *P. phlegethon* was grouped strongly with the Forest lineage of *P. afer* in the molecular analyses. These various molecular based outcomes indicate that several morphological characters did not evolve in a parsimonious manner. In particular, the morphological similarities between *P. quathlambae* and *P. tenuis* seem to be the result of convergent evolution. The trees presented in Fig. 6.10, provides a good summary of all the phylogenetic relationships that were recovered in the present thesis. Individual molecular analyses based on control region, cytochrome *b* and 16S did not resolve the phylogenetic relationships as well as the combined molecular analyses. Even though not all the relationships were resolved, the combined morphological and molecular analysis was the best resolved, especially regarding deeper relationships.

Biogeography and population history

Allopatric differentiation between neighbouring river systems seems to be the dominant process of diversification in *Pseudobarbus*. The earliest divergence within *Pseudobarbus* was between the ancestor of *P. quathlambae* and the ancestor all the *Pseudobarbus* lineages that now occur in river systems associated with the Cape Floristic Region (CFR), and may be associated with the well determined early connection that existed between the Orange and Olifants River systems. The two historically isolated lineages of *P. quathlambae* have diverged within Lesotho between the Mohale catchment and eastern rivers within Lesotho. Within the "Eastern lineage", the Tsoelikane population has become relatively recently isolated. Among the north-eastern populations of the Eastern lineage, both isolation and migration-type processes are inferred to have occurred.

The ancestor of the lineages associated with the CFR, first diverged between lineages of species with two pairs of barbels that currently occur in the south-western river systems of the CFR and the lineages of species with a single pair of barbels that are associated with central and eastern river systems of the CFR, including the Olifants River system. The lineage with two pairs of barbels then diverged into the lineages that now occur in the Verlorenvlei and Berg River systems and an ancestor of the "*P. burchelli*" lineages. It is unsure whether the Berg and Verlorenvlei River systems had a common confluence during the last glacial maximum (LGM) when the sea level was about – 130 m below present levels. The divergence between the Verlorenvlei and Berg lineages seems to suggest an earlier connection. If a connection between the river systems occurred during the LGM, it is possible that ecological speciation had already occurred between these two lineages, since no evidence of introgression exists. If other barriers to dispersal such as waterfalls and sea level transgressions affected migration between the currently isolated river systems, it would necessarily assisted ecological speciation to occur.

Pseudobarbus burchelli currently occurs in river systems that would probably have formed part of western and eastern palaeoriver systems that flowed across the Agulhas bank during the LGM. Differentiation firstly occurred within the western palaeoriver system, between the lineage associated with the Tradou tributary and the ancestor of the Breede and Heuningnes lineages. This "Tradou lineage" may have undergone parapatric speciation, since there does not seem to be evidence of downstream migration and subsequent introgression with the Breede lineage of *P. burchellli*. Within the Tradou lineage, both migration and isolation type processes were inferred.

The Breede, Duiwenhoks and Heuningnes River systems would have been connected during the LGM. However, the differentiation between the Breede lineage and the Heuningnes lineage suggests that sufficient ecological speciation occurred to prevented introgression between these two lineages. Alternatively, the Heuningnes lineage was isolated in the Heuningnes River system by other barriers to dispersal, despite its confluence with the Breede River system. A similar process of ecological differentiation between tributary (more oligotrophic) and mainstream (more eutrophic) habitats could have occurred between the Heuningnes and Breede lineages of *P. burchelli* and between the Verlorenvlei and Berg lineages of *P. burgi*. The latter differentiation event, however, seems to be much older. The Goukou River system probably formed part of the eastern palaeoriver system. The occurrence of the Breede lineage in the Goukou River system may suggests that the Goukou River system either had a common confluence with the western palaeoriver system during the LGM, or alternatively, relatively recent river capture or translocation occurred.

Within the Breede lineage both migration and isolation type processes were inferred. However more sharing of control region alleles were detected between the populations of the Breede lineage of *P. burchelli* than between the populations of the Berg lineage of *P. burgi* (present thesis and Bloomer & Impson, 2000). In the case of the Berg lineage of *P. burgi* these results may suggest low levels of migration between, and possibly preference for, tributary habitats. The Breede lineage of *P. burchelli* may have been able to maintain higher or more frequent migration between tributary streams before recent isolation caused by alien fish species. The larger size of the latter lineage may be an indication of better adaptation to mainstream environments, but a population level nuclear and mitochondrial DNA analysis is needed to investigate this fully.

The ancestor of the lineage with a single pair of barbels in the CFR diverged between the ancestor of *P. asper* and *P. tenuis* in the central regions of the CFR and a complex group that occurs in southern and eastern river systems of the CFR and in the Olifants River system. The central ancestor may have been at the root of the only lineage in which sympatric speciation occurred, since *P. asper* and *P. tenuis* occur in sympatry in the Gourits River system. *Pseudobarbus tenuis* prefers the oligotrophic tributary streams, whereas *P. asper* prefers more eutrophic mainstream areas. Alternatively, they may have speciated allopatrically in the other river systems in which they also occur. Under such a scenario, *P. asper* would have invaded the Gourits River system from the Gamtoos River system. This could have occurred through the central Karoo tributaries of both river systems, since these drain very low gradient areas in close proximity to each other. The slight degree of divergence between the Gamtoos and Gourits River systems for *P. asper* suggests that a recent connection or connections occurred between these two river systems.

Similar levels of divergence suggests that the differentiation between the Gourits and Keurbooms lineages of *P. tenuis* occurred very soon after their divergence from *P. asper*. The Bitou and Keurbooms River systems, in which the Keurbooms lineage of *P. tenuis* occur, share an estuary and would have had a freshwater connection during lower sea levels. This is reflected in the low levels of differentiation between these two systems for the Keurbooms lineage. However, more samples will have to be analysed to investigate whether migration or isolation type evolutionary processes played a role in differentiation within *P. asper* and *P. tenuis*.

According to the present results, the earliest differentiation within the *P. afer* and *P. phlegethon* complex was between the lineage associated with Algoa Bay, a lineage associated with St. Francis Bay and a lineage associated with several southern coastal river systems in Afromontane Forests and the Olifants River system on the west coast. The Algoa lineage is restricted to the Swartkops and Sundays River systems. It is unsure whether these two river systems would have had a common confluence during the LGM based on the available geological and bathymetric evidence, but the molecular evidence does suggest that there has been relatively recent opportunities for exchange between these two river systems. The Baakens River system would have had a common confluence with the Swartkops River system and the expectation is that the *P. afer* population that occurs there would form part of the Algoa lineage. Apart from isolation type processes that have been inferred between the Swartkops and Sundays River systems, sample sizes were too low to investigate evolutionary processes within the Algoa lineage. More samples from more populations including the Baakens River system will have to be analysed to establish better the population history and evolutionary processes of the Algoa lineage.

Differentiation occurred within the palaeoriver system associated with St. Francis Bay between the “Krom” lineage in the Krom River system and the “St. Francis” lineage that was recorded from the Gamtoos, Kabeljous and Swart River systems. From the palaeoriver reconstructions, it seems as if all the river systems that drain into St. Francis Bay would have had a common confluence during the LGM. However, the divergence between the Krom and St. Francis lineages suggest that the Krom River never had a common confluence with the palaeoriver system associated with St. Francis Bay, that there were isolating mechanisms such as waterfalls that prevented gene flow to the Krom River or that the Krom lineage has undergone ecological speciation which prevented introgression with the St. Francis lineage. Allopatric fragmentation was inferred as an evolutionary process within the St. Francis lineage. However, more samples will have to be analysed to investigate evolutionary processes of the fragmented populations of the Gamtoos River system. Further surveys are also needed to obtain samples from *P. afer* from the Maitlands and Seekoei River systems.

The close relationship between *P. phlegethon* in the Olifants River system on the west coast and the Forest lineage of *P. afer* in southern coastal river systems is challenging. Not only does this suggest that *P. afer* is polyphyletic, but the biogeographic explanation is complex. The only logical link between the areas now occupied by these lineages, is through the Gourits River system. It is possible that an ancestor to these lineages occurred in the Gourits River system and that this was extirpated after it gave rise to *P. phlegethon* to the west and the Forest lineage of *P. afer* in the south. Two palaeoriver systems were inferred for the area now occupied by the Forest lineage of *P. afer*. The distribution of the Forest lineage across these two proposed systems suggests that relatively recent river capture occurred between these systems, or that there was a common confluence during the LGM. Minor lineages were found within the Forest lineage, which suggests regional structure within proposed palaeoriver

systems or that more complex confluences occurred during the LGM. Isolation type processes were inferred within the Forest lineage.

Differentiation among the *P. phlegethon* populations is complex. Swartz *et al.* (2004) found seven fixed allelic allozyme differences between the five Olifants and two Doring populations of *P. phlegethon*. However, only one mutational difference occurred between the control region allele found in the Driehoeks population of the Doring River from alleles found in populations from the Olifants River system. From the allozymes, low levels of gene flow and therefore preference for tributary streams was inferred. Further population level analyses are needed to infer evolutionary process among populations of this species, especially in the light of the small mitochondrial differentiation compared to significant allozyme differentiation.

Conservation

Urgent conservation actions are needed to secure the survival of the 15 historically isolated lineages of *Pseudobarbus*. Some of these lineages are much more threatened than others and therefore conservation actions should be prioritised. In terms of the number of existing populations, the lineages that only have a single population or that are currently only known from a single catchment, should receive urgent conservation measures. In order of priority, these are the Heuningnes lineage of *P. burchelli*, Krom lineage of *P. afer*, Verlorenvlei lineage of *P. burgi* and Tradou lineage of *P. burchelli*. The Heuningnes lineage is only known from a single locality in the Heuningnes River, a system that suffers from severe water extraction. Alien fish also occur lower down in the system. The Krom lineage of *P. afer* is also known from a single locality, although previous records from elsewhere in the Krom River system exist, that will have to be surveyed. Alien fish introduction is the main threat to

all these populations. The Verlorenvlei lineage (*P. burgi*) is known from three localities in the Verlorenvlei River system, but alien fish occur in all these localities as well. Two fragmented sub-populations of the Tradou lineage occur in the Tradou Pass and Huis tributary (Tradou catchment of the Breede River system). Alien fish occur in all the localities, except possibly in the upper site of the Huis River.

The Mohale lineage of *P. quathlambae* and Keurbooms lineage of *P. tenuis* are also high priorities for conservation actions, but are more widespread than the lineages mentioned above. The Mohale lineage of *P. quathlambae* may be a single continuously distributed population (Van der Bank *et al.*, 2001), but occur in several tributaries of the Senqunyani catchment. The Mohale dam threatens the survival of this lineage in various ways. In response to the molecular analysis of Chapter 2 and the work of Skelton *et al.* (2001), it was decided to transplant this lineage to three other catchments within Lesotho. For the immediate future this lineage seems to be secure, despite being conservation dependant and requiring monitoring. There are two populations of the Keurbooms lineage of *P. tenuis* in the Palmiet catchment of the Keurbooms River system and one in the Bitou River system, but the populations seem to be small. Records exist of *P. tenuis* in the upper reaches of the Keurbooms River system. Further surveys of the Keurbooms and Bitou River systems are needed, since other populations may exist.

The other historically isolated lineages of *Pseudobarbus* have five or more existing populations. They can be prioritised as those that occur in a single river system with five to ten populations (*P. phlegethon*, the Berg lineage of *P. burgi* and the Eastern lineage of *P. quathlambae*), those that occur in more than one river system with five to ten populations (*P. asper* and the Algoa and St. Francis lineages of *P. afer*) and those that occur in more than one

river system and have more than ten populations (Forest lineage of *P. afer*, Breede lineage of *P. burchelli* and the Gourits lineage of *P. tenuis*. Of these, *P. asper* seems to be experiencing the most rapid recent decline because of introduction of alien fish, particularly the introduction of the sharptooth catfish (*Clarias gariepinus*, Clariidae) may soon cause the extinction of *P. asper* in the Gamtoos River system.

Future research

A revision of morphological characters for all the *Pseudobarbus* lineages and more sequence data, particularly the addition of nuclear genes, will probably improve resolution and confidence in the phylogenetic relationships recovered in the present thesis. In future, it will be important to derive simulation and analytical data to test hypothetical population history scenarios. Climatic and geological information (external to the organism) and behavioural and ecological information (intrinsic to the organism) can be used to decrease the space of possible biogeographic scenarios. Nuclear markers will improve phylogenetic resolution. Nuclear and mitochondrial DNA markers will also play an important role in understanding population level evolutionary processes when sample sizes are increased. Comparative phylogeographic patterns and a better understanding of geo-morphological processes will help to improve our understanding of drainage evolution in the CFR and of the Orange River system.

From a taxonomic and conservation point of view, the next step to be taken must be to describe the new taxa. Further surveys are needed and distributional data will have to summarised and mapped, as a basis for assessing their conservation status. In order to characterise the undescribed taxa and to effectively manage their continued survival,

ecological and behavioural and more genetic data will be needed. Especially population genetic studies will assist conservation management plans. Assessing population dynamics and migration patterns will help identify conservation units and the planning against general effects of inbreeding of such units. Such studies will also shed more light on existing theories of biogeography and evolution of *Pseudobarbus* populations.

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