ORIGINAL ARTICLE

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

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ABSTRACT

Aim The phylogeography of the two closely related species *Pseudobarbus afer* and *Pseudobarbus phlegethon* was investigated to assess the association of evolutionary processes, inferred from mitochondrial DNA (mtDNA) sequence variation, with hypothetical palaeoriver systems and other climatic and landscape changes.

Location One western and several southern river systems in South Africa.

Methods We sampled known populations and confirmed known distribution gaps. This was followed by an assessment of mtDNA control region sequence variation for 31 localities across 17 river systems across the range of the species complex. A map of possible offshore drainage patterns during the last major regression event was constructed based on bathymetry and geological studies.

Results The genetic distinction of four major lineages of *P. afer* strongly correspond with proposed palaeoriver systems. However, a western 'Forest' lineage, is widespread across two such proposed systems and is closely related to *P. phlegethon* on the west coast of South Africa. Both the 'Krom' and 'St Francis' lineages were identified in the single palaeoriver system proposed for St Francis Bay. A fourth 'Mandela' lineage is restricted to the one or two palaeoriver systems proposed for Nelson Mandela Bay. Four minor lineages were identified within the Forest lineage and two within the Mandela lineage.

Main conclusions The close relationship between *P. phlegethon* and the Forest lineage of *P. afer* can only be explained by a series of river captures. We suggest the Gourits River system as a historical link that could account for this relationship. On the south coast, lower sea levels than at present allowed confluence between currently isolated river systems, offering opportunities for dispersal among these populations. At present, isolation between different river systems rather than dispersal appears to have a dominant influence on mtDNA diversity.

Keywords

Evolutionary processes, isolation, last glacial maximum, migration, palaeorivers, phylogeography, population history, South Africa.

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INTRODUCTION

Dispersal of primary freshwater fish species within and among river systems is constrained by physiological tolerance and other biological attributes as well as past and present connectivity of their habitats. Major climatic and geological changes underlie the geomorphological history of rivers, and consequently connectivity within and between systems. 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; Waters & Wallis, 2000; Mesquita *et al.*, 2001). In addition, sea-level changes could have been responsible for many cycles of establishment and disruption of river connections during glacial and interglacial times, respectively (Blum & Tornqvist, 2000).

River systems associated with the Cape Fold Mountains on the southern coast of South Africa provide an appropriate setting to investigate the effects of river capture and sea-level changes on freshwater fish diversity (Figs 1 & 2). The uplift and folding of the mountains that run more or less parallel with the southern coastline has created very complex drainage patterns, which results in a landscape where many river captures may occur. Numerous river systems drain across the coastal plain in close proximity to each other towards a section of the continental shelf that has a relatively gentle gradient. Many of them are therefore likely to share a common confluence with each other during lower sea levels.

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 current South African coastline. The southern coastal area, in particular, has been rising tectonically and became stable only in Quaternary times (Maud, 1990; Figs 1a,b & 2). The last major transgression occurred during the early Pliocene (about 3.4–5.2 Ma)

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 + 30 m above present sea levels (Butzer & Helgren, 1972; Rogers, 1985) and thus would have affected only the smallest and lower-altitude river systems. Several major regressions, however, have occurred since the major transgression of the early Pliocene, notably a regression of *c.* −130 m as recently as the last glacial maximum (LGM) around 18,000 years ago (Tankard, 1976; Rogers, 1985; Ramsay & Cooper, 2002). These regressions would certainly have allowed several different river systems to have a common confluence before reaching the sea. As 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 hybridization, than those occurring between such bays.

In these river systems, major climatic and topographic changes would have influenced the distribution and evolution of two endemic primary freshwater fish species of the south-western rivers of South Africa, the Eastern Cape redfin *Pseudobarbus afer* (Peters, 1864) and the fiery redfin *Pseudo-*
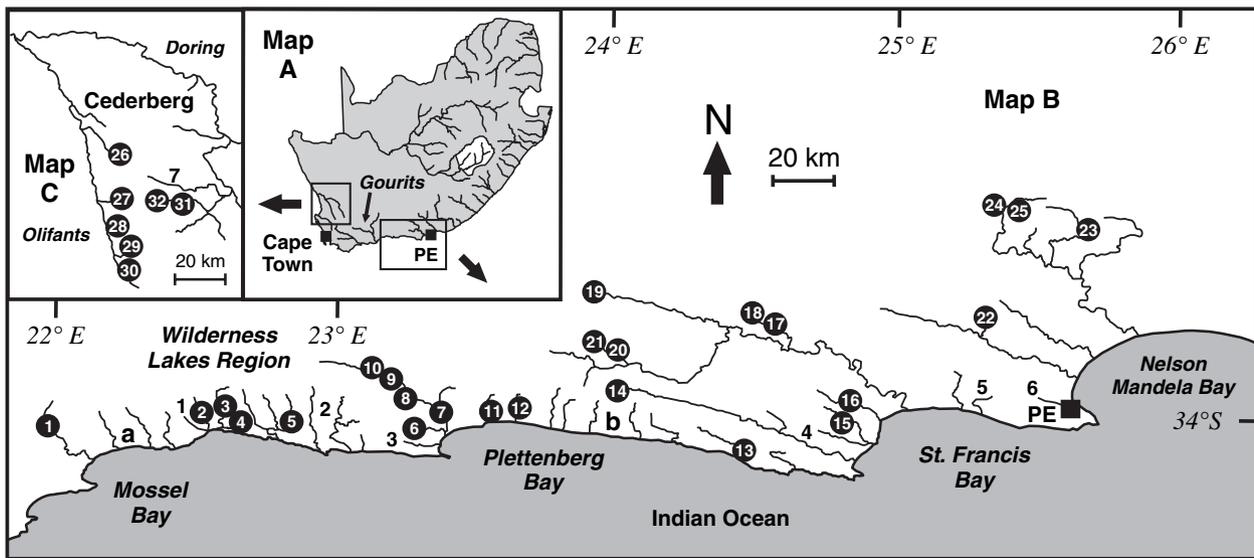

Figure 1 (A) The major river systems in South Africa, the position of the Gourits River system, and the two regions where the present study was conducted on the south coast (area expanded in B; PE indicates the city of Port Elizabeth) and the west coast (C). (B) Map showing where *Pseudobarbus afer* (Peters, 1864) was collected and analysed (circled numbers): Forest lineage (Klein Brak, 1; Kaaimans, 2; Touws, 3; Duiwe, 4; Karatara, 5; Bitou, 6; Keurbooms, 7–10; Groot, 11; Bloukrans, 12; Tsitsikamma, 13); Krom lineage (Krom, 14); St Francis lineage (Swart, 15; Kabeljous, 16; Gamtoos, 17–21); Mandela lineage (Swartkops, 22; Sundays, 23–25). Numbers without circles show other river systems where *P. afer* have been recorded (Swart [George], 1; Goukamma and Knysna, 2; Piesang, 3; Seekoei, 4; Maitlands, 5; Baakens, 6). Distribution gaps are across the Groot Brak, Malgas and Gwaing river systems (a) and from the Bloukrans to Tsitsikamma river systems (b). (C) *Pseudobarbus phlegethon* (Barnard, 1938) sampling and analysis sites within the Olifants River system on the west coast of South Africa, indicated with circled numbers (Rondegat, 26; Boskloof, 27; Noordhoeks, 28; Thee, 29; Oudste, 30; Breekkrans, 31–32). This species has also been recorded from the Driehoeks River (7, not circled).
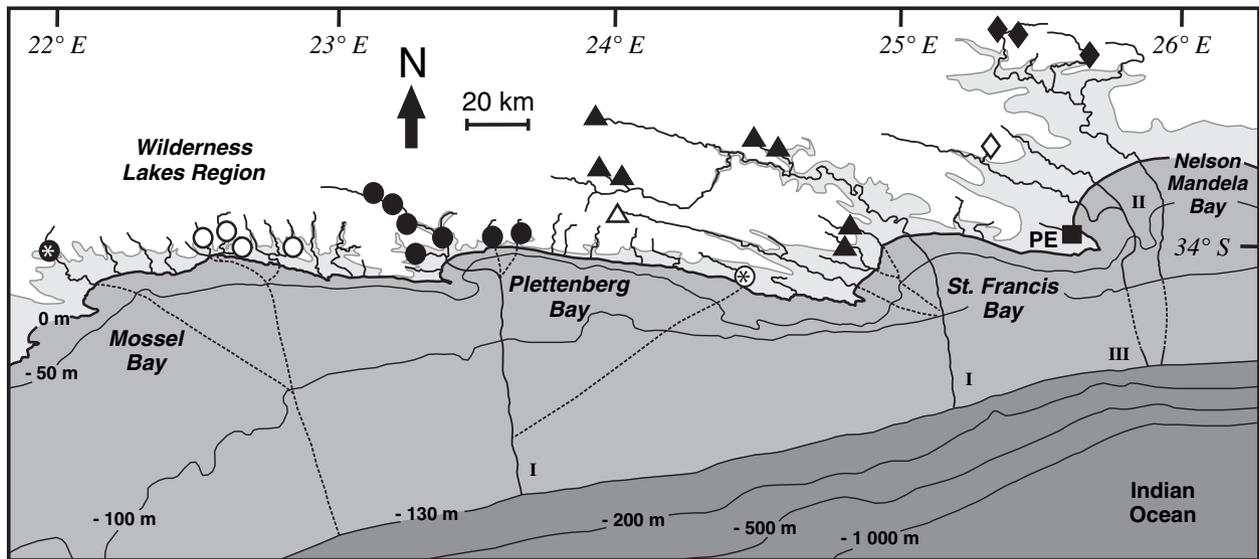
Figure 2 *Pseudobarbus afer* lineages in relation to inferred palaeoriver systems along the south coast of South Africa (modification of Fig. 1b). Symbols show distribution of the Forest (circles), Krom (white triangle), St Francis (black triangle) and Mandela (diamond) lineages. The Klein Brak (black circle, white asterisk), Wilderness Lakes region (white circle), Plettenberg Bay (black circle) and Tsitsikamma (white circle, black asterisk) minor lineages within the Forest lineage; the Swartkops (white diamond) and Sundays (black diamond) minor lineages within the Mandela lineage are also shown. Light shaded areas indicate habitat that may have been unavailable for *P. afer* during the early Pliocene transgression (at the +200 contour). Possible LGM palaeoriver courses extending up to the −130 m contour based on the geological literature are shown by solid lines in the moderate shaded area (I, Dingle & Rogers, 1972; II, Bremner & Day, 1991; III, Dingle *et al.*, 1987) and those inferred mostly from the available bathymetry are shown by dashed lines. Dark shaded area indicates offshore areas beyond the −130 m contour.

barbus phlegethon (Barnard, 1938). *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 *c.* 380 km (Fig. 1b). They have been recorded in as many as 22 river systems (Skelton, 1988, 1994; Rippon, 1996; Russell, 1998/1999, 1999). 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 (Fig. 1c).

Pseudobarbus afer has been recognized 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 (Fig. 1b), 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 with *P. afer* (in *Barbus* at that stage) or any other described species of redfin. Jubb (1965) later synonymized *B. senticeps* with *Barbus afer*, but the taxonomic confusion persisted.

There was confusion, especially, between *P. afer* and *P. asper* (Boulenger, 1911), 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. Apart from suggesting a sister relationship between *P. afer* and *P. asper*, Skelton (1988) placed *P. phlegethon* as the sister species to two other redfin species (*P. quathlambae* and *P. tenuis*). However, recent phylogenetic analysis of mitochondrial DNA (mtDNA) data (cytochrome *b*, 16S rRNA and control region) and a combined data set surprisingly grouped *P. phlegethon* within the *P. afer* clade (unpublished data). This suggests that *P. afer* is paraphyletic.

Here we report on the phylogeography of *P. afer* and *P. phlegethon* with the aim of evaluating the association between evolutionary processes inferred from mtDNA data and hypothetical palaeoriver systems and other landscape changes. To this end, we constructed a map of the possible offshore drainage patterns during the last major regression event to assess which river systems were most likely to have been connected at that time. We further assessed the geographical genetic structuring and differentiation within the *P. afer* and *P. phlegethon* complex by analysing mtDNA

control region variation among as many of the existing populations as possible. Although mtDNA represents a single maternally inherited locus, its high rate of variation and lack of recombination has made it an ideal marker for inferring the genealogical history within species (Moritz *et al.*, 1987). We used nested clade analysis to infer the evolutionary processes (migration and isolation processes) that played an important role in shaping the genetic patterns we found. Finally, these evolutionary processes were associated with the geological and climatic processes of river capture and confluence of river systems during low sea levels to assess their role in the evolution of these two redfin species and their populations.

MATERIALS AND METHODS

Sampling

Pseudobarbus afer and *P. phlegethon* specimens were caught with a 3 m 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 on returning to the laboratory, or muscle, fin-clips or whole fish samples were placed in EtOH. 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 geographical distance measurement

Maps of possible palaeoriver courses were constructed based on the bathymetry as suggested by the South African Navy Charts, the bathymetry proposed by Birch *et al.* (1978) for the Wilderness Lakes region, seismic profiling of offshore sediments (Birch *et al.*, 1978; Birch, 1980; Bremner & Day, 1991) and reviews published on offshore stratigraphical, sedimentological and bathymetric studies (Dingle & Rogers, 1972; Dingle *et al.*, 1987). Geographical distances 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 (Fig. 2). If it was assumed that certain river systems did not connect before reaching the continental shelf at any stage, then the geographical distance measurement followed the −200 m contour line between the proposed palaeoriver systems, as 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, geographical distances among sampled localities were also measured along current river courses and along the current coastline. The +200 m contour was used as a surrogate for the high sea level 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 cyprinid fish. These were L16560 (5′-CCAAAGCCAGAATTCTAAC-3′) in the tRNA (Thr) on the 5′ end of mtDNA control region and H677 (5′-GTCGCG-CAAAAACCAAAG-3′) within the 3′ area of the control region. The primer names were given according to the positions of the 3′ base of each primer in the complete mtDNA genome sequence of *Cyprinus carpio* (Linnaeus, 1758) published by Chang *et al.* (1994). Amplification was performed in 50 μL volumes containing 1× buffer, 2 mM MgCl$_2$, 0.2 mM of each of the four nucleotides (Promega, Madison, WI, USA), 25 pmol of each primer, 1.5 U Super-Therm DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa) and 100–200 ng template DNA. Conditions for amplification in a Geneamp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) were 2 min at 94°C, then 35 cycles of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C, finishing with 5 min at 72°C. PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany), followed by elution in ddH$_2$O.

Cycle sequencing was performed in 10-μL volumes containing 100 ng purified DNA as template, 1.6 pmol primer (either L16560 or H677 mentioned above) and 2 μL ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). 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 bp were obtained from the forward and reverse sequences and by comparing these with other sequences through alignment and inspection in SEQUENCE NAVIGATOR ver. 1.01 (Applied Biosystems). Consensus sequences were aligned using CLUSTALX (Thompson *et al.*, 1997) and checked manually. Sequences were deposited in GenBank under accession numbers EF376194–EF376254.

Genetic analysis

The model of nucleotide substitution that best fits the data was selected from 56 models with hierarchical likelihood ratio and Akaike tests in MODELTEST ver. 3.06 (Posada & Crandall, 1998). With the same program, base frequencies, $T_i : T_v$ 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 (Saitou & Nei, 1987) and maximum likelihood (Felsenstein, 1981) estimation of phylogenetic relationships using PAUP* (Swofford, 2002). In addition, a genealogy was estimated using 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 ver. 2.000 (Schneider *et al.*, 2000). In the same program, an AMOVA (Excoffier *et al.*, 1992) was performed and the significance of the variance components was determined with permutation tests (10,000 replicates). Three predefined hierarchical structures were tested, namely four 'regions', five 'lineages' and six 'bays'. Four regions were defined to test only the major and well defined bays, leaving the distribution of *P. afer* west of St Francis Bay unstructured (Fig. 1). Groups were therefore defined as: (1) *P. afer* from the Klein Brak to Tsitsikamma River system, (2) the Krom to Gamtoos River system, (3) the Swartkops and Sundays river systems, and (4) *P. phlegethon* from the Olifants River system. The five lineages were based on preliminary data, with (1) *P. afer* from the Klein Brak to Tsitsikamma River system, (2) the Krom River system, (3) the Swart to Gamtoos River system, (4) the Swartkops and Sundays river systems, and (5) *P. phlegethon* from the Olifants River system as groups. Based on whether the river systems flowed into the same bay, six bays were defined with the following groups: (1) *P. afer* from the Klein Brak, (2) lakes region, (3) Plettenberg Bay to the Tsitsikamma River system, (4) the Krom to Gamtoos River system, (5) the Swartkops and Sundays river systems, and (6) *P. phlegethon* from the Olifants River system (Fig. 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 (Fig. 1b,c). Pairwise φ_{ST} 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 geographical location could be rejected (Templeton & Sing, 1993). This was done by comparing observed χ^2 values with distributions of χ^2 generated from 10,000 random permutations of the original data in the program GEODIS ver. 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 geographical 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 geographical distribution of individuals in a clade compared with all individuals in the nested clade. Different historical processes influence these geographical distance measures (D_c, D_n, IT_c and IT_n) in particular ways, and may allow the inference of the underlying evolutionary processes that 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 broadly as associated with either migration or isolation.

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 disperse to other river systems under normal circumstances, as they are primary freshwater species. A total of 49 *P. afer* specimens were collected and analysed from 16 such river systems and 25 localities (see Appendix S1 in Supplementary Material; circled numbers in Fig. 1b). Apart from these, *P. afer* was not recorded from four localities in the Maitlands River system near Port Elizabeth or at single localities each in the Swart (near George) and Piesang (flowing into Plettenberg Bay) river systems, despite previous records of their presence (Skelton, 1988; Rippon, 1996). It might still occur in the Seekoei (St Francis Bay) and Baakens (Nelson Mandela Bay) river systems (Skelton, 1988), but these were not visited during recent surveys. In addition, *P. afer* was recorded from the Knysna and Goukamma river systems (between Mossel Bay and Plettenberg Bay), but not included in the analysis. This species was not recorded, however, from a further six river systems and 14 localities within its possible range (Fig. 1b). In contrast to *P. afer*, the distribution of *P. phlegethon* has been well established (Bills, 1999; Swartz, 2000; Swartz *et al.*, 2004). Of the seven remaining populations, only the Driehoeks population was not included in the present study. A total of 11 specimens was analysed from the other six tributaries of the Olifants River system (Appendix S1; Fig. 1c).

Map reconstructions

Four major historical river systems could be inferred on the south coast from the reconstructions of a −130 m sea level (moderate shaded area in Fig. 2). 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 Skelton (1980), Dingle *et al.* (1987) and Bremner & Day (1991), it is unclear whether a historical Baakens–Swartkops–Coega River system (see dashed lines in Nelson Mandela Bay region, Fig. 2) would have had a common confluence with the Sundays River system before reaching the −130 m LGM coastline. However, it seems as if all the river systems flowing into St Francis Bay would have had a common confluence before reaching the LGM coastline. Specimens of *P. afer* were collected at altitudes ranging from almost sea level to 560 m asl (Appendix S1). 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
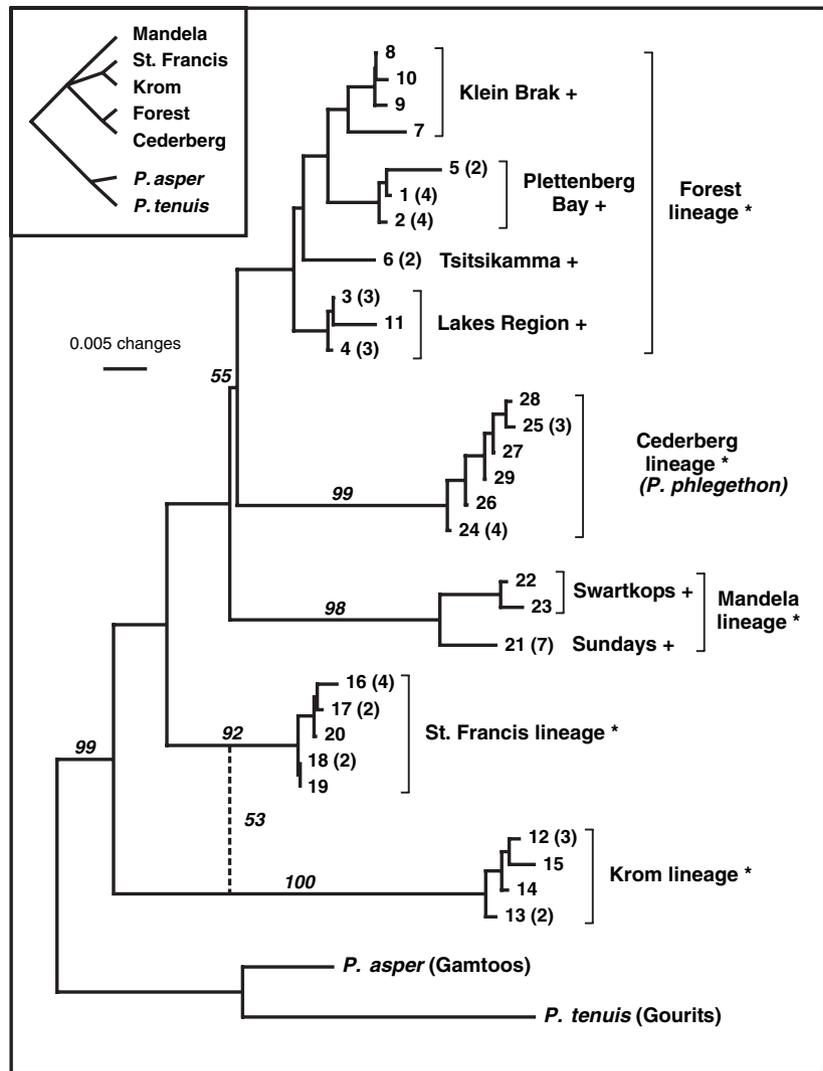
Figure 3 Neighbour-joining tree that shows the differentiation and relationships among five major (*) and six minor (+) lineages of *Pseudobarbus afer* and *Pseudobarbus phlegethon*. The tree is based on the HKY85 substitution model of Hasegawa *et al.* (1985) with a T_i/T_v ratio of 4.378, $I = 0.697$ and $\alpha = 0.592$. Bootstrap support (italic text indicated at nodes) is based on a maximum likelihood analysis with the above-mentioned model parameters and 1000 replicates. Allele numbers (regular text) and allele sample size (in brackets) are also shown. The insert summarizes the relationship among lineages in a simplified cladogram based on the present study and unpublished phylogenetic analyses of all *Pseudobarbus* species (unpublished data).

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) (light shaded area, Fig. 2). 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 to 1–2014 km, depending on whether the −200 m contour (and proposed palaeoriver courses) or the current sea level, respectively, was used in the measurements (Appendix S2).

Sequence variation and diversity

The total of 605 bp analysed yielded 73 variable sites defining 29 alleles among 60 individuals. The HKY85 substitution model (Hasegawa *et al.*, 1985) with a $T_i{:}T_v$ ratio of 4.378,

$I = 0.697$ and $\alpha = 0.592$ was selected with MODELTEST based on the hierarchical likelihood ratio and Akaike tests, which gave almost identical results.

Five major lineages (Forest, Krom, St Francis, Mandela and Cederberg) are evident from the neighbour-joining and maximum likelihood analyses (Fig. 3), which show 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 with between them ($D = 0.9$–2.3%) (Figs 1–3). The range of genetic distances within the Mandela 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%) as this lineage was recorded only from a single locality. The circled numbers in Fig. 1b,c show the localities from where the Forest (1–13), Krom (14), St Francis (15–21), Mandela (22–25) and Cederberg (26–32) lineages were collected, and the symbols in Fig. 2 show the distribution of *P. afer* lineages in relation to proposed palaeoriver systems.

Maximum likelihood bootstrap support was high for all the major lineages (92–100%), but low for the Forest lineage of *P. afer* (Fig. 3). There was also low bootstrap support for a close relationship between the Krom and St Francis lineages of *P. afer*, and for *P. phlegethon* being closely related to the Forest lineage of *P. afer*. Further phylogenetic studies with larger data sets and more taxa have, however, confirmed these relationships (unpublished data; see inserted cladogram in Fig. 3). The relationships between these lineage groupings and the Mandela lineage of *P. afer* remain unresolved (present study and unpublished data).

Gene diversity did not differ significantly between the Cederberg, Forest, Krom and St Francis lineages, but all these lineages showed significantly larger gene diversity in comparison with the Mandela lineage (Fig. 4a). Nucleotide diversity was lowest in the Krom and St Francis lineages. The Cederberg and Mandela lineages did not differ significantly from any of the other lineages, but the Forest lineage showed significantly larger nucleotide diversity compared with the Krom and St Francis lineages (Fig. 4b).

Genetic structuring

Only four of the alleles were shared between river systems, and a further four alleles were shared among different localities but within the same river system (Table 1; Figs 1b,c & 2). When the five lineages were specified as 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 populations within groups and within populations, respectively (all P values < 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 with the structure above (Table 2). High and significant pairwise φ_{ST} values among the specified regions indicated that much more structuring exists within the species complex 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).

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, as all the alleles within this clade are from a single locality in the Klein Brak River (Fig. 5). None of the five major lineages could be connected by the program TCS, as there were more than 10 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), therefore exact contingency tests for geographical association could not be done. Using the –200 m contour for geographical distances, clades 1-2, 1-8, 1-9, 1-12, 2-1, 2-5, 2-7, 3-1 and 3-3 did not show a significant association between their clades or alleles and geographical 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 geographical position was detected in clades 2-9, 3-4, 3-5, 3-6, 4-1, 4-2, 5-1, 6-1 and 7-1 ($9.000 < \chi^2 < 59.000$; $0.000 < P < 0.027$).

The four divergent lineages identified in the neighbour-joining and maximum likelihood analyses were restricted to clades 5-1 (Forest *P. afer*), 2-8 (Krom *P. afer*), 2-9 (St Francis *P. afer*), 3-6 (Mandela *P. afer*) and 3-4 (Cederberg *P. phlegethon*). Most of the inferences based on the key of Templeton (2004) refer to isolation processes and, more specifically, historical isolation (Table 4). 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
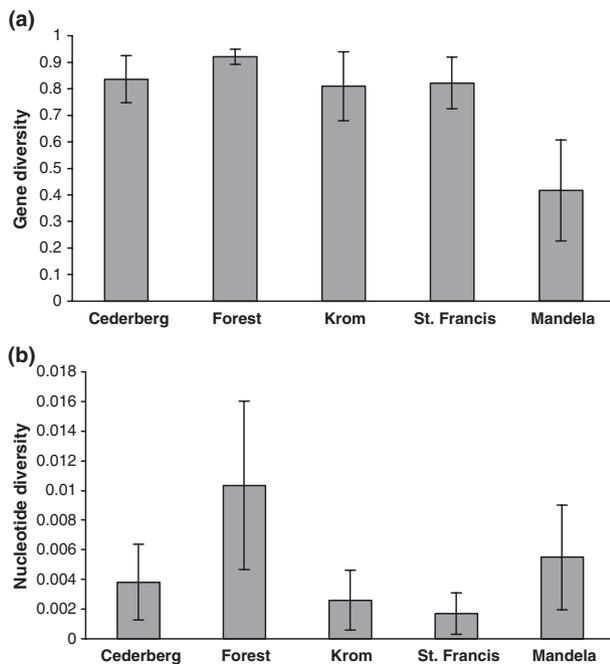
Figure 4 Gene diversity ±SE (a) and nucleotide diversity ±SE (b) within the one *Pseudobarbus phlegethon* (Cederberg) and four *Pseudobarbus afer* lineages (Forest, Krom, St Francis and Mandela).

Table 1 Frequency of alleles among sampled localities of 49 *Pseudobarbus afer* and 11 *Pseudobarbus phlegethon* individuals (locality codes follow Appendix S1).

| Allele number | 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	–	–	–	–	–	–	–	–

| Allele number | N | Krom | Gamtoos | | | | | | | Mandela | | | |
		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	–	–	–

| Allele number | 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 Appendix S1 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.

colonization, fragmentation and range expansion could have played a role to a more simple inference of migration due to restricted gene flow with isolation by distance. The difference in the two conclusions results from much shorter geographical distances (Appendix S2) when the current coastline is used compared with when the –200 m contour line is used.

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

Table 2 AMOVA results for *a priori* structures among *Pseudobarbus afer* and *Pseudobarbus phlegethon* populations.

	Variance components		
Source of variation	Four regions	Five lineages	Six 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

All the estimated values of variance components were significant ($P < 0.05$) based on 10,000 permutations. See Fig. 1 and Methods for details on *a priori* groupings.

Table 3 Pairwise φ_{ST} for the *Pseudobarbus afer* and *Pseudobarbus phlegethon* localities, grouped in regions (see Fig. 1 and Methods describing regions defined for AMOVA analysis).

	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. St Francis (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*

Comparisons that were significant ($P < 0.05$) are indicated with an asterisk. Sample sizes are in parentheses.
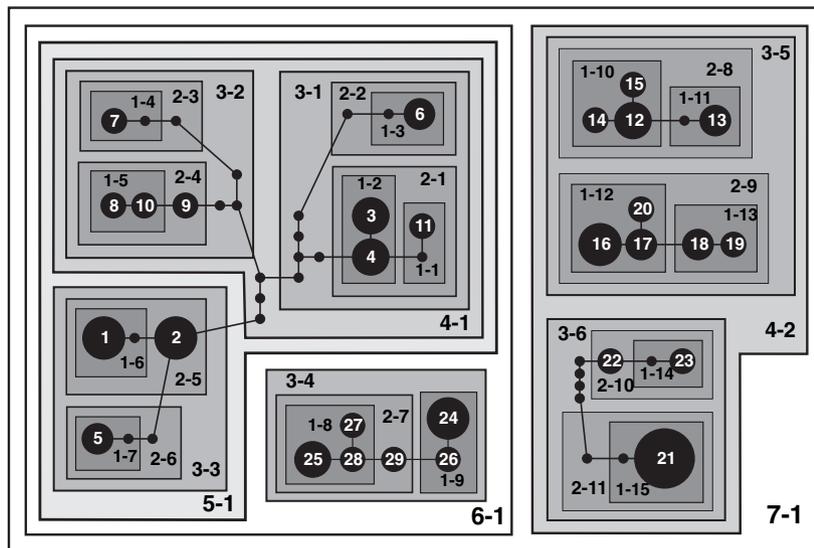

Figure 5 Nested clade design of the control region alleles of *Pseudobarbus afer* and *Pseudobarbus phlegethon*. The five major lineages identified within the species complex are restricted to clades 5-1 (Forest), 2-8 (Krom), 2-9 (St Francis), 3-6 (Mandela) and 3-4 (*P. phlegethon*). The six minor lineages 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). See Fig. 1 for the geographical location of these lineages.

history of connection, and therefore migrants, is partly supported, especially in the eastern section of the range (Fig. 2). Within Nelson Mandela Bay (previously known as 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 are 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

Table 4 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 –200 m 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	Nelson Mandela 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 Nelson Mandela 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.

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 the lineage. More surveys will have to be done, but from our current knowledge the two large gaps in distribution (Fig. 1b) appear to be real, probably for historical reasons rather than because of recent anthropogenic disturbance.

The Krom lineage is the most restricted, and it seems as if it is only surviving in a very short section of the upper Krom River, mainly because of the impact of alien bass (*Micropterus* sp.). All the other river systems of St Francis Bay probably share a 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, however, care must be taken in making such an assumption. Recent surveys failed to find *P. afer* in the Maitlands, raising fears that it 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 as they share a common estuary. The Mandela 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).

There was little differentiation within *P. phlegethon*, and the species is thus represented by a single mtDNA lineage. This lack of mtDNA differentiation contrasts with the seven fixed allelic differences reported from an analysis 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; Fig. 1c). At least one population has

been extirpated in the Jan Dissels River system because of the introduction of North American bass species (E. Swartz, R. Bills and M. Cunningham, unpublished data). In addition, 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.

Cederberg (*P. phlegethon*) and Forest (*P. afer*) lineages

The close relationship between the widespread Forest lineage of *P. afer* and *P. phlegethon* is unexpected given the previously suggested relationships of the two species (Skelton, 1988) and the large geographical separation of the Olifants River system on the west coast relative to the rivers on the south coast of South Africa (Fig. 1a). Our data lead to an inference of recent geographical contact between the two lineages. Given the latter, the mtDNA allele tree could thus either reflect recent speciation or may not accurately reflect the underlying species tree due to introgressive hybridization. The mtDNA alleles of *P. phlegethon* may for example have been replaced by *P. afer* alleles. Nuclear DNA markers are needed to resolve the latter. Nevertheless, the two lineages have been in recent contact suggesting that representatives of one or both species or a common ancestor occurred in the Gourits River system in the recent past followed by subsequent lineage extinction in the Gourits. 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 our mtDNA analysis. Only one mutational step separates the allele that was found in the Breekkrans tributary of the Doring River from the

allele that was identified in the Rondegat tributary of the Olifants River (Fig. 1c). 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 mtDNA data sets will have to be investigated further with larger sample sizes and different genetic markers.

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 (Fig. 2). Historical isolation or allopatric fragmentation was inferred as a possible evolutionary process within the Forest lineage (Table 4; Fig. 5). 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 divergences 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 could have occurred via 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 colonize 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 for the Forest lineage of *P. afer*, 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 within this lineage, 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 (Fig. 2).

The Krom and St Francis lineages

In contrast to the relatively low divergence of 0.9–2.3% 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 System and other river systems of this bay (Krom and St Francis lineages). From the offshore map reconstructions (Fig. 2), 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 indicate 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 (Skelton, 1974; Marriott, 1998), that would have disrupted contact with their sister species (*B. calidus*) which occupies most of the remainder of the Olifants River system. Why *B. erubescens* has not spread throughout the river system remains unexplained (Skelton, 1974; 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 Tradouw catchment, with a much more widespread lineage occupying the rest of the Breede River system, as well as two neighbouring river systems (unpublished data). 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 Kabeljous river systems within the St Francis lineage, which could be due to recent genetic drift since the LGM.

Mandela 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, as these two river systems would have had a common confluence within Nelson Mandela Bay before reaching the −130 m LGM sea level (Fig. 2). 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 palaeoriver systems may have remained separate within Nelson Mandela 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 and evolution of populations

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 colonization 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 1–3.6 Myr if one assumes the relatively slow mutation rate of about 3% per Myr 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 rate 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 reflects 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 found across the range of the Forest lineage (lack of major divergence between possible historical river systems). The divergence of control region alleles 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.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:

Appendix S1 Localities where *Pseudobarbus afer* (Peters, 1864) and *P. phlegethon* (Barnard, 1938) specimens were collected and analysed.

Appendix S2 Geographical 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).

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2699.2007.01768.x.

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