Mitochondrial DNA Differentiation in the Critically Endangered Berg River Redfin (*Pseudobarbus burgi*)

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The Berg River redfin (*Pseudobarbus burgi*) is a critically endangered endemic cyprinid from South Africa. We investigated mitochondrial DNA control region variation among specimens representative of five populations drawn from two adjacent river systems. Phylogenetic analyses, a minimum spanning network, and an analysis of molecular variance underscore the pronounced genetic separation of redfins originating from the geographically closely allied Verlorevlei and Berg Rivers, two populations that may have remained isolated since the Pleistocene. Despite a lack of geographic structuring within the Berg River, historic female gene flow among the upper and middle/lower parts of the river appears to be limited and the contemporary populations are probably isolated due to deterioration of the mainstream of the river. Our results suggest that the Berg and Verlorevlei populations should be managed as distinct conservation units. We encourage the use of sanctuaries, particularly by private landowners within both river systems, as this approach may contribute effectively to preserving genetic diversity within the species.

*Pseudobarbus burgi* is a threatened small cyprinid that is endemic to a few rivers within the south coastal drainage system of the Western Cape Province, South Africa (Figure 1; Jubb 1964; Skelton et al. 1995). The species is critically endangered (Baillie and Groombridge 1996) and has already disappeared from the Eerste River (Gaigher et al. 1980). Once widespread in the Berg River system (Harrison 1952), it now only exists as isolated populations confined to a few mountain tributaries with clear flowing water and rocky substrates. These habitats are usually in good ecological condition and lack invasive alien fish. The species also occurs in the adjacent Verlorevlei River system where it seems to prefer turbid, well-vegetated lowland habitat (Skelton 1987).

The main threats to *P. burgi* are predation by invasive alien predatory fishes [in particular, smallmouth bass (*Micropterus dolomieu*)] and habitat degradation and destruction by industrial and agricultural pollution, unsustainable water resource development, channelization of streambeds, and introduction of alien aquatic and riparian plants (Skelton 1987). Unfortunately studies on the biology and ecology of this highly threatened species are lacking (Skelton 1987). An understanding of both its demography and genetic variability is also essential—a critical consideration for the future conservation of any species (Lande 1988).

Mitochondrial DNA (mtDNA) is a widely used marker for studying population differentiation because of its maternal, non-recombining mode of inheritance and rapid rate of evolution (Wilson et al. 1985). Phylogeographic studies (Avise et al. 1987) have shown that most species have hierarchical and sometimes deep genetic structures (Avise 1994) and have highlighted the dominant role of historical biogeographic and demographic events in shaping observed patterns of mtDNA variation (Bernatchez and Wilson 1998). The distributions of strictly freshwater fish species are influenced by the often restricted and discrete nature of freshwater systems, as well as physical barriers (e.g., waterfalls) and physicochemical tolerance levels (Skelton 1993); consequently most freshwater species exhibit higher levels of genetic population structuring than marine and anadromous fish (Ward et al. 1994).

The value of molecular phylogenies for conservation are twofold. First, Moritz (1994a,b) has suggested that two types of conservation units can be distinguished, namely management units (MUs), representing populations that are currently de-
mographically independent, and evolutionary significant units (ESUs), which represent historically isolated sets of populations. While MUs are important for short-term conservation management, ESUs should form part of long-term strategies (Moritz 1995). Second, population processes such as gene flow and trends in population size can be inferred from molecular phylogenies, although it may be difficult to distinguish between current and historical processes (Moritz 1995).

In the present investigation we chose to analyze part of the mtDNA control region for two reasons: (1) the control region is often the most variable part of the mitochondrial genome (Brown 1985), and (2) the variable region I of the control region has been used to investigate population structure in a wide variety of freshwater fish species (Bowers et al. 1994; Chubb et al. 1994; Duvernall and Turner 1998; Giuffra et al. 1994). The aims of the investigation were to assess genetic variability among isolated and fragmented P. burgi populations and to identify ESUs and MUs for conservation management of this highly threatened species.

Materials and Methods

Sample Collection

Twenty-eight Berg River redfinns were collected from five populations, four in the Berg River system and one in the adjacent Verlorevlei River system (Figure 1). The populations analyzed were as follows: V = Verlorevlei River (N = 8, two sites about 4 km apart 32°28'S, 18°41'E); L = Leeu River (N = 4, middle Berg system tributary 33°09'S, 19°03'E); H = Hugo River (N = 4, upper Berg River tributary 33°44'S, 19°02'E), and O = Olifants River (N = 3, upper Berg system tributary, two sites approximately 300 m apart, 33°50'S, 19°06'E). Burchell’s redfin (P. burchelli; Breede River 33°34'S, 19°08'E), the whitefish (Barbus andrewi; Berg River 33°42'S, 18°58'E), and the carp (Cyprinus carpio; Chang et al. 1994) were included as outgroups.

DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted from fin clips or gills using standard protocols of chemical digestion (50 mM Tris pH 7.6, 100 mM NaCl, 1 mM Na EDTA pH 8.0, 0.5% SDS, 1 mg/ml Proteinase K, 0.1 mg/ml RNAse A) followed by phenol/chloroform extraction and ethanol/ammonium acetate precipitation. Air-dried DNA pellets were eluted in TE.

The 5' region of the mtDNA control region was amplified via the polymerase chain reaction (PCR) using conserved vertebrate primers L15925 (5' TACACTGTCTTGTAAACC 3'), Kocher et al. 1989) and H16499 (5' CTGGAAGTGAAACAGAT 3'; Southern et al. 1988); these primers span part of the tRNAthr gene, the entire tRNApro gene and the variable region I of the control region. Amplification was performed in 50 μl volumes containing 1.25 units of Promega Taq polymerase enzyme, reaction buffer, 2.5 mM MgCl2, 2 mM dNTPs, and approximately 500 ng template DNA. The thermocycler (PE Biosystems 2400) profile consisted of an initial denaturation cycle of 2 min at 94°C, followed by 35 cycles of denaturation (93°C, 30 s), primer annealing (50°C-55°C, 30 s), and polymerase extension (72°C, 45 s). A final extension cycle of 5 min at 72°C completed the reaction. Amplification products were separated through 1.5% agarose gels and excised gel fragments were purified using the Nucleotrap extraction kit (Macherey-Nagel). The two terminal PCR primers and a custom-made internal P. burgi primer (5' TGTTGAGCAAATAACTTAC 3'; position 176-193 in the C. carpio sequence; Chang et al. 1994) were employed for chain termination sequencing of both strands using Sequenase version 2.0 (United States Biochemicals). Sequences were aligned using ClustalW (Thompson et al. 1994) and the computer alignment was checked manually. Sequences have been deposited in GenBank under accession numbers AF093066-AF093080.

Data Analysis

Genotypic diversity was estimated following Nei and Tajima (1981) and estimates of nucleotide sequence divergence between unique mtDNA haplotypes were calculated using the HKY85 model (Hasegawa et al. 1985). Nucleotide diversity within populations was calculated as the mean of pairwise divergence values between all specimens within each population. Phylogenetic analyses were performed using PAUP4.0.6d4*.

The HKY85 distances were determined using 1000 bootstrap iterations with random replacement (Felsenstein 1985) and phylogenetic signal was assessed by evaluating tree length distribution of 1000 randomly generated trees (g statistic; Hillis and Huelsenbeck 1992). The transition:transversion (TT:TV) ratio was estimated using the maximum likelihood option of PAUP4.0.6d4. Unweighted analysis as well as transition weighting were used in the parsimony analysis. Indels were scored as a fifth character. The number of substitutions between haplotypes was used to construct a minimum spanning tree using MINSNET (Excoffer and Smouse 1994).

The degree of among-population genetic variation (f) was calculated using an analysis of molecular variance (AMOVA; Excoffer et al. 1992). The significance of variance components and φ statistics were tested using 1000 nonparametric random permutations. Female gene flow among populations was estimated based on pairwise φST values (Takahata and Palumbi 1985).

Results

The sequence for the 94 bp of the tRNAthr and tRNApro genes and the 344–349 bp of region I of the control region generated for P. burgi, P. burchelli, and B. andrewi were aligned to the published carp sequence (Table 1). In the 448 bp alignment, 115 sites (25.7%) showed variation among these cyprinid taxa and 14 insertion/deletion (indel) events were inferred. Thirty-five variable characters (25 Ts, 9 Tvs, and 1 indel) delimit 13 unique P. burgi lineages.

Table 2 shows the geographic distribution and frequency of the P. burgi haplotypes among 28 specimens from five populations. No lineages were shared between the Verlorevlei and Berg River populations and haplotype diversity was high in both
Table 1. Sequence alignment of 448 bp of the mtDNA control region

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
</tr>
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<tbody>
<tr>
<td>Cyprinus</td>
<td>GAGGTTATATCCCTCTAGCAACGAGAAAAAATACGTCTCAAGGTGCTCAACACCAAGATCCCTTTATCGGGAG</td>
</tr>
</tbody>
</table>
rivers. Nucleotide diversity was generally higher in the Berg River populations. Among Berg River populations, two lineages were shared between sites while the remaining six were confined to specific parts of the river. Genotypic diversity was slightly higher for populations from the upper parts of the river (O and H) compared to the middle (L) and lower populations (P).

Sequence divergence among the Berg River redfin lineages ranged from 0.23% to 7.03%. Of 35 sites that varied among P. burgi haplotypes, 23 were diagnostic between fish from the Verlorevlei and Berg Rivers, and sequence divergence values of 5.27–7.03% between lineages from the two rivers approximate those between fish from the Verlorevlei and Berg Rivers, respectively. During the Cenozoic are thought to have had a profound influence on the rivers of the Cape Fynbos region (Hendey 1983). During the Cretaceous to early Tertiary, the Berg River followed the valley presently occupied by the Verlorevlei River.

An analysis of molecular variance supported the distinction between Verlorevlei and Berg River redfin populations and significant population and regional structuring were indicated ($\phi_{ST} = 0.902, P < .001; \phi_{CT} = 0.902, P < .001$). In the hierarchical analysis, 90.17% of the overall variance was due to variation between the two groups, 8.52% due to variation within populations, and only 1.31% to variation among populations within the groups. There was a lack of significant structure among Berg River populations ($\phi_{ST} = 0.098, P = .176$); 91.12% of the variance was attributed to variation within populations. Small sample sizes limit the reliability of gene flow estimates. Pairwise estimates of $\phi_{ST}$ between the four Berg River populations revealed more gene flow between the two upper populations than between the upper and middle/lower parts of the river, while the middle and lower populations appeared to be isolated from each other.

Discussion

mtDNA Control Region Variation

Mitochondrial DNA control region sequence variation revealed significant genetic differentiation in P. burgi and high levels of genotypic and nucleotide diversity in nearly all populations. Within the Berg River system, most of the variation was within rather than between populations, resulting in a lack of significant population structure. Some of the Berg River populations were polymorphic for divergent haplotypes and pairwise comparisons of the four localities showed female gene flow from the upper parts of the river to its middle/lower reaches. These trends may, however, indicate historic association rather than contemporary dispersal (Bernatchez and Wilson 1998).

The most striking result was the genetic distinction between P. burgi from the Verlorevlei and Berg River systems which is of the same magnitude as that detected between P. burgi and P. burchelli. The sequence divergence of 5.3–7.03% between the lineages from the two rivers represents one of the highest intraspecific mtDNA divergences reported for freshwater fish species (see the review by Bernatchez and Wilson 1998). Moreover, these genetic differences are reinforced by morphological differences. Skelton (1988) reported that the Verlorevlei River P. burgi differ from Berg River populations by having a distinctive color and pigment pattern, lacking head or body tubercles, and by having a much longer intestine. When taken together these observations clearly question the robustness of the existing taxonomy.

The rate of control region sequence variation has not been calibrated in cyprinids. For salmonids, Bernatchez and Danzmann (1993) and Giuffra et al. (1994) reported the rate to be less than twice that of the entire mtDNA molecule (±3%), whereas Brown et al. (1993) found the rate in white sturgeon to be comparable to that of mammals, being four to five times that of the entire molecule (±8–10%). Therefore if we use a tentative rate of 3–10% per million years, the maternal lineages from the two rivers diverged between 0.5 and 2.3 million years ago, while diversification within each of the rivers was more recent, 23,000–230,000 years for the Verlorevlei River lineages and 23,000–620,000 years for the Berg River haplotypes.

Phylogeography

The phylogeographic separation of P. burgi from the Verlorevlei and Berg River systems may be related to the drainage history of the region. At present the two river systems are independent (Skelton 1980), however, sea level and climatic changes during the Cenozoic are thought to have had a profound influence on the rivers of the Cape Fynbos region (Hendey 1983). The Verlorevlei to its middle/lower reaches. These trends
and during the time of the maximum sea level regression (approximately 30 million years ago), with the sea level more than 200 m below the present level, the Clanwilliam Olifants River and the Berg/Verlorevlei Rivers converged (Dingle et al. 1983).

It has been suggested that a common *Pseudobarbus* ancestor evolved or became established in the southwestern river drainages of South Africa during temperate conditions 45 to 25 million years ago (corresponding to the sea level regression described above) and that it became widely distributed in the upper Orange River and rivers of the south coastal drainage basin (Skelton 1980). During the late Oligocene, with a rise in sea level, the Berg River took up its westerly path in the direction of Saldanha Bay (Hendey 1983) and *P. burgi*, which is basal within *Pseudobarbus* (Skelton 1980), may have become isolated in the Berg and/or Verlorevlei Rivers at that time. The course of the Berg River has, however, not remained constant since the Oligocene (Hendey 1983), and it is conceivable that similar convergences between the Verlorevlei and Berg Rivers were possible during the regressions of the late Miocene and late Pliocene (although the drops in sea level were not as extreme as during the middle Oligocene). During the Pleistocene there were only minor fluctuations in sea level (Hendey 1983) and the separation between *P. burgi* from the Berg and Verlorevlei Rivers may therefore reflect the independence of the two rivers since the late Pliocene to early Pleistocene (2 million years ago).

**Conservation Recommendations**

Moritz (1994b) proposed that ESUs should be reciprocally monophyletic for mtDNA alleles and also differ significantly for the frequency of alleles at nuclear loci. Reciprocal monophyly of control region haplotypes of *P. burgi* from the Verlorevlei and Berg Rivers is evident from our study (Figure 2). Morphological differences between redfinns from the two rivers (Skelton 1980, 1988) provide indirect evidence of differences in nuclear genes. The recognition of the two populations as separate ESUs is further supported by ecological differences, with Berg River populations confined to clear flowing mountain streams in contrast to the Verlorevlei population, which appears to inhabit turbid, well-vegetated lowland habitats (Skelton 1987).

Our results clearly suggest that the Berg and Verlorevlei River populations should be managed as distinct conservation units and that the Verlorevlei population in particular warrants increased protection. This small nonperennial river system will, however, be difficult to conserve, as most of the catchment area is privately owned and is being further developed for orchards and irrigated crops. Although the Berg River populations are not graphically independent, they have significant conservation value because of their fragmented nature and their high haplotype diversity. Genetic exchange between these populations is likely to be minimal in the future, as the mainstream of the Berg River has been extensively altered by anthropogenic activities and is dominated by alien fish species, including several highly destructive predators such as *M. dolomieu*. Alternative and innovative conservation strategies involving private landowners will need to be investigated to ensure the preservation of *P. burgi* habitat.

**Table 2. Geographic distribution and frequency of *Pseudobarbus burgi* mtDNA haplotypes based on 448 bp of the control region**

<table>
<thead>
<tr>
<th>River system</th>
<th>Population</th>
<th>mtDNA control region haplotypes</th>
<th>Percent nucleotide diversity (±SD)</th>
</tr>
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<tbody>
<tr>
<td>Verlorevlei</td>
<td>V</td>
<td>2 1 1 2 2</td>
<td>0.893 (±0.03)</td>
</tr>
<tr>
<td>Berg</td>
<td>P</td>
<td>— — — 2 7</td>
<td>0.826 (±0.12)</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>— — — 4</td>
<td>0.547 (±0.18)</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>— — — 2 2 1 1 1 1</td>
<td>0.667 (±0.05)</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>— — — 3 2 1 — — —</td>
<td>1.000 (±0.15)</td>
</tr>
</tbody>
</table>

Genotypic diversity was estimated following Nei and Tajima (1981) and nucleotide diversity as the mean of all pairwise sequence divergence (HKY85) estimates within each population. Population designations follow Figure 1.

**References**


Dingle RV, Siesser WG, and Newton AR, 1983. Mesozoic...
and Tertiary geology of southern Africa. Rotterdam: A.A. Balkema.


