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ORIGINAL ARTICLE

A freshwater fish defies ancient mountain ranges and drainage divides: extrinsic and intrinsic influences on the evolutionary history of a recently identified galaxiid

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ABSTRACT

Aim A river hierarchy model has been proposed for stream-dwelling taxa, where genetic structure is expected to reflect geographical proximity and connectivity of river systems. However, many exceptions and deviations from this model have been detected. The present study tested three biogeographical hypotheses (River Hierarchy, Palaeoriver Systems and Interdrainage Dispersal) to assess how a recently identified galaxiid, *Galaxias* sp. 'nebula', came to have a wide distribution across multiple currently isolated river systems in the Cape Floristic Region of South Africa.

Location Seventeen river systems in the Cape Floristic Region at the southern tip of Africa.

Methods We analysed mitochondrial cytochrome *c* oxidase subunit I and cytochrome *b* sequences from across the entire distribution of *Galaxias* sp. 'nebula' and compared divergence times between populations with known geological and climatic events to explain the observed geographical patterns of genetic diversity.

Results The data revealed historical divergence between the Olifants, Berg and southward-draining river systems. The phylogeographical analyses revealed that range expansion occurred across currently isolated river systems, with some haplotypes being shared between geographically distant river systems. Molecular dating revealed recent divergence times between populations from isolated river systems (*c.* 4000–1,200,000 years ago).

Main conclusions The phylogeographical pattern of *Galaxias* sp. 'nebula' indicates that drainage basin boundaries have historically not played a dominant long-term role in structuring this lineage. River captures are not widespread and frequent enough to explain the observed genetic patterns. *Galaxias* sp. 'nebula' has been able to disperse between proposed palaeoriver systems. Interdrainage dispersal via episodic freshwater connections during periods of increased rainfall during the Pleistocene and Holocene epochs seems to have played an important role in allowing this lineage to attain and maintain its wide contemporary distribution.

Keywords

Cape Floristic Region, *Galaxias*, intermittent freshwater connections, palaeorivers, phylogeography, river capture, river hierarchy, sea-level changes, South Africa.

INTRODUCTION

A hierarchical pattern of genetic variation has traditionally been expected for primary freshwater taxa due to their limited ability to disperse across marine and terrestrial barriers (Meffe & Vrijenhoek, 1988). While this model has been supported in some studies (reviewed by Hughes *et al.*, 2009), departures have also been reported, with some taxa displaying extensive genetic subdivision within drainage basins (e.g. Huey *et al.*, 2010; Hughes *et al.*, 2012), while others exhibit

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unexpectedly low levels of genetic structuring between isolated river systems (e.g. Hurwood & Hughes, 2001). Understanding the processes that shaped the geographical patterns of genetic variation in extant taxa is a continuing challenge in evolutionary biology. River confluences during periods of lower sea levels and drainage rearrangements (river captures) have often been invoked as the principal factors for the occurrence of obligate freshwater fishes across multiple currently isolated river systems (Waters & Wallis, 2000; Burridge *et al.*, 2006, 2007; Swartz *et al.*, 2007, 2009). Thus far, the role of alternative mechanisms, such as intermittent freshwater connections across low relief terrain, in influencing the evolutionary history of freshwater fishes has received little attention (Burridge *et al.*, 2008a), yet some freshwater taxa have intrinsic ecological attributes that may allow them to exploit these mechanisms to disperse across drainage divides (e.g. Hurwood & Hughes, 2001; Thacker *et al.*, 2007).

The Cape Floristic Region (CFR) located at the southern tip of Africa provides a unique opportunity for studying the processes that influenced the evolutionary history of freshwater taxa that occur across multiple currently isolated river systems. The CFR is clearly demarcated from surrounding areas by the Cape Fold Mountains and the Great Escarpment and it has a high diversity of endemic freshwater biota (Barnard, 1943; Skelton, 1986; Wishart & Day, 2002). The region has experienced a complex geological and climatic history marked by extensive uplifting and mountain-building processes (Hendey, 1983; Cowling *et al.*, 2009), periodic sea-level regressions and transgressions (Tankard, 1976; Siesser & Dingle, 1981; Dingle *et al.*, 1983; Rogers, 1985; Ramsay & Cooper, 2002), as well as periods of extreme wet and dry conditions (Partridge *et al.*, 1999; Marlow *et al.*, 2000; Zachos *et al.*, 2001; Cowling *et al.*, 2009).

Major uplifting of the CFR landscape has occurred at least three times in the past. A strong uplift during the early Cenozoic (*c.* 65 Ma) led to the formation of the Cape Fold Mountains and the establishment of the major present-day drainage systems (Hendey, 1983). Two further uplift events occurred after the early Cenozoic (Partridge & Maud, 1987, 2000). The first, the Post-African I uplift event, occurred in the Miocene (*c.* 22 Ma). The second, the Post-African II uplift event, occurred as late as the Pliocene (*c.* 5.3–2.6 Ma). As the region has been stable since the Pliocene (reviewed by Cowling *et al.*, 2009), contemporary landscape features of the CFR are likely to have been unaltered since then.

The region has also been subjected to major changes in climatic conditions. During the late Oligocene (*c.* 25 Ma) to middle Miocene (*c.* 15 Ma), southern Africa is thought to have been characterized by wetter and more mesic climatic conditions, and the landscape was probably covered by extensive wetlands and widespread subtropical forests (Zachos *et al.*, 2001; Cowling *et al.*, 2009). Intermittent connections of adjoining drainages might have occurred during periods of heavy flooding. The gradual transition of the CFR climate towards steeper moisture and thermal gradients and the loss of connectivity among river systems began in the middle Miocene (Zachos *et al.*, 2001). Deacon *et al.* (1992) and Tyson & Partridge (2000) suggest that the contemporary drier conditions of much of the CFR were established by the end of the Pliocene (*c.* 2.6 Ma), but some sections are thought to have experienced wetter climatic conditions as recently as the Holocene Altithermal (*c.* 8000–6000 years ago; Partridge *et al.*, 1999).

Southern Africa also experienced repeated fluctuations in sea levels throughout the Tertiary period (Siesser & Dingle, 1981; Hendey, 1983), although there are uncertainties regarding global sea-level amplitudes (reviewed by Miller *et al.*, 2005). The last major sea-level regression occurred *c.* 22,000–18,000 years ago when continental coastlines are estimated to have been about 120 ± 5 m below present levels (Miller *et al.*, 2005). These fluctuations could have produced recurrent connection and separation of adjacent river systems (Hendey, 1983).

Molecular studies have revealed both highly restricted and widely distributed genetic lineages of freshwater-dependent fish species in the CFR (Waters & Cambray, 1997; Wishart *et al.*, 2006; Swartz *et al.*, 2007, 2009). This suggests that some species of fish have been isolated for long periods of time, while others have been able to maintain relatively recent gene flow across the CFR despite the apparent isolation of contemporary river systems. The evolution of range-restricted lineages can be explained by vicariance and specialized habitat preferences, but the occurrence of widespread lineages in a landscape where the dominant process is expected to be isolation between different river systems challenges conventional views on how obligate freshwater fishes can disperse.

Galaxias sp. 'nebula' (see Chakona *et al.*, 2011), a recently identified lineage of the Cape galaxias, *Galaxias zebratus* (Castelnau, 1861) (Galaxiidae), is ideal for testing biogeographical questions in the CFR, because it is currently understood to be the most widespread obligate freshwater fish lineage in this region. It occurs across 17 isolated river systems from the Olifants on the west coast to the Bitou River system on the south coast (Fig. 1). *Galaxias zebratus* was previously thought to be the only representative of the freshwater fish family Galaxiidae in Africa. This species was thought to be widely distributed across several coastal streams and rivers from the Gamtoos on the south coast to the Olifants on the west coast (Barnard, 1943). However, molecular studies have uncovered deep genetic subdivision within *G. zebratus*, indicating that it is a species complex (Waters & Cambray, 1997; Wishart *et al.*, 2006). The complex is currently represented by at least 10 historically isolated lineages (Linder *et al.*, 2010). Individuals of *Galaxias* sp. 'nebula' attain around 70 mm total length and are capable of surviving out of water for a prolonged period of time (Chakona *et al.*, 2011). Due to its cryptic nature and small size, the lineage is not exploited and its distribution is unlikely to have been influenced by human-mediated translocation.

For this study, we employed a predictive hypothesis-testing approach to assess the extent to which three alternative (but

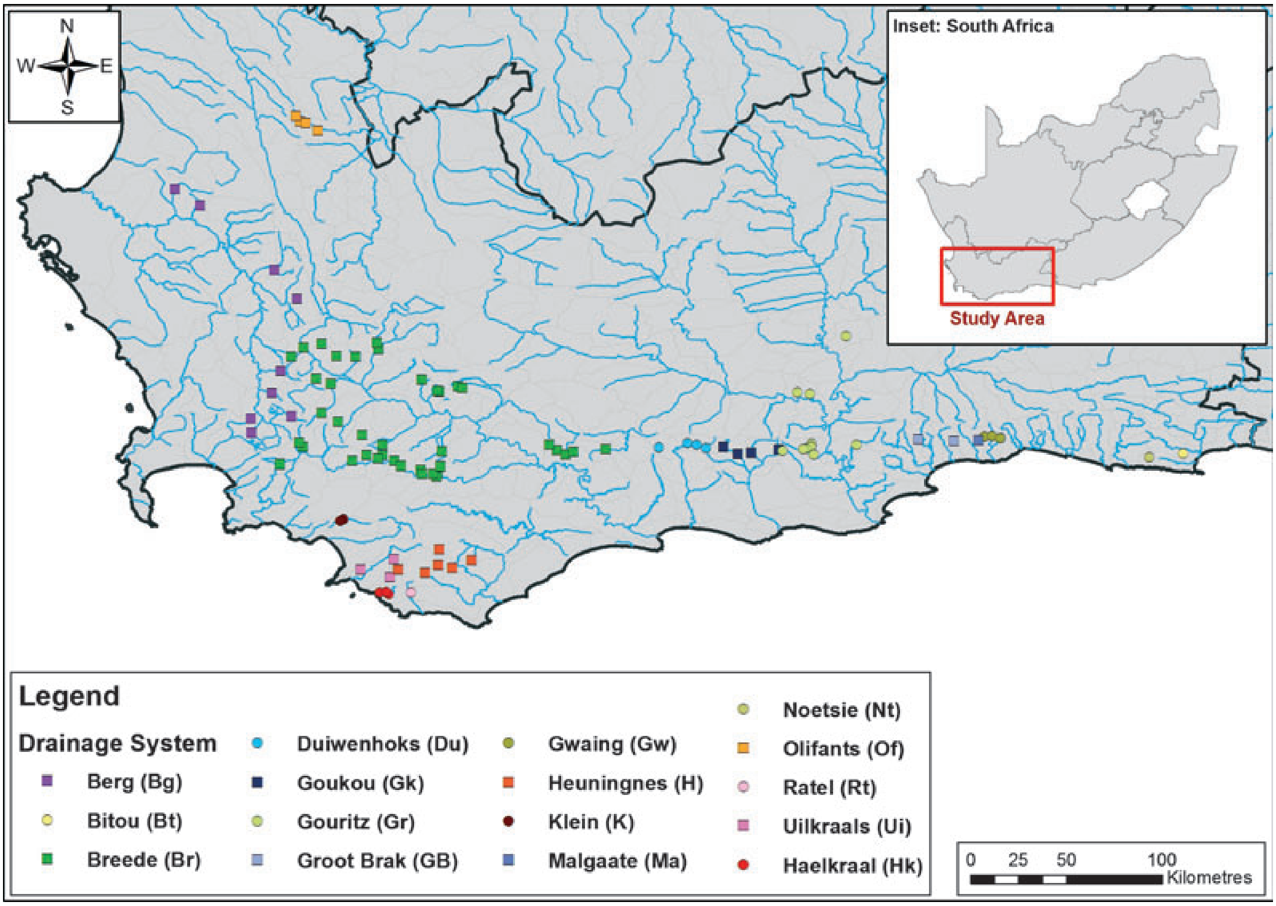

Figure 1 Sampling localities in the Cape Floristic Region of South Africa for *Galaxias* sp. 'nebula' used for the present study. See Appendix S1 for locality descriptions. The colour codes represent the different contemporary river systems (see insert for key to river names).

non-exclusive) biogeographical hypotheses (River Hierarchy, Palaeoriver Systems and Interdrainage Dispersal) could explain genetic patterns in *Galaxias* sp. 'nebula'. The River Hierarchy hypothesis considers present-day river systems as the main drivers of genetic patterns. Under this hypothesis, the lineage is expected to have genetic structuring that reflects the current drainage basin boundaries (Fig. 2a) with shallower divergences within current river systems and deep divergences among them (e.g. Meffe & Vrijenhoek, 1988). This would be consistent with our current understanding that present-day drainage systems have been unaltered since the landscape became stable after the Post-African II uplift event. Divergence dates among populations from the current river systems are expected to coincide with the Pliocene epoch.

The Palaeoriver hypothesis considers that the confluence of rivers during low sea levels of the Last Glacial Maximum (LGM) may have facilitated movement of freshwater fishes between currently isolated river systems. Swartz *et al.* (2007, 2009) recently provided empirical evidence that the confluence of rivers during the LGM influenced the distribution of genetic lineages of cyprinid minnows in the genus *Pseudobarbus* in the CFR. Under this hypothesis, little genetic subdivision is expected among *Galaxias* sp. 'nebula' populations from river systems that coalesced during the LGM (Fig. 2b), with comparatively greater genetic structure among populations from rivers that belonged to different palaeoriver systems.

The Interdrainage Dispersal hypothesis considers that *Galaxias* sp. 'nebula' could have experienced recent range expansion across the CFR, defying drainage divides and proposed palaeoriver systems. Finding shallow genetic structuring, low divergences and sharing of haplotypes among *Galaxias* sp. 'nebula' populations in river systems that belonged to different palaeoriver systems would support this hypothesis (Fig. 2c).

MATERIALS AND METHODS

Sample collection

Comprehensive sampling was carried out between 2008 and 2010 across the distribution of *Galaxias* sp. 'nebula' (Fig. 1) using a combination of methods: electrofishing, seine netting or snorkelling with a hand net. Fish were anaesthetized using clove oil (0.2‰). Small pieces of muscle tissue or whole fish

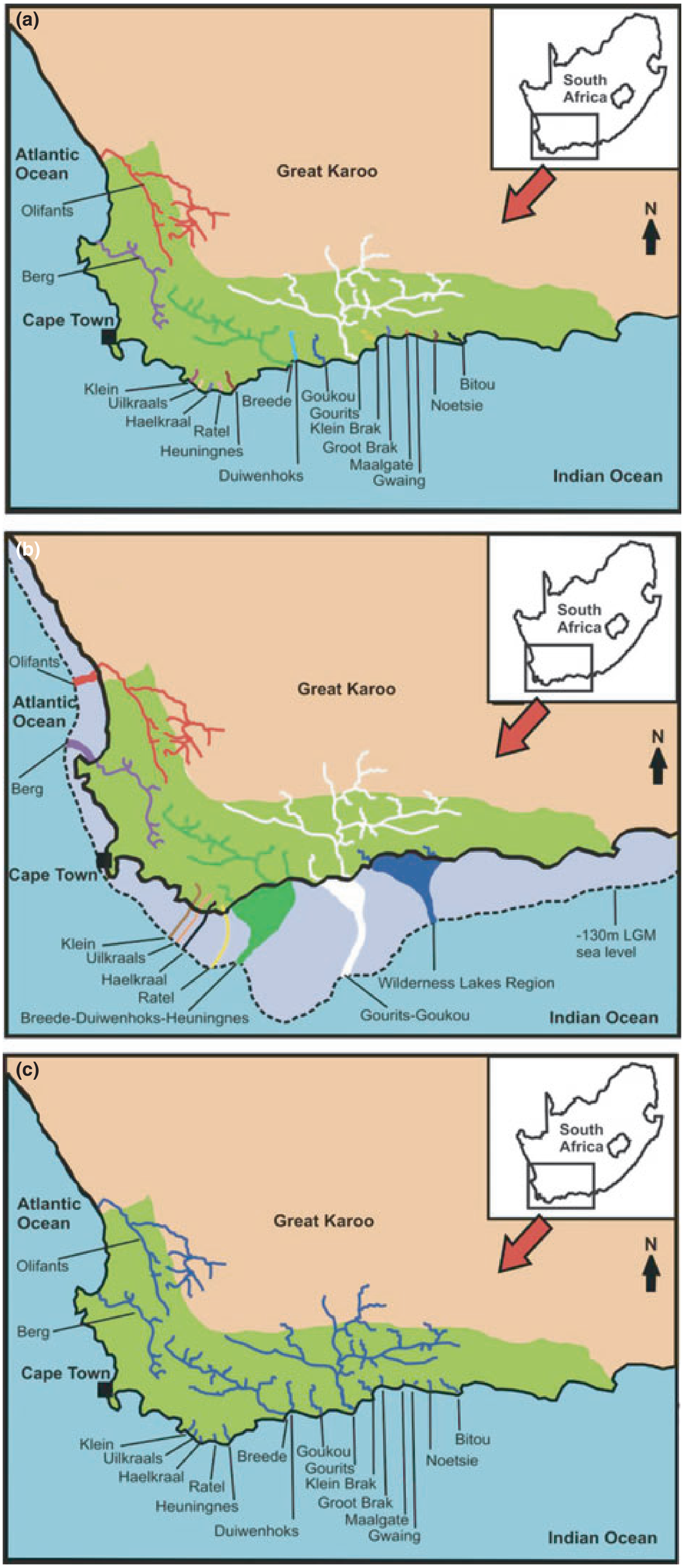

Figure 2 Hypotheses of factors that could have influenced genetic diversity and structure in *Galaxias* sp. 'nebula' in the Cape Floristic Region of South Africa. Panel (a) describes the hypothesis that genetic variation has been strongly influenced by contemporary river systems (River Hierarchy hypothesis). This model predicts significant partitioning of genetic variation among river systems. Panel (b) shows the river systems that would have coalesced forming the palaeoriver systems of the Last Glacial Maximum (LGM). The colours indicate the predicted partitions of genetic variation (Palaeoriver hypothesis). Panel (c) illustrates the hypothesis that more recent connections have occurred among populations, resulting in low genetic differentiation and no structuring across the lineage's distribution (Interdrainage Dispersal hypothesis).

samples were placed in 95% ethanol in the field, and then transferred to –20 °C in the laboratory. Voucher specimens and tissue samples have been deposited in the South African National Fish Collection (South African Institute for Aquatic Biodiversity, Grahamstown). Additional samples collected during previous field surveys (2004 and 2007) were also included. A total of 181 individuals from 91 localities were sequenced for both mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) (see Appendix S1 in Supporting Information).

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from preserved tissue using the Wizard Genomic DNA purification kit (Promega, Madison, WI). A fragment of the COI gene was amplified using the primers Vf2-T1 and Vr1-T1 from Ivanova *et al.* (2007). The primers cytb-Glu and cytb-Thr (Waters & Wallis, 2001) were used to amplify a fragment of the cyt *b* gene. Polymerase chain reactions (PCR) were performed with an Eppendorf Mastercycler (Eppendorf, Hamburg) and each reaction mixture (50 μL) contained 1× reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP [Kapa Biosystems (Pty) Ltd, Cape Town], 20 pmol of each primer, 1 U Super-Therm Taq Polymerase (Southern Cross Biotechnology, Cape Town) and 100–200 ng of template DNA. The profile for amplification of the COI gene was 94 °C for 3 min, followed by 38 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 50 s, and then final extension at 72 °C for 7 min. The profile for amplification of the cyt *b* gene was similar but included 35 cycles with an annealing step of 51 °C for 45 s. PCR products were sent to Macrogen Inc. (Seoul, Korea) where purification was carried out using Montáge PCR filter units (Millipore, Bedford, MA, USA). Some samples were purified at the South African Institute for Aquatic Biodiversity using a QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) and cycle-sequenced using BigDye Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Both gene regions were sequenced with only the forward primers. Automated sequencing was performed at Macrogen Inc. (Seoul, Korea) using an ABI 3730xl DNA Analyzer (Applied Biosystems) and at the sequencing facility at Rhodes University using an ABI Prism 3100 Genetic Analyzer. Sequences were cleaned, aligned and trimmed to equal lengths using the program SeqMan 7.2.1 (DNASTAR, Madison, WI, USA). Sequences were submitted to GenBank (accession numbers KC821787-KC821968). The program DnaSP 5.10 (Librado & Rozas, 2009) was used to translate nucleotide sequences to amino acid sequences to check for stop codons and errors.

Sequence diversity analysis

Concatenated sequences of the two gene fragments were used for all analyses. DnaSP was used to identify shared and unique haplotypes, to determine the number of polymorphic sites and the number of haplotypes (N_H) in the entire sample of 181 sequences. Prior to analysis, a model of sequence evolution that best fitted the data was estimated in Modeltest 3.7 (Posada & Crandall, 1998) using Akaike's information criterion (AIC) (Burnham & Anderson, 2002). The best model of sequence evolution selected was the TrN+G (Tamura & Nei, 1993). Model-corrected distances between unique haplotypes were calculated using paup* (Swofford, 2003). These values were converted to sequence divergences between river systems and the clades that were resolved using tcs 1.2.1 (Clement *et al.*, 2000).

Genetic structure and evolutionary history

Analysis of molecular variance (AMOVA) implemented in Arlequin 3.5 (Excoffier *et al.*, 2005) was used to explore how genetic variation is partitioned across the geographical landscape. The AMOVA was carried out using 20,000 permutations to assess the significance of differences between estimated values and those expected when haplotypes are randomly assigned to populations. The parameters of the model of sequence evolution identified above were implemented for these analyses. To evaluate the role of current river hierarchies, sampling sites were first divided into 17 groups corresponding to currently isolated drainage systems. AMOVA was also carried out to assess the structure among the southward-draining river systems without the influence of the divergent clades from the westward-draining rivers (see Results). To evaluate the influence of sea-level regression on *Galaxias* sp. 'nebula' genetic variation, the sites were divided into nine groups based on five palaeoriver systems (Olifants, Berg, Breede–Duiwenhoks–Heuningnes, Gouritz–Goukou and Wilderness Lakes Region) proposed by Swartz *et al.* (2007, 2009) and an additional four hypothetical palaeoriver systems (Klein, Uilkraals, Haelkraal and Ratel) based on the bathymetry of South African Navy charts. Pairwise Φ_{ST} values were also calculated in Arlequin to examine genetic differentiation between palaeoriver systems and between contemporary river systems.

Nested clade phylogeographical analysis (NCPA) (Templeton *et al.*, 1995) was used to make inferences about the evolutionary history of *Galaxias* sp. 'nebula'. Although NCPA has been criticized in recent years (e.g. Petit, 2008), Templeton (2008) argued that NCPA is still a useful method for making phylogeographical inferences, especially when used in conjunction with other methods (Templeton, 2009). A 95% minimum-spanning network was constructed using the statistical parsimony method implemented in tcs 1.21 (Clement *et al.*, 2000). Haplotypes in the network were manually converted into a hierarchical nested clade design following the rules of Templeton *et al.* (1987, 1995) and Templeton (1998). Ambiguities (represented by loops) in the haplotype network were resolved using predictions based on coalescent theory (Posada & Crandall, 2001) and the criteria outlined by Pfenninger & Posada (2002).

Distances between sampling localities were measured along the river courses and the current coastline using ArcView

GIS 3.3 (ESRI, Redlands, CA). These distances were used in Geodis 2.6 (Posada *et al.*, 2000) to calculate the different NCPA distances and to test whether the null hypothesis of no association between haplotypes or clades and their geographical distribution (i.e. panmixia) could be rejected. The latest Geodis inference key (26 April, 2009) was used to infer the processes responsible for the observed distributions of haplotypes for each clade where a significant geographical association was found (Posada & Templeton, 2005). Evolutionary relationships among haplotypes were inferred using the neighbour-joining method based on the selected model.

Estimates of divergence times

To interpret the observed patterns in relation to documented geological and climatic events, divergence times among clades resolved with tcs and among populations from contemporary river systems were estimated using two different approaches. The first approach, mdiv (Nielsen & Wakeley, 2001), uses Bayesian inference to estimate the time to the most recent common ancestor (TMRCA), posterior distributions of scaled migration rates ($M = 2N_em$), time since divergence ($T = T_{div}/2N_e$) and a measure of genetic diversity ($\theta = 2N_e\mu$) for two populations specified a priori. The parameter estimates are the modes of the respective posterior distributions. Analyses were run under the HKY model of sequence evolution using the CBSU Web Computing Resources (http://cbsuapps.tc.cornell.edu/mdiv.aspx). For every sequence set, default priors ($M = 10$ and $T = 5$) were first used with 2,000,000 Markov chain generations with 500,000 generations as burn-in, to identify appropriate parameter values for further analyses (Nielsen & Wakeley, 2001). The program was then re-run for 2,000,000 generations (with the first 500,000 generations discarded as burn-in) across five replicate runs. Values of T and θ were estimated for each run. The estimates from the five runs were then used to calculate mean T and θ. These averages of T and θ were then used to estimate divergence time as

$$T_{\text{pop}} = \left[\frac{T\theta}{2L} \times \frac{1}{\mu g}\right]$$

where T and θ represent the mode of the posterior distribution, L is the length of the sequence used, μ is the mutation rates or substitution rate and g is the generation time (Brito, 2005). To account for time-dependent rate of molecular evolution, the slowest (0.011 changes/site) and fastest (0.125 changes/site) rates estimated by Burridge *et al.* (2008b) for New Zealand galaxiids were used. As the generation time for African galaxiids is unknown, a generation time of one year was used based on studies of New Zealand galaxiids following Burridge *et al.* (2008b).

A second approach used a variable molecular clock recently developed by Craw *et al.* (2008). The clock was calibrated using sequence divergences among galaxias populations with known geological isolation histories in New Zealand (Craw *et al.*, 2008). The authors proposed that this could be a potential dating tool for evolutionary processes in the late Quaternary in the Southern Hemisphere (Craw *et al.*, 2008). Divergence times (T_{div}) were computed using the following equation.

$$\text{Divergence}(\%) = -2.2\mathrm{e}^{(-9T_{\text{div}})} + 2.5T_{\text{div}} + 2.2$$

Arlequin (Excoffier *et al.*, 2005) was used to calculate Fu's (1997) F_S for the overall sample (181 sequences) to test for evidence of recent population expansion as predicted by the Interdrainage Dispersal hypothesis. Fu's F_S value was also calculated using only samples from the southward-draining river systems to test for evidence of recent population expansion without the influence of the divergent clades from the westward-draining rivers (see Results). The significance of the F_S parameter was assessed using 10,000 simulations.

RESULTS

Genetic variation and phylogeographical structure

A total of 1227 base pairs of the concatenated sequences (627 base pairs of cyt *b* and 600 bases of COI) were used for all analyses. The combined COI–cyt *b* sequences yielded 161 polymorphic sites and 91 unique haplotypes. Two of these haplotypes were shared between river systems: haplotype 21 was shared between the Ratel and the Heuningnes, while haplotype 32 occurred in the Breede and Klein Brak (Fig. 3).

The statistical parsimony network revealed three disjointed clades (Fig. 3): clade 3-1 comprised all the Olifants River system samples, clade 3-2 comprised all the Berg River system samples and clade 5-1 comprised all the samples from the southward-draining river systems. The neighbour-joining phylogram (Fig. 4) reflected the same clades as the tcs network (Fig. 3). Sequence divergence was high between the Olifants and Berg clades (4.1–5.5%), between the Olifants and southern clades (2.2–4.3%), and between the Berg and the southern clades (2.1–3.9%) (Table 1). In contrast, sequence divergences among the southward-draining river systems were relatively low (0.0–2.5%) (Table 1).

Pairwise Φ_{ST} values revealed significant ($P < 0.05$) genetic heterogeneity among most of the river systems (Table 1), substantiating that there is little ongoing gene flow across the landscape. However, some southward-draining river systems recorded significantly low pairwise Φ_{ST} values, suggesting more recent connectivity between them. Low sample sizes for some of the river systems could have, however, reduced the power of the Φ_{ST} test to estimate the degree of structuring among populations. Pairwise Φ_{ST} values for the palaeoriver systems also revealed significant ($P < 0.05$) genetic heterogeneity between systems, although many of the southward-draining palaeoriver systems recorded values below 0.5 (Appendix S2: Table S1). This is further support for more recent connectivity across the southward-draining river systems.

AMOVAs provided additional support for deep genetic structuring across the lineage's range, with an overall Φ_{ST} of

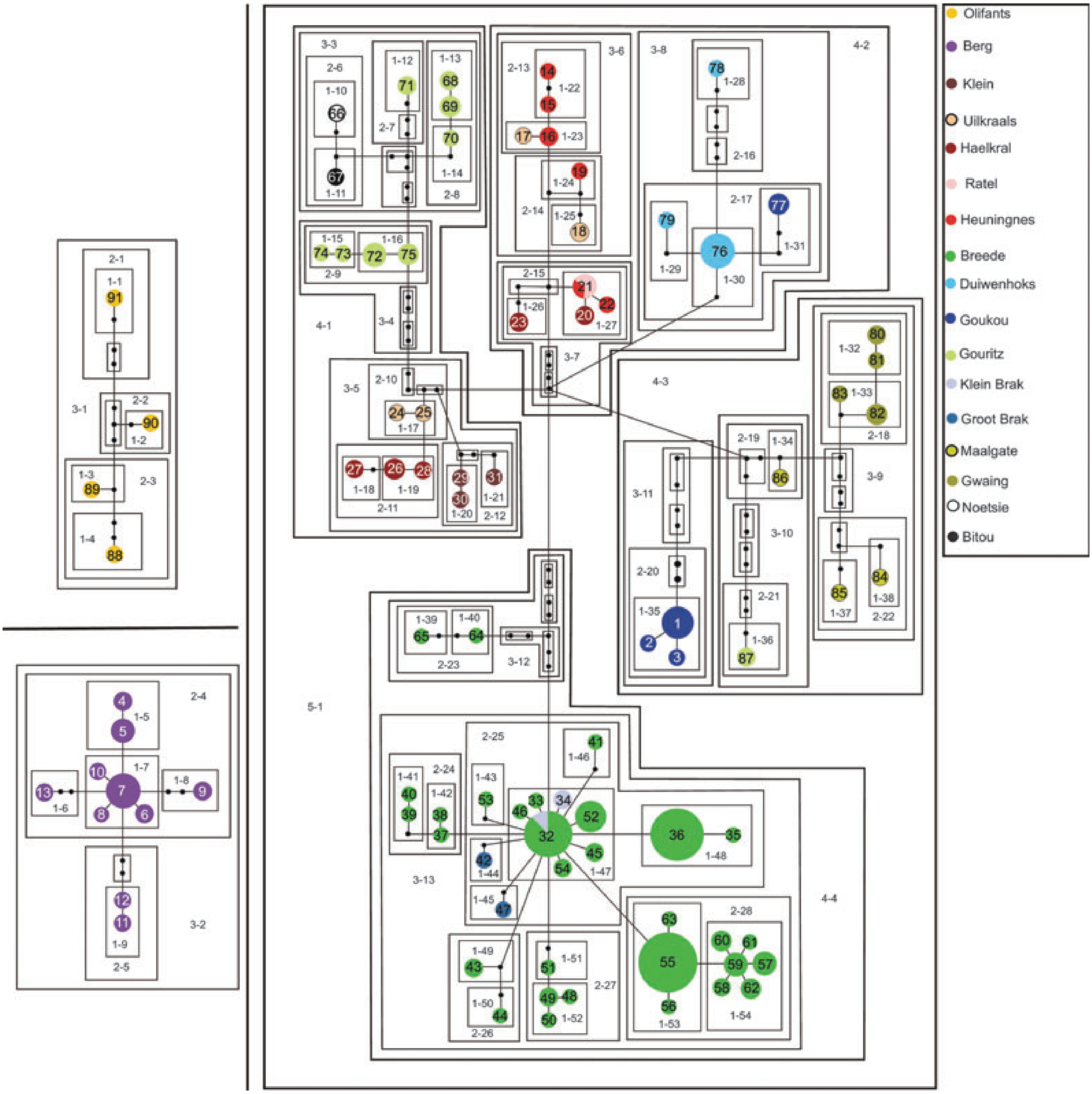

Figure 3 Combined mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) TCS haplotype network for *Galaxias* sp. 'nebula' in the Cape Floristic Region of South Africa showing nesting levels used in nested clade phylogeographical analysis (NCPA).

0.91 ($P < 0.001$). Much of the structuring was due to differences among contemporary river systems, $\Phi_{CT} = 0.75$ ($P < 0.001$) (Table 2). Significant genetic differentiation was also detected when palaeoriver systems were used as groups in the analysis, but this grouping explained only 55% of the variation (Table 2). An AMOVA for the 15 southward-draining river systems revealed that this grouping explained 58% of the variation, while an AMOVA for the seven southern palaeorivers revealed that this grouping explained only 23% of the variation (Table 2). This is further evidence of the close genetic relationships between populations from the southward-draining river systems.

Divergence time estimates

The MDIV estimates (T_{div}) indicated that divergences between populations occurred during the Pleistocene and Holocene epochs (range *c.* 4000–1,200,000 years ago) (Appendix S2: Table S2). The oldest split was between the Olifants and the Berg and was estimated to have occurred between *c.* 110,000

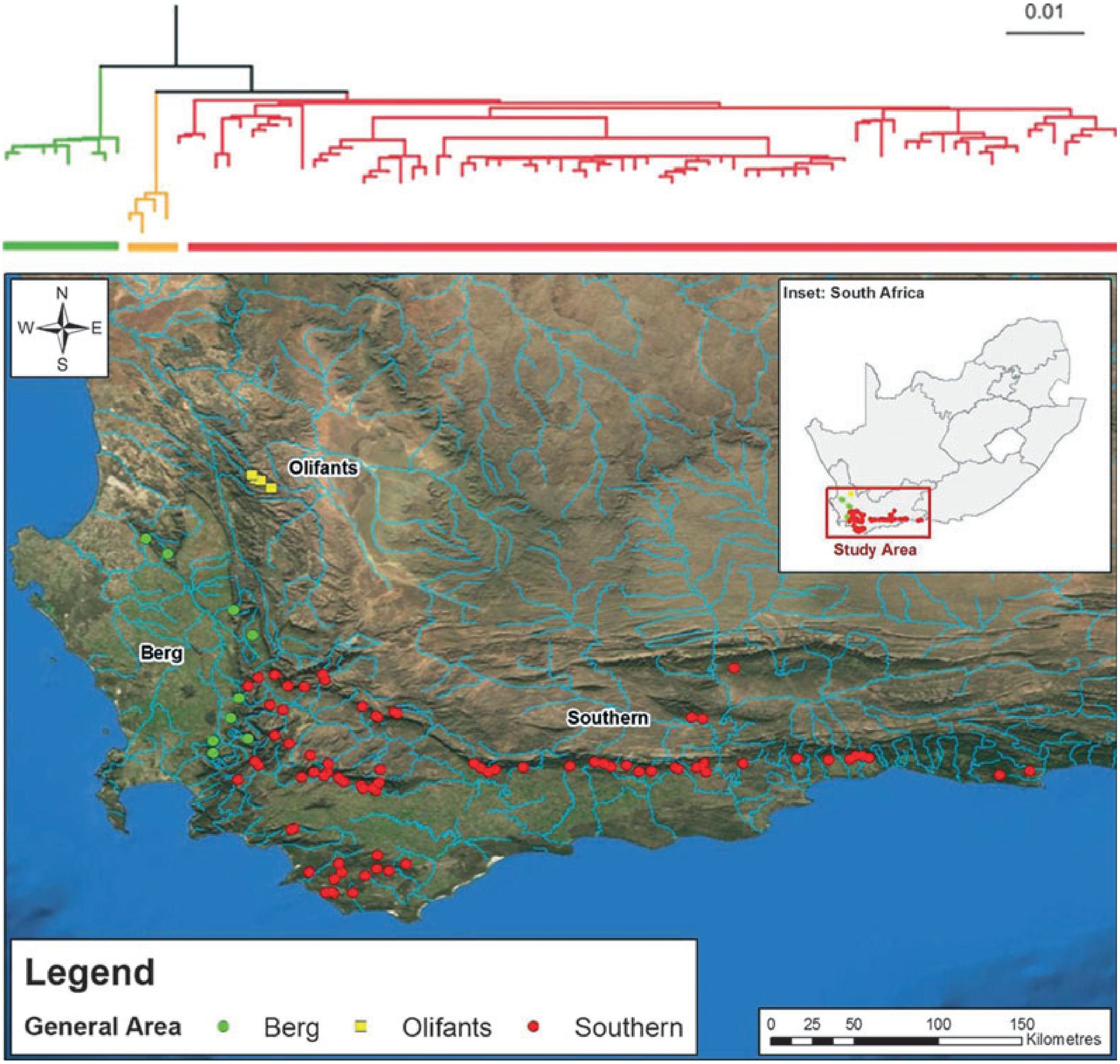

Figure 4 Neighbour-joining phylogram of *Galaxias* sp. 'nebula' in the Cape Floristic Region of South Africa based on 1227 nucleotides of concatenated cytochrome *b* (627 base pairs) and cytochrome *c* oxidase subunit I (600 base pairs) from 181 individuals. Coloured dots on the map represent sampling localities and the distribution of the three main clades.

and 1,200,000 years ago (Table S2). More recent divergence times were found among the southern populations, with estimates ranging from *c.* 4,000 to 470,000 years ago. However, low sample sizes for some of the populations may have reduced the power of the MDIV test to estimate the timing of divergence. The variable molecular clock confirmed that divergences between populations occurred during the Pleistocene and Holocene epochs (*c.* 4000–1,300,000 years ago; Table S2). Similar to the MDIV results, the oldest split was found between the Olifants and the Berg clades (*c.* 750,000–1,300,000 years ago), followed by divergence between the Olifants and the southern clade (*c.* 220,000–810,000 years ago), with the Berg and southern clades splitting last (160,000–650,000 years ago). More recent divergence times were found among the southern populations (*c.* 4000–230,000 years ago; Table S2).

Evolutionary history

Nested clade phylogeographical analysis indicated significant geographical associations of haplotypes for nine clades. Allopatric fragmentation was inferred for the geographical distribution of haplotypes belonging to three major clades within the lineage's range (total cladogram; Appendix S2: Table S3).

Table 1 Ranges of divergences (%) (below the diagonal) and Φ_{ST} values (above the diagonal) for pairwise comparisons of *Galaxias* sp. 'nebula' from 17 river systems in the Cape Floristic Region of South Africa, based on combined mitochondrial COI and cyt *b* sequences.

	River system	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	Olifants		0.945	0.856*	0.806*	0.808**	0.884*	0.877**	0.924**	0.900**	0.817**	0.773**	0.897*	0.860	0.824*	0.897**	0.836	0.827
2	Berg	4.10–5.54		0.915**	0.884**	0.869**	0.924**	0.913**	0.925**	0.916**	0.885**	0.835**	0.940**	0.933**	0.909**	0.921**	0.934	0.938
3	Klein	2.86–3.56	2.20–2.96		0.346*	0.291*	0.823	0.692*	0.788**	0.659**	0.555*	0.485**	0.855	0.767	0.551	0.770*	0.802	0.818
4	Uilkraals	2.83–3.66	2.18–3.06	0.34–1.20		0.087	0.222	0.167*	0.769**	0.503**	0.492**	0.492**	0.644*	0.574	0.490*	0.611**	0.580	0.611
5	Haelkraal	2.83–3.80	2.29–3.18	0.43–1.10	0.08–1.39		0.256	0.362*	0.768**	0.435**	0.511**	0.497**	0.629**	0.591*	0.454**	0.525**	0.593	0.624
6	Ratel	2.83–3.24	2.19–2.67	0.71–0.90	0.34–0.80	0.08–1.09		0.262	0.823**	0.810**	0.656**	0.563**	0.978	0.918	0.751	0.886*	1.000	1.000
7	Heuningnes	2.84–3.67	2.19–2.94	0.71–1.31	0.08–1.19	0.08–1.50	0.00–0.52		0.822**	0.723**	0.671**	0.607**	0.837*	0.804*	0.729*	0.791**	0.828	0.839
8	Breede	3.23–4.25	2.66–3.88	12.00–1.63	0.99–1.83	1.09–1.83	1.19–1.61	1.19–2.06		0.779**	0.800**	0.735**	0.053	0.134	0.835**	0.816**	0.834	0.847
9	Duiwenhoks	2.73–3.72	2.09–2.86	0.44–1.22	0.43–1.31	0.52–1.42	0.61–1.00	0.61–1.42	0.90–1.97		0.553**	0.532**	0.861*	0.830*	0.723*	0.772**	0.868	0.879
10	Goukou	2.21–3.57	2.21–3.05	0.72–1.20	0.71–1.40	0.80–1.40	0.90–1.19	0.90–1.61	1.19–1.94	0.25–1.52		0.592**	0.733**	0.711*	0.604**	0.618**	0.729	0.748
11	Gouritz	2.57–3.85	2.41–3.49	0.89–1.77	0.79–2.09	0.79–1.98	1.08–1.75	1.08–2.22	0.98–2.32	0.80–2.13	1.10–2.10		0.565**	0.541*	0.577**	0.570**	0.098	0.017
12	Klein Brak	3.23–3.80	2.79–3.45	1.00–1.30	0.99–1.50	1.09–1.50	1.19–1.29	1.19–1.72	0.17–0.89	0.90–1.63	1.20–1.61	0.98–1.96		0.272	0.793	0.908**	0.964	0.967
13	Groot Brak	3.38–4.00	2.79–3.64	1.20–1.43	1.19–1.63	1.29–1.63	1.40–1.41	1.40–1.85	0.17–0.99	1.10–1.76	1.41–1.74	1.18–2.10	0.17–0.25		0.708	0.878*	0.794	0.809
14	Maalgate	2.73–3.84	2.09–3.50	0.61–1.32	0.61–1.85	0.71–1.63	0.81–1.52	0.80–1.97	1.10–1.97	0.53–1.88	0.80–1.63	1.00–2.49	1.10–1.97	1.31–2.11		0.589*	0.672	0.692
15	Gwaing	2.44–3.14	2.06–2.73	0.61–1.01	0.61–1.31	0.70–1.20	0.80–1.00	0.80–1.42	1.09–1.74	0.52–1.33	0.79–1.10	0.98–1.66	1.09–1.41	1.29–1.54	0.34–1.01		0.904	0.912
16	Noetsie	3.13–3.84	2.95–3.39	1.31–1.53	1.30–1.84	1.40–1.73	1.51	1.51–1.96	1.40–1.84	1.21–1.87	1.53–1.84	0.52–2.21	1.40–1.51	1.62–1.64	1.42–2.22	1.30–1.42		1.000
17	Bitou	3.00–3.70	3.08–3.53	1.42–1.64	1.40–1.95	1.51–1.84	1.62	1.62–2.07	1.51–1.95	1.31–1.98	1.64–1.95	0.43–2.33	1.51–1.62	1.73–1.75	1.53–2.34	1.40–1.53	0.25	

Significant Φ_{ST} values are indicated with asterisks.
*$P < 0.05$.
**$P < 0.005$.

Table 2 Results of the analysis of molecular variance (AMOVA) tests evaluating biogeographical hypotheses to explain the partitioning of genetic variation in *Galaxias* sp. 'nebula' from 17 river systems in the Cape Floristic Region of South Africa, based on combined COI and cyt *b* sequences.

Level of partitioning (or hypothesis)	% Variance	Phi-value	*P*-value
Seventeen contemporary river systems			
Among river systems	74.5	$\Phi_{CT} = 0.75$	< 0.001
Among localities within river systems	14.2	$\Phi_{SC} = 0.56$	< 0.001
Within all localities	11.3	$\Phi_{ST} = 0.89$	< 0.001
Fifteen southward-draining river systems			
Among river systems	57.9	$\Phi_{CT} = 0.58$	< 0.001
Among localities within river systems	24.5	$\Phi_{SC} = 0.58$	< 0.001
Within all localities	17.6	$\Phi_{ST} = 0.82$	< 0.001
Nine palaeoriver systems			
Among palaeorivers	55.0	$\Phi_{CT} = 0.55$	< 0.001
Among drainages within palaeorivers	34.1	$\Phi_{SC} = 0.76$	< 0.001
Within drainages	10.9	$\Phi_{ST} = 0.89$	< 0.001
Seven southern palaeorivers			
Among palaeorivers	23.0	$\Phi_{CT} = 0.23$	0.053
Among drainages within palaeorivers	59.0	$\Phi_{SC} = 0.77$	< 0.001
Within drainages	18.0	$\Phi_{ST} = 0.82$	< 0.001

The process inferred for clade 5-1, which was composed of haplotypes from all the southward-draining river systems (Fig. 3), was contiguous range expansion. Contiguous range expansion was also inferred for clade 4-2, which was composed of haplotypes from the Haelkraal, Ratel, Heuningnes, Duiwenhoks and Goukou river systems, and clade 3-6, which was composed of haplotypes from the Uilkraals, Haelkraal, Ratel and Heuningnes river systems. Migration type processes coupled with isolation by distance were inferred for clades 4-1, 3-13, 2-28 and 1-54 (Fig. 3, Table S3). The neutrality test suggested that historical demographic expansion occurred both within the southern clade ($F_S = -24.16$, $P = 0.001$) and across the lineage's distribution ($F_S = -23.87$, $P = 0.002$).

DISCUSSION

The phylogeographical pattern identified in *Galaxias* sp. 'nebula' is partly consistent with expectations of the River Hierarchy hypothesis. The high Φ_{ST} values suggest a high degree of structure among populations in different river systems, with further support from NCPA inferences that suggest restricted gene flow across the lineage's range, as well as the results of the AMOVA, which indicate that partitioning of the samples by river system explains close to 60% of the variation. However, inter-basin divergence times that postdate the Pliocene and the occurrence of haplotypes 21 and 32 in isolated river systems suggest recent connectivity between populations in different river systems. Daniels *et al.* (2006) also found haplotypes that were shared among currently isolated river systems on the south coast of the CFR in the Cape river crab. The occurrence of closely related freshwater fishes in currently isolated river systems across the CFR has traditionally been attributed to drainage rearrangements or river capture events (Barnard, 1943; Skelton, 1986). More recently, Swartz *et al.* (2007) considered river capture to be the most plausible explanation for the close relationships between *Pseudobarbus phlegethon* restricted to the Olifants River system and a lineage of *Pseudobarbus afer* on the south coast. However, river capture alone is unlikely to adequately explain the recent divergence between populations of *Galaxias* sp. 'nebula' across the CFR. River captures would have had to occur relatively recently, across a wide area and at a high frequency. This is unlikely because drainage rearrangements are rare in geologically stable landscapes (Bishop, 1995). The CFR landscape has been stable for a long period of time, with the last major tectonic activities occurring during the Pliocene (Partridge & Maud, 1987, 2000; Cowling *et al.*, 2009), thereby making it unlikely that the region experienced frequent river captures in recent times.

It is clear that ancient mountain ranges and drainage divides have not formed an effective barrier to prevent dispersal in *Galaxias* sp. 'nebula'. This finding is rather unusual for an obligate freshwater fish, but is not unique to this region. Šlechtová *et al.* (2004) found close relationships between representatives of *Cottus ferrugineus* across the European Alps, suggesting connectivity of populations on either side of this prominent mountain range from the glacial cycles to more recent times. There is also evidence of closer genetic relationships between a number of species pairs of freshwater taxa inhabiting both sides of the Great Dividing Range in Australia (Jerry, 2008; Faulks *et al.*, 2010; Unmack & Dowling, 2010). Burridge *et al.* (2008a) also provide evidence of recent dispersal of *Gobiomorphus breviceps* across the Southern Alps in New Zealand through intermittent freshwater connections.

The confluence of river systems during periods of lowered sea levels can be an important mechanism of facilitating range expansion of freshwater-restricted taxa (Bermingham & Martin, 1998; Swartz *et al.*, 2007, 2009). The phylogeographical pattern observed in *Galaxias* sp. 'nebula' is, however, not fully consistent with expectations of the Palaeoriver hypothesis. This taxon has clearly not been contained by the proposed palaeoriver systems. Both NCPA and F_S results suggest that range expansion occurred across river systems that did not coalesce during the LGM. Recent divergence times were also found between rivers that belonged to different palaeoriver systems, which can only be explained by alternative and relatively recent dispersal mechanisms. While these results certainly do not exclude the role of sea-level regression in aiding migration between currently isolated river systems, they provide evidence that this mechanism alone is not sufficient to explain the observed genetic patterns in *Galaxias* sp. 'nebula'.

The close relationship of *Galaxias* sp. 'nebula' populations across its distribution is likely to be a result of inter-drainage range expansion following episodic drainage connections, possibly during periods of heavy flooding in the Pleistocene and Holocene epochs. There are several low-lying mountain passes that could act as dispersal corridors when they become inundated (drainage over-topping) during heavy flooding events. *Galaxias* sp. 'nebula' is the only freshwater fish lineage that appears to have recently utilized such connections to move across drainages. Many of the newly identified lineages of freshwater fishes in the CFR are narrow endemics (Linder *et al.*, 2010). The ability of *Galaxias* sp. 'nebula' to exploit dispersal corridors across drainage divides can be attributed to a number of factors. This lineage penetrates to higher elevations in mountain tributaries of the CFR than other native fishes (Chakona & Swartz, 2012). Opportunities for temporary inter-basin connections are more likely at higher elevations than in lower parts of the basin. Experimental evidence shows that *Galaxias* sp. 'nebula' can tolerate aerial exposure for prolonged periods of time (Chakona *et al.*, 2011) and, based on field observations, it can maintain viable populations in very shallow habitats. Such intrinsic ecological attributes can facilitate movement across low drainage divides during wet and humid conditions, for example during the Holocene Altithermal (*c.* 6000–8000 years ago) (Partridge *et al.*, 1999). The close relationship between southern populations of *Galaxias* sp. 'nebula' is not surprising, because the south coast of the CFR experienced relatively recent wetter conditions while the western parts have been drier for a much longer period (Partridge *et al.*, 1999). As the Berg and Olifants are unlikely to have coalesced during periods of low sea levels because of the large distance between their river mouths, any connections are likely to have occurred via their headwaters. The inferred earlier fragmentation of *Galaxias* sp. 'nebula' populations between the Olifants, Berg and southward-flowing river systems can therefore be attributed to loss of connectivity among river systems due to higher aridity on the west coast than the south coast.

Galaxias sp. 'nebula' shows more recent and extensive dispersal than any other freshwater fish species in the CFR. River capture, confluence of rivers during lower sea levels and isolation in contemporary river systems must have had an influence, but individually they do not adequately explain the phylogeographical patterns of *Galaxias* sp. 'nebula'. Inter-basin dispersal via intermittent freshwater connections (through either over-topping of low drainage divides or low-lying mountain passes and headwater connections) during Pleistocene and Holocene wetter periods, enabled by the intrinsic ecological features of *Galaxias* sp. 'nebula', is the most plausible explanation for the extensive distribution and genetic pattern in *Galaxias* sp. 'nebula'. The biogeographical significance of this mechanism in explaining present-day distribution patterns of freshwater taxa has clearly been underestimated.

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REFERENCES

Barnard, K.H. (1943) Revision of the indigenous freshwater fishes of the S.W. Cape Region. *Annals of the South African Museum*, **36**, 101–263.

Bermingham, E. & Martin, A.P. (1998) Comparative mtDNA phylogeography of neotropical freshwater fishes: testing shared history to infer the evolutionary landscape of lower Central America. *Molecular Ecology*, **7**, 499–517.

Bishop, P. (1995) Drainage rearrangement by river capture, beheading and diversion. *Progress in Physical Geography*, **19**, 449–473.

Brito, P.H. (2005) The influence of Pleistocene glacial refugia on tawny owl genetic diversity and phylogeography in western Europe. *Molecular Ecology*, **14**, 3077–3094.

Burnham, K.P. & Anderson, D.R. (2002) *Model selection and multi-model inferences: a practical information-theoretic approach*, 2nd edn. Springer, New York.

Burridge, C.P., Craw, C. & Waters, J.M. (2006) River capture, range expansion, and cladogenesis: the genetic signature of freshwater vicariance. *Evolution*, **60**, 1038–1049.

Burridge, C.P., Craw, C. & Waters, J.M. (2007) An empirical test of freshwater vicariance via river capture. *Molecular Ecology*, **16**, 1883–1895.

Burridge, C.P., Craw, C., Jack, D.C., King, T.M. & Waters, J.M. (2008a) Does fish ecology predict dispersal across a river drainage divide? *Evolution*, **62**, 1484–1499.

Burridge, C.P., Craw, D., Fletcher, D. & Waters, J.M. (2008b) Geological dates and molecular rates: fish DNA sheds light on time dependency. *Molecular Biology and Evolution*, **25**, 624–633.

Chakona, A. & Swartz, E.R. (2012) (2012) Contrasting habitat associations of imperilled endemic stream fishes from a global biodiversity hot spot. *BMC Ecology*, **12**, 19.

Chakona, A., Swartz, E. & Magellan, K. (2011) Aerial exposure tolerance of a newly discovered galaxiid fish. *Journal of Fish Biology*, **78**, 912–922.

Clement, M., Posada, D. & Crandall, K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Cowling, R.M., Procheş, Ş. & Partridge, T.C. (2009) Explaining the uniqueness of the Cape Flora: incorporating geomorphic evolution as a factor for explaining its diversification. *Molecular Phylogenetics and Evolution*, **51**, 64–74.

Craw, D., Burridge, C., Norris, R. & Waters, J. (2008) Genetic ages for Quaternary topographic evolution: a new dating tool. *Geology*, **36**, 19–22.

Daniels, S.R., Gouws, G. & Crandall, K.A. (2006) Phylogeographic patterning in a freshwater crab species (Decapoda: Potamonautidae: *Potamonautes*) reveals the signature of historical climatic oscillations. *Journal of Biogeography*, **33**, 1538–1549.

Deacon, H.J., Jury, M.R. & Ellis, F. (1992) Selective regime and time. *The ecology of fynbos: nutrients, fire and diversity* (ed. by R.M. Cowling), pp. 6–22. Oxford University Press, Cape Town.

Dingle, R.V., Siesser, W.G. & Newton, A.R. (1983) *Mesozoic and Tertiary geology of southern Africa*. Balkema, Rotterdam.

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.

Faulks, L.K., Gilligan, D.M. & Beheregaray, L.B. (2010) Evolution and maintenance of divergent lineages in an endangered freshwater fish, *Macquaria australasica*. *Conservation Genetics*, **11**, 921–934.

Fu, Y-X. (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking, and background selection. *Genetics*, **147**, 915–925.

Hendey, Q.B. (1983) Cenozoic geology and palaeogeography of the fynbos region. *Fynbos palaeoecology: a preliminary synthesis* (ed. by H.J. Deacon, Q.B. Hendey and J.J.N. Lambrechts), pp. 35–60. South African National Scientific Programmes Report 75. Council for Scientific and Industrial Research, Pretoria.

Huey, J.A., Baker, A.M. & Hughes, J.M. (2010) High levels of genetic structure in the Australian freshwater fish, *Ambassis macleayi*. *Journal of the North American Benthological Society*, **29**, 1148–1160.

Hughes, J.M., Schmidt, D.J. & Finn, D.S. (2009) Genes in streams: a molecular approach to understanding movement of freshwater fauna and their riverine habitat. *BioScience*, **59**, 573–583.

Hughes, J.M., Real, K.M., Marshall, J.C. & Schmidt, D.T. (2012) Extreme genetic structure in a small-bodied freshwater fish, the purple spotted gudgeon, *Mogurnda adspersa* (Eliotridae). *PLoS ONE*, **7**, e40546.

Hurwood, D.A. & Hughes, J.M. (2001) Nested clade analysis of the freshwater shrimp, *Caridina zebra* (Decapoda: Atyidae), from north-eastern Australia. *Molecular Ecology*, **10**, 113–125.

Ivanova, N., Zemlak, T.S., Hanner, R.H. & Hebert, P.D.N. (2007) Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*, **6**, 998–1002.

Jerry, D.R. (2008) Phylogeography of the freshwater catfish *Tandanus tandanus* (Plotosidae): a model species to understand evolution of the eastern Australian freshwater fish fauna. *Marine and Freshwater Research*, **59**, 351–360.

Librado, P. & Rozas, J. (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.

Linder, H.P., Johnson, S.D., Kuhlmann, M., Matthee, C.A., Nyffeler, R. & Swartz, E.R. (2010) Biotic diversity in the Southern African winter-rainfall region. *Current Opinion in Environmental Sustainability*, **2**, 109–116.

Marlow, J.R., Lange, C.B., Wefer, G. & Rosell-Melé, A. (2000) Upwelling intensification as part of the Pliocene–Pleistocene climate transition. *Science*, **290**, 2288–2291.

Meffe, G.K. & Vrijenhoek, R.C. (1988) Conservation genetics in the management of desert fishes. *Conservation Biology*, **2**, 157–169.

Miller, K.G., Kominz, M.A., Browning, J.V., Wright, J.D., Mountain, G.S., Katz, M.E., Sugarman, P.J., Cramer, B.S., Christie-Blick, N. & Pekar, S.F. (2005) The Phanerozoic record of global sea-level change. *Science*, **310**, 1293–1298.

Nielsen, R. & Wakeley, J. (2001) Distinguishing migration from isolation: a Markov chain Monte Carlo approach. *Genetics*, **158**, 885–896.

Partridge, T.C. & Maud, R.R. (1987) Geomorphic evolution of southern Africa since the Mesozoic. *South African Journal of Geology*, **90**, 179–208.

Partridge, T.C. & Maud, R.R. (2000) Macro-scale geomorphic evolution of southern Africa. *The Cenozoic of southern Africa* (ed. by T.C. Partridge and R.R. Maud), pp. 3–18. Oxford University Press, New York.

Partridge, T.C., Scott, L. & Hamilton, J.E. (1999) Synthetic reconstructions of southern African environments during the Last Glacial Maximum (21–18 kyr) and the Holocene Altithermal (8–6 kyr). *Quaternary International*, **57/58**, 207–214.

Petit, R.J. (2008) The coup de grâce for the nested clade phylogeographic analysis? *Molecular Ecology*, **17**, 516–518.

Pfenninger, M. & Posada, D. (2002) Phylogeographic history of the land snail *Candidula unifasciata* (Helicellinae, Stylommatophora): fragmentation, corridor migration, and secondary contact. *Evolution*, **56**, 1776–1788.

Posada, D. & Crandall, K.A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Posada, D. & Crandall, K.A. (2001) Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution*, **16**, 37–45.

Posada, D. & Templeton, A.R. (2005) *GeoDis revised inference key*. Available at: http://darwin.uvigo.es/download/geodisKey_11Nov05.pdf (accessed 7 February 2007).

Posada, D., Crandall, K.A. & Templeton, A.R. (2000) GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Ramsay, P.J. & Cooper, J.A.G. (2002) Late Quaternary sea-level change in South Africa. *Quaternary Research*, **57**, 82–90.

Rogers, J. (1985) Geomorphology, offshore bathymetry and Quaternary lithostratigraphy around the Bot River estuary. *Transactions of the Royal Society of South Africa*, **45**, 211–227.

Siesser, W.G. & Dingle, R.V. (1981) Tertiary sea-level movements around southern Africa. *The Journal of Geology*, **89**, 83–96.

Skelton, P.H. (1986) Distribution patterns and biogeography of non-tropical southern African freshwater fishes. *Palaeoecology of Africa*, **17**, 211–230.

Šlechtová, V., Bohlen, J., Freyhof, J., Persat, H. & Delmastro, G.B. (2004) The Alps as barrier to dispersal in cold-adapted freshwater fishes? Phylogeographic history and taxonomic status of the bullhead in the Adriatic freshwater drainage. *Molecular Phylogenetics and Evolution*, **33**, 225–239.

Swartz, E.R., Skelton, P.H. & Bloomer, P. (2007) 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. *Journal of Biogeography*, **34**, 2086–2099.

Swartz, E.R., Skelton, P.H. & Bloomer, P. (2009) Phylogeny and biogeography of the genus *Pseudobarbus* (Cyprinidae): shedding light on the drainage history of rivers associated with the Cape Floristic Region. *Molecular Phylogenetics and Evolution*, **51**, 75–84.

Swofford, D.L. (2003) *PAUP**: phylogenetic analysis using parsimony (*and other methods)*. Version 4. Sinauer Associates Inc., Sunderland, MA.

Tamura, K. & Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512–526.

Tankard, A.J. (1976) Cenozoic sea-level changes: a discussion. *Annals of the South African Museum*, **71**, 1–17.

Templeton, A.R. (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381–397.

Templeton, A.R. (2008) Nested clade analysis: an extensively validated method for strong phylogeographic inference. *Molecular Ecology*, **17**, 1877–1880.

Templeton, A.R. (2009) Statistical hypothesis testing in intraspecific phylogeography: nested clade phylogeographical analysis *vs*. approximate Bayesian computation. *Molecular Ecology*, **18**, 319–331.

Templeton, A.R., Boerwinkle, E. & Sing, C.F. (1987) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics*, **117**, 343–351.

Templeton, A.R., Routman, E. & Phillips, C. (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767–782.

Thacker, C.E., Unmack, P.J., Matsui, L. & Rifenbark, N. (2007) Comparative phylogeography of five sympatric *Hypseleotris* species (Teleostei: Eleotridae) in south-eastern Australia reveals a complex pattern of drainage basin exchanges with little congruence across species. *Journal of Biogeography*, **34**, 1518–1533.

Tyson, P.D. & Partridge, T.C. (2000) Evolution of Cenozoic climates. *Cenozoic of southern Africa* (ed. by T.C. Partridge and R.M. Maud), pp. 371–387. Oxford University Press, New York.

Unmack, P.J. & Dowling, T.E. (2010) Biogeography of the genus *Craterocephalus* (Teleostei: Atherinidae) in Australia. *Molecular Phylogenetics and Evolution*, **55**, 968–984.

Waters, J.M. & Cambray, J.A. (1997) Intraspecific phylogeography of the Cape galaxias from South Africa: evidence from mitochondrial DNA sequences. *Journal of Fish Biology*, **50**, 1329–1338.

Waters, J.M. & Wallis, G.P. (2000) Across the Southern Alps by river capture? Freshwater fish phylogeography in South Island, New Zealand. *Molecular Ecology*, **9**, 1577–1582.

Waters, J.M. & Wallis, G.P. (2001) Cladogenesis and loss of the marine life-history phase in freshwater galaxiid fishes (Osmeriformes: Galaxiidae). *Evolution*, **55**, 587–597.

Wishart, M.J. & Day, J.A. (2002) Endemism in the freshwater fauna of the south-western Cape, South Africa. *Verhandlungen der Internationalen Vereinigung für theoretische und angewandte Limnologie*, **28**, 1762–1766.

Wishart, M.J., Hughes, J., Stewart, B. & Impson, D. (2006) Extreme levels of intra-specific divergence among Cape Peninsula populations of the Cape galaxias, *Galaxias zebratus* Castelnau 1861, reveals a possible species complex. *African Journal of Aquatic Science*, **31**, 99–106.

Zachos, J., Pagani, M., Sloan, L., Thomas, E. & Billups, K. (2001) Trends, rhythms, aberrations in global climate 65 Ma to present. *Science*, **292**, 686–693.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Sample and locality information for sampled individuals of *Galaxias* sp. 'nebula' from the Cape Floristic Region of South Africa.

Appendix S2 Additional results from analyses based on mitochondrial cytochrome *c* oxidase subunit I and cytochrome *b* sequences of *Galaxias* sp. 'nebula' from the Cape Floristic Region of South Africa (Tables S1–S3).

BIOSKETCH

This paper formed part of Albert Chakona's PhD research on the ecology and biogeography of freshwater fishes in the CFR supervised by Ernst Swartz, Gavin Gouws and Paulette Bloomer. A.C.'s research interests are in the biogeography, ecology and conservation of freshwater taxa.

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