

Contrasting signals of genetic diversity and historical demography between two recently diverged marine and estuarine fish species

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ABSTRACT: Estuaries, at the confluence of marine and freshwater systems, are mostly of geologically recent origin and as such make excellent models for understanding recent speciation events. Using molecular approaches, we compared genetic diversity and demographic histories in 2 closely related southern African klipfish species, the marine *Clinus superciliosus* and the estuarine *C. spatulatus*. Strong genetic differentiation was identified using both mtDNA control region and nDNA *S7* sequencing, despite some haplotype sharing. Coalescent-based modelling suggests that species divergence occurred during the Late Pleistocene or, more likely, during the Early Holocene, when present-day estuaries formed. Analyses of population demography suggest that *C. superciliosus* has undergone historical population expansion, whereas *C. spatulatus* is characterized by a population decline, potentially driven by repeated cycles of population crashes linked to the opening and closing of estuarine systems. This is also reflected in values of genetic diversity, which are almost an order of magnitude lower in the estuarine than in the marine species. Given the unique evolutionary history of *C. spatulatus*, a species that is restricted to only 2 South African estuaries, we highlight the need for a better understanding of the processes that have shaped the evolution of estuarine populations. The identification of unique genetic lineages in estuaries can help to better guide conservation and management efforts for some of South Africa's most fragile habitats.

KEY WORDS: Connectivity · Estuarine systems · Anthropogenic impacts · Evolution · Adaptive divergence · Biodiversity · Conservation planning

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INTRODUCTION

Speciation in the sea is complex, and unravelling the mechanisms behind population divergence can give fascinating insights into the evolution of marine biodiversity. The scarcity of absolute barriers in the oceans, coupled with biological traits such as large effective population sizes, the potential to disperse

over vast distances and the extensive geographical ranges that many marine species inhabit, suggest that opportunities for speciation might be reduced. However, numerous studies have shown that even in taxa with high dispersal capacity, speciation rates can be high (Palumbi 1994) and that allopatric divergence is not necessarily the most common mode of speciation (Bernardi 2013, Bowen et al. 2013). Estuar-

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ies, the confluence of marine and freshwater systems, represent an interesting case. Here, speciation could be promoted in a variety of ways, either through allopatric events caused by the formation of estuaries, or through sympatric or parapatric models where differences in ecological variables, such as salinity, temperature or turbidity, drive rapid adaptation leading to ecological, physiological and behavioural differentiation that may result in assortative mating and, eventually, speciation. However, fish diversity in estuaries is often low, probably because estuaries are usually geologically short-lived and because few species are able to tolerate the wide fluctuations of abiotic conditions that can occur over short temporal scales (Whitfield 1994). Therefore, the processes that drive population and species divergence in estuaries are poorly understood.

Localities in which marine and estuarine taxa occur in sympatry provide a good model for investigating the evolutionary processes driving population demography and speciation in such disparate environments. Fish species with internal fertilization provide additional complexity; unlike broadcast-spawning species whose eggs and sperm provide more opportunities for hybridization and gene flow between populations and genetic lineages, internal fertilization should be more likely to drive reproductive isolation. Southern African fishes of the family Clinidae are unique, not only in that they are live-bearing, but also that all species examined to date show super-embryonation, where the female broods larvae at different developmental stages, before releasing them as fully formed post-flexion larvae (Veith 1979, Prochazka 1994, Moser 2007). As the planktonic larval duration is relatively short, dispersal over greater distances is unlikely; further, the adults are relatively sedentary and often show low levels of gene flow, even between populations in close geographical proximity (von der Heyden et al. 2008, 2011, 2013).

The majority of clinids in southern Africa are found along rocky coastlines, with up to 98% of the intertidal community dominated by clinid fishes (Prochazka & Griffiths 1992). Of these, the super klipfish, *Clinus superciliosus*, is among the largest and most

abundant intertidal fishes on the coast, with an extensive geographical distribution that ranges from southern Namibia to the South African east coast. By contrast, the Bot River klipfish, *C. spatulatus*, is the only estuarine resident clinid known in southern Africa, with an extremely restricted geographic range that is limited to 2 estuaries on the southwestern Cape coast, the Klein and Bot-Kleinmond systems (Fig. 1). Unlike many other fishes that are estuarine-associated, *C. spatulatus* is truly restricted to estuaries and may possibly be the only live-bearing obligate estuarine resident species in the world. Given its rarity, it is currently classified as Endangered on the IUCN Red List of Threatened Species (www.iucnredlist.org/details/4982/0). The 2 species are rarely, if ever, caught together, suggesting that they occupy different habitats within the estuaries.

C. superciliosus and *C. spatulatus* belong to a complex of 6 closely related species (Holleman et al. 2012). On the basis of combined DNA sequence data from 3 mitochondrial DNA (mtDNA 16S rRNA, 12S rRNA and control region) markers and 1 nuclear (rhodopsin) marker, each of these species

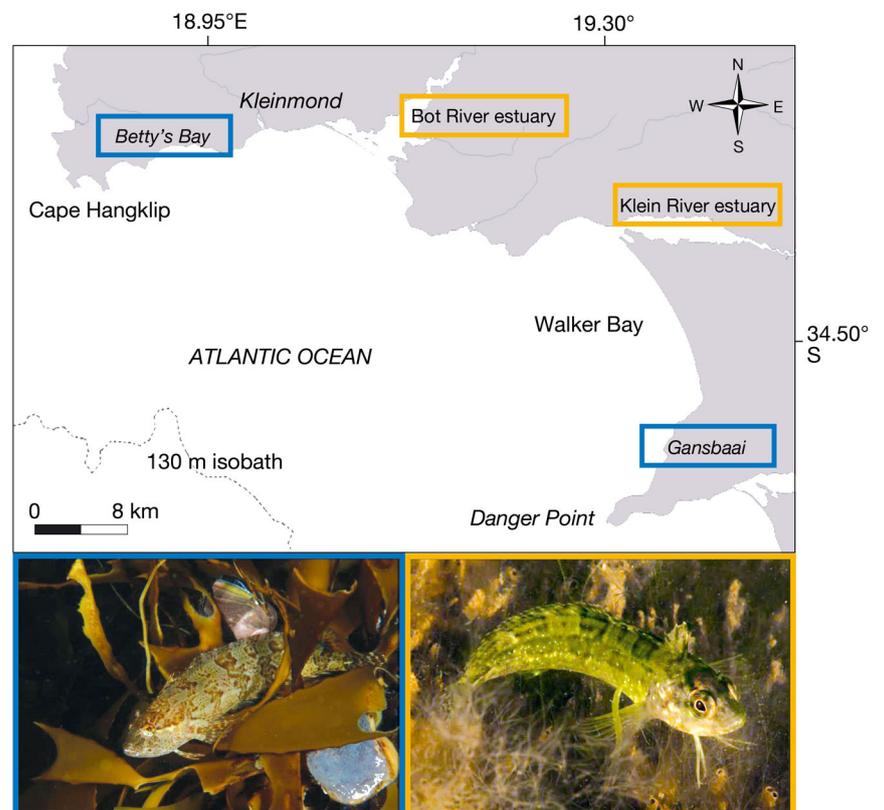


Fig. 1. Sampling area for *Clinus spatulatus* (yellow) and *C. superciliosus* (blue). Photos detailing morphological differentiation of the 2 species are also shown (see also Holleman et al. 2012)

forms a reciprocally monophyletic clade, an exception being that some marine *C. superciliosus* group with the estuarine *C. spatulatus*. Analyses of only the mtDNA control region recovered completely distinct clades, whereas the 2 species' mtDNA 16S and 12S sequences are identical (Holleman et al. 2012). This suggests not only incomplete lineage sorting for the more slowly evolving markers, but also that the split between marine and estuarine *Clinus* species was probably quite recent, potentially resulting from sea-level changes associated with glaciations, especially the Last Glacial Maximum (LGM), approximately 28 000 to 19 000 yr ago (Toms et al. 2014). In South Africa, post-LGM changes in sea levels resulted in the formation of new drainage channels and estuaries, with the current topography of the coastline being approximately 6000 yr old (Compton 2001). Elsewhere, events around the LGM caused populations to expand and contract (Hewitt 2000), contributing to, for example, the diversification of marine and estuarine populations of fishes (see Beheregaray & Sunnucks 2001). How changes in the topography and composition of the coastline affected coastal and estuarine species in southern Africa is not well understood, but may have involved vicariant events (Toms et al. 2014), and the close sister relationship between *C. superciliosus* and *C. spatulatus* provides a suitable model system to shed light on speciation processes potentially linked with the LGM.

Using a phylogeographical approach with both mitochondrial and nuclear DNA markers, we tested the hypothesis that *C. spatulatus* underwent a recent speciation event coupled to the LGM, as the latter had a significant impact on numerous other marine organisms in the region (von der Heyden et al. 2010, Teske et al. 2011, Toms et al. 2014). To do this, we used a coalescent-based method to estimate divergence time between the 2 lineages. We also tested whether genetic diversity values for estuarine fishes are lower than for the marine species, as has been shown in other studies (Ward et al. 1994), and characterized the population genetic structure between these 2 closely related species. We also used this opportunity to examine patterns of demographic change and link these to census population size changes related to the opening and closing of estuarine systems. Given that *C. spatulatus* is restricted to only 2 estuaries in southwestern Africa, we use our results to highlight this unique genetic fish lineage and discuss some of the biodiversity management and conservation implications for South African estuaries.

MATERIALS AND METHODS

Study species and ecology

Clinus spatulatus and *C. superciliosus* have distinct morphological differences and are easily distinguishable in the field (Holleman et al. 2012: Fig. 1). Ecological differences in estuaries are more pronounced between the 2 species. *C. superciliosus* is found in the lower reaches of estuaries in and amongst rocky habitats, seaweed and seagrass beds with salinities above 5 ppt, suggesting that low salinities may exclude this species from the higher reaches of estuaries (Lamberth et al. 2008). Depending on freshwater inflow and evaporation, salinities in South African estuaries may exceed or drop far below those of seawater (Whitfield et al. 1980). Mass mortality events of all species in the Bot River estuary were only survived by *C. spatulatus* (Bennett 1985), suggesting that this species is euryhaline, i.e. a species that is well adapted to tolerate the considerable fluctuations of environmental variables in estuaries, and whose distribution within these systems is largely independent of salinity (Teske & Wooldridge 2003).

Collections and DNA extractions

To test genetic differentiation and demographic changes between marine and estuarine clinid species, 55 and 65 estuarine *C. spatulatus* were collected from 2 estuaries that comprise their known geographic range, the Klein and Bot-Kleinmond estuaries, respectively, which are located on the southwestern coast of South Africa (Fig. 1). Marine *C. superciliosus* were sampled from localities close to these estuaries; 46 and 45 fishes were collected from Betty's Bay and Gansbaai, respectively (Fig. 1, Table 1). DNA was extracted from muscle or fin tissue with the NucleoSpin Tissue kit (Machery Nagel) following the manufacturer's instructions.

Further, as the ichthyofauna of the Bot and Klein estuaries have been sampled at least twice annually since 2000 as part of a long-term monitoring programme of priority South African estuaries, we used this opportunity to monitor population size changes according to physico-chemical variables over an extended period in the Bot system. The estimated number of *C. spatulatus* was plotted against mean water level and is indicative of an open or closed system. See the Supplement at www.int-res.com/articles/suppl/m526p157_supp.pdf for further explanation on the methodology, as well as the data set.

Table 1. Sampling localities and genetic diversity indices for *Clinus spatulatus* and *C. superciliosus*; n = number of individuals sampled, Nh = number of haplotypes per locality (numbers in brackets denote haplotypes that were unique to a particular site), h = haplotype diversity, π = nucleotide diversity

Sampling localities	— Control region —				— S7 second intron —			
	n	Nh	h	π	n	Nh	h	π
<i>C. spatulatus</i>								
Klein River Estuary	55	5 (1)	0.77	0.0029	55	3 (1)	0.088	0.00013
Bot River Estuary	65	6 (2)	0.85	0.0021	64	1	1	0.00000
<i>C. superciliosus</i>								
Betty's Bay	47	18 (8)	0.90	0.0096	23	17 (9)	0.889	0.0035
Gansbaai	46	18 (12)	0.85	0.0100	29	12 (4)	0.838	0.0032
Total	213				171			

PCR amplification and sequencing

The mtDNA control region and the nuclear second intron of the S7 ribosomal protein gene were targeted. Additionally, 10 fish from each locality were sequenced for the mtDNA 16S rRNA and NADH subunit 2 gene (ND2), as well as the nuclear rhodopsin fragment. PCR cycles for control region, ND2, 16S rRNA and S7 followed protocols as given by Holleman et al. (2012) and von der Heyden et al. (2008); rhodopsin was amplified as per Levy et al. (2013). Sequencing was carried out on an ABI 3100 automated sequencer (ABI Biosystems) at the Central Analytical Facility at Stellenbosch University (<http://academic.sun.ac.za/saf/>). ND2 fragments were sequenced in both directions; for all other genes, 20% of samples were reverse sequenced to check for *Taq* or sequencing errors. All sequences generated in this study were submitted to GenBank (*C. spatulatus*: accession numbers KP222917–KP223036; *C. superciliosus*: accession numbers KP223037–KP223129).

Genetic diversity and phylogeographic analyses

Sequences were edited and aligned in BioEdit v7.0.5.3 (Hall 1999). S7 and rhodopsin alleles were identified using a 95% probability criterion using Phase2.1 as implemented in DNAsp v5.10.01 (Librado & Rozas 2009). Modeltest v3.7 (Posada & Crandall 2001) was used to estimate the best model of sequence evolution under Akaike's information criterion, which was then incorporated into subsequent analyses. For each species and sampling locality, haplotype (h) and nucleotide (π) diversity for both mtDNA and nDNA data sets were estimated in Arlequin v3.5 (Excoffier & Lischer 2010). To investigate population genetic structure, analyses of molecular variance

(AMOVA) and pairwise Φ_{ST} were calculated in Arlequin for both the control region and S7 data sets. Two separate analyses were carried out; first, both species were analysed in one data set. For the second analysis, the species were treated separately. Significance was assessed using a non-parametric permutation approach with 10 000 iterations. Further, for all data sets, statistical parsimony haplotype networks were constructed using the program TCS v1.21 (Clement et al. 2000).

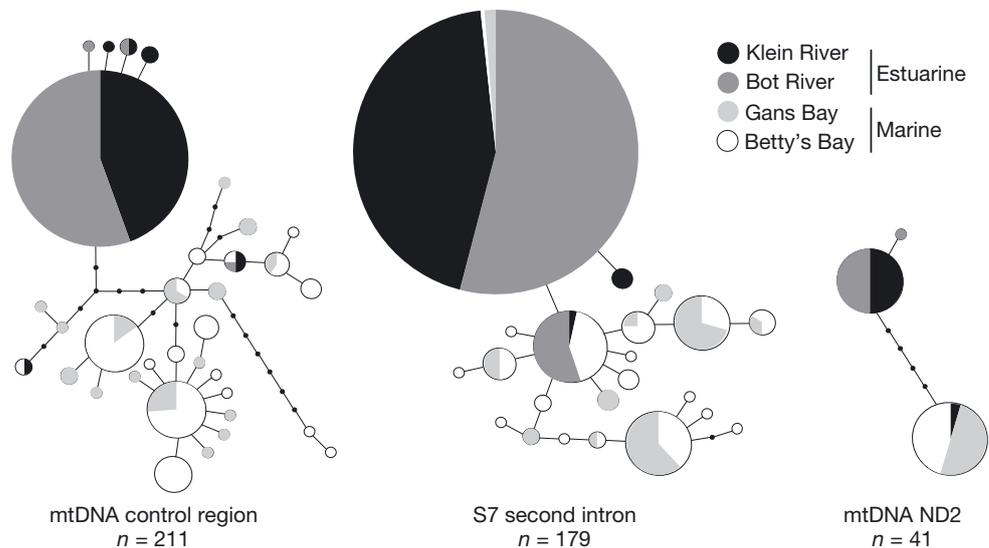
In addition, 10 individuals from each population were sequenced for an additional 3 markers, the mtDNA ND2 and 16S rRNA markers and the nDNA rhodopsin. These datasets were included to provide additional information on the level of divergence between marine and estuarine populations.

Estimating demographic change and divergence time

Comparisons in evolutionary rates of both mtDNA and nuclear markers suggest that the faster mutation rate of mtDNA compared to nuclear sequences makes it more sensitive for detecting recent divergence or demographic changes (see for example Moritz et al. 1987, Bowen et al. 2014). While pitfalls of estimating demographic change in the absence of calibrated mutation rates remain, these are reasonably well understood (Grant et al. 2012, Karl et al. 2012). In the absence of a calibrated tree for clinid fishes, we used the highly conserved teleost control region rate of 3.6% per million years of Donaldson & Wilson (1999) for analyses of demographic change and divergence time. This allows us to estimate not only relative rates of change between *C. spatulatus* and *C. superciliosus*, but, as recent species divergences can be characterized by elevated rates of mutation (Grant et al. 2012), also to estimate the oldest likely date of divergence. In order to estimate demographic change, mismatch distributions and Fu's F_s were calculated in Arlequin.

The growth parameter, g , was calculated for estuarine and marine populations in Lamarc v2.1.8 (Kuhner 2006) under a likelihood scenario using the HKY model options. An initial 10 chains were run and 10 000 trees sampled; the first 1000 trees were discarded as burn-in. The program IMA2 (Hey 2010), which is a coalescent-sampler that implements the

Fig. 2. Haplotype networks for 3 gene regions that show population genetic differentiation between *Clinus spatulatus* and *C. superciliosus*. The size of the circles is proportional to the frequency of each haplotype; the smallest circles represent extinct or unsampled haplotypes. Each line represents one mutational step



isolation-with-migration model of Nielsen & Wakeley (2001), was used to estimate the divergence time between *C. superciliosus* and *C. spatulatus*. Several test runs showed that running this program with the complete data set was problematic, as effective sample size (ESS) values remained below 200. Given that the accuracy of divergence time estimates depends more on the number of loci and less on the number of samples (Felsenstein 2006), we randomly selected 100 individuals (50 from each species) from the control region data set, and 50 individuals (25 from each species, each represented by 2 sequences for a total of 100 sequences) from the S7 data set. We then included some additional sequences that had not been randomly selected from each species on the basis of being closely related or identical to sequences from the other species (control region data: 4 sequences of *C. superciliosus* and 3 sequences of *C. spatulatus*; S7: 2 sequences of *C. superciliosus* that were identical to those of one of the common *C. spatulatus* haplotypes). This approach ensured that divergence time was not overestimated as an artefact of sub-sampling the original data set. Three replicate analyses that differed only in terms of starting seeds were run using the following settings: -b (burn-in, i.e. the number of initial trees discarded) 100 000; -l (number of trees sampled every 100 generations following burn-in) 10 000; -hn (number of chains) 200; -hfg (geometric increment model); -ha 0.96; -hb 0.9 (terms of the geometric increment model). To convert time in coalescent units to time in years, the formula BX/U was used (where B is the estimate of divergence time in coalescent units, X is the geometric mean of the estimates of the mutation rate scalars for the loci for

which mutation rates are available, and U is the geometric mean of the per year mutation rates for those loci). We only specified the 3.6% per million years mutation rate for the control region and a generation time of 1 yr.

RESULTS

Diversity indices and population genetic structuring

For the 2 largest data sets (mtDNA control region: 213 individuals; nuclear S7 intron: 171 individuals), genetic diversity values were considerably lower for the estuarine *Clinus spatulatus* than for the marine *C. superciliosus* (Table 1), which is also reflected in the total number of haplotypes recovered for each species. For *C. spatulatus*, 7 control region haplotypes were recovered compared to 29 for its marine sister species. Only 3 nuclear intron haplotypes were found for the estuarine fishes, out of a total of 22 haplotypes (Table 1, Fig. 2). For each marker, 1 and 2 haplotypes, respectively, were shared between estuarine and marine fishes, although most haplotypes were confined to either *C. spatulatus* or *C. superciliosus* (Fig. 2). The number of hetero- and homozygous fishes for the S7 intron also differed significantly between estuarine and marine fishes; only 1 out of 119 individuals sampled from the estuaries was heterozygous, compared to 24 out of 52 marine fishes.

AMOVA showed strongest genetic differentiation between marine and estuarine fishes (mtDNA con-

Table 2. Pairwise Φ_{ST} values between sampling localities for the mtDNA control region (below dashed line) and S7 nuclear intron (above dashed line). *Significance at $p < 0.05$. Klein: Klein River Estuary, Bot: Bot River Estuary

Habitat	Sampling locality	Klein	Bot	Betty's Bay	Gansbaai
Estuarine	Klein		0.02*	0.65*	0.63*
Estuarine	Bot	-0.01		0.70*	0.67*
Marine	Betty's Bay	0.78*	0.81*		0.00
Marine	Gansbaai	0.74*	0.76*	0.13*	

control region $\Phi_{ST} = 0.7$, $p < 0.001$; S7 intron $\Phi_{ST} = 0.54$, $p < 0.001$); pairwise Φ_{ST} values support differentiation between marine and estuarine fishes (Table 2) for both marker types. Marine sites showed shallow but significant population differences at the mtDNA control region, whereas estuarine fishes showed no significant differences for the mtDNA control region, but had slight but significant genetic structure for the S7 intron (Table 2). As expected from the Φ_{ST} values, haplotype networks for both markers show a clear separation between estuarine and marine fishes, with 4 and 1 nucleotide substitutions separating *C. spatulatus* and *C. superciliosus* for mtDNA and nuclear markers, respectively (Fig. 2).

For the additional, smaller data sets (mtDNA 16S rRNA, ND2 and nDNA rhodopsin), we found congruence with Holleman et al. (2012), with no genetic differences recovered for both the mtDNA 16S rRNA and nDNA rhodopsin despite increased sample sizes; for the ND2 gene, however, we found structure that correlated with that recovered for both the control region and S7 intron (Fig. 2).

Demographic change and estimation of divergence time

Analyses used to estimate demographic change show signals of population expansion for the marine *C. superciliosus* (Fu's $F_s = -12.8$, $p < 0.001$), but do not support a similar scenario for the estuarine *C. spatulatus* (Fu's $F_s = -1.2$, $p = 0.72$). Further, the growth parameter g estimated in Lamarc showed strong population growth for *C. superciliosus* ($g = 206$) and decreasing population size for *C. spatulatus* ($g = -108$). Mismatch distributions based on the control region data ($\tau = 4.992$ for *C. superciliosus*; $\tau = 3.086$ for *C. spatulatus*) suggest that using mutation rates of 1.1% and 15% per million years, *C. superciliosus* underwent a population expansion between 600 000 and 44 000 yr ago. Given the signals of population decline, we did not attempt similar analyses for *C. spatulatus*.

The mean \pm SD estimate for the time of divergence between the 2 species based on 3 runs (in coalescent units) was 0.234 ± 0.003 . Converted into time in years, this corresponds to a divergence time of $40\,992 \pm 472$ yr ago (mean 95% highest posterior density interval, HPD: $13\,567 \pm 144$ to $187\,754 \pm 2184$ yr ago). Effective population size estimates differed considerably, with that for *C. superciliosus* being 2 orders of magnitude (28.93 ± 0.46) greater than that of *C. spatulatus* (0.83 ± 0.03), while the population size of their common ancestor was intermediate (9.41 ± 0.06). Similar results (as indicated by comparatively low SD values) for all 3 runs, as well as very high ESS values ($>27\,000$) suggest that the program IMA2 was run sufficiently long for starting parameters not to impact the divergence time and effective population size estimates.

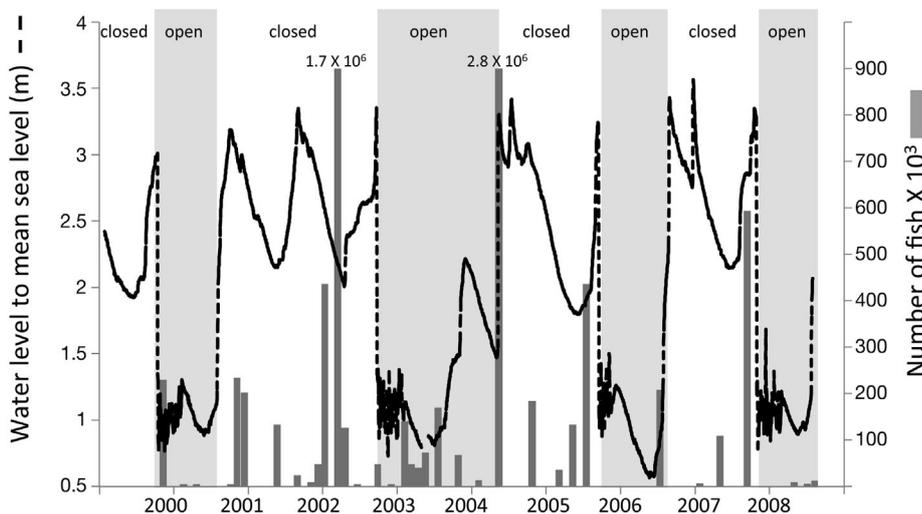


Fig. 3. Water levels and *Clinus spatulatus* population size estimates in the Bot River Estuary for the years 2000 to 2010. Open-mouth periods are shaded. Water-level drops during closure are related to evaporation and seepage

In order to better understand the impact of ecological events on *C. spatulatus*, population size estimates were plotted against mean water level. Population size estimates varied between open and closed estuary states. The Bot and Klein estuaries are temporarily open/closed systems that open to the sea at about 1.5 yr intervals, but some closed periods, especially the Bot Estuary during drought, may be 3 to 4 yr long. The Bot Estuary breached on 4 occasions from 2000 to 2010 (Fig. 3). On each occasion, in less than 24 h, water levels dropped at least 2 m and habitat area available to *C. spatulatus* shrank to as little as 4% of the full estuary. Population estimates after each breaching were significantly lower than during estuary closure (normal approximation to the Mann-Whitney Test $Z = 3.6391 > t_{0.05(2),\infty} = 1.9600$, $p < 0.05$, Fig. 3). Mean population size immediately after opening events was 10% of pre-breaching values.

DISCUSSION

The evolutionary history of estuarine-associated species remains largely unresolved, but hypotheses suggest that most of these taxa probably have a more recent evolutionary origin than their marine congeners, given that many estuaries are relatively young, having arisen in the late Pleistocene or Holocene (Whitfield 1994, Bilton et al. 2002). In southern Africa, the rise in sea level since the LGM has resulted in most extant estuaries having been in their current position for less than 15 000 yr (Whitfield 1994). There are, however, opposing views on estuaries and lagoons as agents of speciation: one notion is that highly variable environments such as estuaries do not encourage speciation (Whitfield 1994), whereas a second model proposes that such variable habitats select for generalist genotypes and drive rapid adaptive divergence (Bamber & Henderson 1988, Beheregaray & Sunnucks 2001). However, few studies examining the molecular relationships between marine and estuarine congeners have been carried out, and it is probable that some radiations predate the Holocene (Bilton et al. 2002). This requires further testing, especially in the light that species diversity for estuarine-restricted species (as has been shown for fishes) is significantly lower than for freshwater or marine systems globally (Whitfield 1994).

The results of our multiple-marker study support a recent divergence (~41 000 yr ago) of the estuarine *Clinus spatulatus* from the marine *C. superciliosus* for a number of reasons: (1) we found few mutational differences for both mtDNA and nDNA markers and

(2) estuarine and marine fishes are identical at the mtDNA 16S rRNA and nDNA rhodopsin loci. While our estimate predates the LGM, the mutation rate used is somewhat conservative. Given that the evolutionary event studied falls within the past 2 million years, a time-dependent mutation rate an order of magnitude greater is likely more realistic (Ho et al. 2005), resulting in a 95% HPD of 1000 to 14 000 yr ago. This suggests that the divergence between the 2 species may have occurred after the LGM and was probably linked to the establishment of contemporary coastlines during the early Holocene, when new estuarine habitats became available for colonization by marine species. Despite uncertainties in molecular dating approaches, it is becoming clearer that the last 100 000 yr played a significant role in determining the molecular fate of southern African marine species (Teske et al. 2011, Toms et al. 2014).

Population structure and recent speciation between estuarine and marine species

Phylogeographic analyses revealed considerable differences between marine and estuarine *Clinus* species (Table 2, Fig. 2) for 3 of the 5 markers analysed; the more conserved markers (mtDNA 16S and nDNA rhodopsin) showed no differentiation between the 2 species (Holleman et al. 2012). Most previous studies describing recent speciation processes recovered mtDNA genealogies as distinct monophyletic clades (Terry et al. 2000, Pinho et al. 2008), whereas nuclear genealogies show discordant scenarios as a result of low but ongoing gene flow or ancestral polymorphism as a result of lower mutation rates (Pinho et al. 2008). Others again show that both mtDNA and nDNA genealogies may not reliably separate populations inhabiting different ecological niches into monophyletic groups (Beheregaray & Sunnucks 2001), as reflected in this study of southern African clinids.

Phylogenetic analyses show that *C. spatulatus* is most likely derived from *C. superciliosus*, particularly because all *C. spatulatus* S7 alleles cluster as a monophyletic group within the tree of *C. superciliosus* sequences (Holleman et al. 2012). The combination of low molecular, but high morphological, divergence (Fig. 1) suggests rapid adaptation by *C. spatulatus* to the estuarine environment. As recently diverged fish populations in disparate environments have been shown to have distinct morphological and physiological adaptations (Ohlberger et al. 2008, Dijkstra et al. 2011), it is therefore not surprising that *C. spatulatus* and *C. superciliosus* are morphologi-

cally, but not yet genetically, distinct. For example, Beheregaray & Sunnucks (2001) proposed a 'divergence with gene flow model' to explain the divergence of populations of *Odontesthes argentinensis* and invoked rapid adaptive divergence and reproductive isolation between populations, which is also a likely scenario for our clinid study system. Recent studies have implicated several gene regions that contribute to adaptive divergence between marine fish populations, because they code for functionally relevant genes. These include heatshock protein 90 (Basu et al. 2002) and rhodopsin (Larmuseau et al. 2010); in sticklebacks, genome scans resolved genetic clusters based on allele frequency differences of selected loci which showed associations with temperature and salinity, despite neutral markers showing high levels of gene flow between populations (DeFaveri et al. 2013). These studies further highlight the difficulty of disentangling the ecological, geographical and evolutionary processes contributing to the genetic divergence and maintenance of structure in marine and estuarine species.

The few papers examining the evolutionary relationships between marine, brackish and freshwater fishes—including atherinids (Klossa-Kilia et al. 2002), silversides (*O. argentinensis* and *O. perugiae*; Beheregaray & Sunnucks 2001, Beheregaray et al. 2002) and sticklebacks (Reusch et al. 2001)—show that relationships between such populations and species are relatively recent and probably arose in the Holocene. However, the haplotype networks for *C. spatulatus* and *C. superciliosus* show that only 2 haplotypes are shared for mtDNA. Similarly low levels of divergence were also identified for the coastal clinid species *C. cottoides* by Toms et al. (2014), who related this to changes in the topography and composition of rocky and sandy shores along the South African coastline between 70 000 and 15 000 yr ago. Shifting coastlines might have separated some *C. superciliosus* in the Bot/Klein system, resulting in a rapid adaptation and divergence of fishes in these estuarine systems. Despite secondary contact following a return of sea-level to stands of pre-70 000 yr ago, behavioural, physiological and ecological adaptations probably maintain reproductive isolation between the 2 species.

Contrasting signals of genetic diversity and population demography

The 2 species studied here show distinct differences in their levels of genetic diversity, which is

significantly lower in *C. spatulatus* (Table 1, Fig. 2), especially with nucleotide diversity being 10-fold lower in the estuarine than in the marine fish. Further, we found only one estuarine fish that was heterozygous for the S7 intron, whereas almost half of the marine fish were heterozygotes. This suggests 2 potential scenarios; the first is a recent origin of *C. spatulatus* coinciding with or driven by the formation of south-western South African estuaries. This was also shown for Australian barramundi *Lates calcarifer*, where populations that recolonized estuaries after the LGM had lower genetic diversity than older populations (Keenan 1994). Secondly, the reduction in genetic diversity in *C. spatulatus* may be a signal of fluctuating population sizes corresponding to environmental stochasticity (see below). Although it is not possible to disentangle these 2 scenarios using the marker systems and sample sizes of our study, it is likely to represent a combination of both. In general, marine fishes tend to have higher genetic variability than those in freshwater environments or diadromous fishes (Ward et al. 1994, DeWoody & Avise 2000), as a consequence of more stable environments. Differences in genetic diversity levels and population stability may also stem from contrasting effective population sizes. For example, the presence of numerous heterozygote *C. superciliosus* for the S7 intron, compared to predominantly homozygous *C. spatulatus*, suggests larger effective population sizes for the marine fish, which was also confirmed by the population numbers estimated by coalescent analysis.

Demographic analyses also show some interesting disparities between *C. superciliosus* and *C. spatulatus*. The control region haplotype network, and to a lesser degree the S7 intron network, show signals of population expansion for *C. superciliosus*, with several high-frequency haplotypes surrounded by closely related low-frequency ones. In contrast, *C. spatulatus* is characterized by one dominant haplotype for both markers, with few associated low-frequency haplotypes. This scenario is also reflected in the positive growth parameter ($g = 208$) estimated for *C. superciliosus*. In contrast, *C. spatulatus* showed negative growth ($g = -106$). These results may well reflect differences in the fishes' habitats; estuaries and lagoons are much less stable than coastal environments. For example, the *C. spatulatus* population in the Bot River undergoes severe population declines (by as much as 90%) when the estuary is breached (Fig. 3). Within specific sites, occurrence can drop from 55% to less than 10%, with fish concentrating in a few refugia (van Niekerk & Turpie

2012), and such cumulative die-off events may well contribute to maintaining genetically depauperate populations. It is unlikely that *C. superciliosus* experiences similarly severe population declines, as it is the most numerically abundant fish inhabiting the intertidal and shallow sub-tidal on the South African south-west coast (Prochazka & Griffiths 1992). At most, these declines are very rare, such as the near 100% mortality experienced by this species over 30 km of coastline during a low-oxygen, hydrogen-sulphide 'black tide' event on South Africa's west coast (Lamberth et al. 2010).

How can molecular approaches highlight estuarine conservation priorities?

Estuaries are among the most threatened environments globally; in South Africa, around 60% of estuarine ecosystem types are not protected and only 14 systems have full protection, including no-take status (van Niekerk et al. 2011). In terms of conservation priority, the Bot and Klein systems are ranked in the top 10 of more than 300 South African estuaries, partly due to the presence of *C. spatulatus* (Turpie et al. 2002, van Niekerk & Turpie 2012). Accordingly, both systems are within the core set of priority estuaries that need to be protected to meet biodiversity targets in South Africa. This is further underscored by the findings of this study that the estuarine *C. spatulatus* comprises a unique genetic lineage and also shows low levels of genetic diversity.

Key pressures for South African estuaries are similar to those for all estuaries and lagoons globally and include flow modification, pollution and exploitation of living resources, land-use changes, development (habitat modification) and river mouth manipulation (van Niekerk et al. 2013). Both the Klein and Bot estuaries have seen a 10 to 20% reduction in mean annual runoff, with a concomitant decrease in the frequency of floods, which in addition to other factors has resulted in a loss of connectivity to the sea and adjacent estuaries (van Niekerk & Turpie 2012). In addition, resident estuary-dependent marine species have become severely depleted through illegal gill-netting, and *C. spatulatus* may be a small but inadvertent bycatch of this fishery, further adding to the pressures facing fish assemblages in estuarine systems. Our results show that not only do the 2 estuaries studied here have unique biodiversity, but that the fundamental genetic signature of this unique lineage differs from that of the marine congener. Molecular work on estuarine invertebrates (Teske et

al. 2006, 2009) and the goby *Glossogobius callidus* (Maake et al. 2013) also revealed patterns of genetically structured populations. This suggests that, at least for some species, connectivity between estuaries is limited, which may have driven the evolution of divergent genetic lineages. Disparate lineages may well show physiological or other adaptations to particular localities (Teske et al. 2008, Papadopoulos & Teske 2014), thus further driving population divergence that could eventually lead to speciation. Genetic isolation and genetically unique lineages therefore become important considerations when setting biodiversity targets and maintaining and protecting potentially important genetic adaptations, especially in the light of future climate change scenarios.

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