Habitat requirements affect genetic variation in three species of mayfly (Ephemeroptera, Baetidae) from South Africa

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Abstract

This study investigates genetic diversity in three species of Ephemeroptera, one eurytopic and therefore widespread (Afroptilum sudafricanum) and two stenotopic and thus endemic (Demoreptus natalensis and Demoreptus capensis) species, all of which co-occur in the southern Great Escarpment, South Africa. Mitochondrial DNA was analysed to compare the genetic diversity between the habitat generalist and the two habitat specialists. Afroptilum sudafricanum showed no indication of population genetic structure due to geographic location, while both Demoreptus species revealed clear genetic differentiation between geographic localities and catchments, evident from phylogenetic analyses and high FST values from AMOVA. In addition, the phylogenetic analyses indicate some deeper haplotype divergences within A. sudafricanum and Demoreptus that merit taxonomic attention. These results give important insight into evolutionary processes occurring through habitat specialisation and population isolation. Further research and sampling across a wider geographic setting that includes both major mountain blocks of the Escarpment and lowland non-Escarpment sites will allow for refined understanding of biodiversity and associated habitat preferences, and illuminate comparative inferences into gene flow and cryptic speciation.

Keywords
cytochrome oxidase 1, genetic diversity, habitat specialisation, haplotype, phylogeography, mayfly

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Introduction

Greater genetic diversity within a lineage is regarded as increasing its resilience to environmental change (Jump et al. 2009, Razgour et al. 2019), which gives contemporary relevance to insights into the mechanisms shaping genetic diversity of populations. Genetic diversity between populations is, in part, a reflection of their members’ dispersal activity through space and time (Slatkin 1985, Bohonak 1999, Avise 2009). Theoretically, if widespread intermigration between populations of a species occurs, then levels of genetic differentiation will be relatively low, whereas if dispersal is restricted by physical barriers or limitations to mobility, then genetic differentiation is likely to be higher (Slatkin 1993). The relationship between dispersal ability and genetic population structure of a species can provide important insights into micro-evolutionary processes, phylogeography (Hanski and Gaggiotti 2004, Avise 2009), and resilience to environmental change.

Aquatic insects have a winged adult stage that is generally considered to have relatively strong dispersal ability (Hughes and Mather 1995, Bunn and Hughes 1997). This is reflected in the ability of stream organisms to recover from disturbance (Wallace 1990, Yount and Niemi 1990) and the widespread geographic distribution of many aquatic species across catchments. Consequently, many such insects show low levels of genetic differentiation among populations, both within and between catchments, attributed to the extensive dispersal of adults by flying (Schmidt et al. 1995, Hughes et al. 1998, 2000, Miller et al. 2002, Monaghan et al. 2002, Pereira-da-Conceicao et al. 2012, Gattolliat et al. 2018). Despite the apparent mobility of these species, their need for persistent waters for breeding tends to fragment their distribution into metapopulations (Avise 2009). The patchiness of lakes, the linear, unidirectional, hierarchical character of rivers, and the topographical structure of catchments tend to structure the dispersal of aquatic organisms between breeding sites or local populations (Wishart et al. 2003, Kaltenbach and Gattolliat 2018). The population genetic variance of certain species is structured significantly according to drainage basin, especially in mountainous landscapes with rugged topography (Hughes et al. 1999, 2003, Wishart and Hughes 2001, 2003, Monaghan et al. 2002, Price et al. 2010, Toussaint et al. 2013, 2014, Barber-James and Pereira-da-Conceicao 2016). Ecologically, aquatic habitats within terrestrial landscapes can therefore be conceptualised as functional islands for some aquatic organisms.

Genetic variation between populations is related to the ability of their members to disperse, and a high degree of genetic structure has been observed among populations of some South African winged aquatic (Wishart and Hughes 2001, 2003) and terrestrial (Price et al. 2007, 2010) insects. This has been attributed to habitat-specificity that imparts a high cost to unsuccessful dispersal, so that stronger associations with restricted habitats, such as particular aquatic conditions, result in increasingly limited potential for successful dispersal (Price et al. 2007). Aquatic invertebrate species, including Ephemeroptera, show varied degrees of habitat-specificity, with some species being completely restricted to a certain habitat and others occurring in a range of habitat types (Barber-James and Lugo-Ortiz 2003).
The aim of this study is to use three model species of mayfly to test the hypothesis that habitat-restricted taxa have greater phylogeographical structure than habitat-generalist species. *Afroptilum sudafricanum* Lestage is a common, widespread African species, occurring in a range of ecological conditions, including different flow regimes and a wide altitude range (Barber-James and Lugo-Ortiz 2003). *Demoreptus natalensis* Crass and *Demoreptus capensis* Barnard have very specific habitat requirements, being most commonly found on rock faces associated with waterfalls in fast-flowing mountain streams (Barber-James and Lugo-Ortiz 2003).

### Materials and methods

#### Study region

The southern Great Escarpment forms an 800-km-long stretch of mountain complexes extending from the Nuweveldberge in the west to the Eastern Cape Drakensberg in the east. Ancient erosional features divide the mountains into five main blocks that range in altitude from 1 600–3 000 m a.s.l., making the area interesting for study of dispersal-limited groups.

#### Taxon sampling

Nymphs of *A. sudafricanum*, *D. capensis*, and *D. natalensis* were collected from 21 rivers in the Eastern Cape Great Escarpment, relating to 12 study areas within the Escarpment and non-Escarpment sites (Table 1). An additional six rivers were sampled for *A. sudafricanum* in lower-altitude (non-Escarpment) areas in the Eastern Cape and KwaZulu-Natal (Table 1). All specimens were preserved in 80% ethanol.

A related species of Baetidae, *Baetis rhodani* Pictet, was used as the outgroup for phylogenetic analyses, and relevant sequence data (Rutschmann et al. 2014) were obtained through Genbank (Benson et al. 2012) for both cytochrome c oxidase subunit I (COI) (KP438135 and KP438160) and 16S rRNA (16S) (KP438109 and KP438119) gene regions.

#### DNA extraction, amplification, and sequencing

DNA was extracted using the Invisorb Spin Tissue Mini Kit following manufacturer’s protocol (Invitek, Berlin, Germany). Extraction was non-destructive, using internal body digestion, which ensured the preservation of the exoskeleton for future morphological analysis (housed in the Albany Museum, Makhanda, South Africa, along with additional material that is stored in the collection, listed under the GEN catalogue.)
Two mitochondrial gene regions were amplified: cytochrome c oxidase subunit I (COI) and small subunit ribosomal RNA (16S). A 528-bp section of the COI regions of *D. natalensis* and *D. capensis* was successfully amplified with the standard ‘universal’ primer pair, LCO1490 and HCO2198 (Folmer et al. 1994), which worked with only limited initial success with *A. sudafricanum*. A new forward primer (5’–GGT GGA TGG GCA GGA ATG GTA GGA–3’) was designed and used with HCO2198 to successfully sequence the remaining samples of *A. sudafricanum*. The 16S region was amplified with the primer pair 16Sar (5’–CGC CTG TTT ATC AAA AAC AT–3’) and 16Sbr (5’–CCG GTC TGA ACT CAG ATC ACG T–3’) (Palumbi 1996). However, these primers proved problematic for the *Demoreptus* samples, and this region is thus excluded from subsequent analyses for this taxon.

The polymerase chain reaction (PCR) was performed in a 50 μl volume using the following thermal regime: 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 50 °C for 45 s, and 72 °C for 90 s, followed by a final extension period of 72 °C for 5 min. PCR amplifications were checked for the presence of amplified PCR products by gel electrophoresis (0.5% agarose gel stained with SYBR green) and viewed with a UV-transilluminator. Successful PCR products were cleaned up using the Invisorb PCRa pace® Quick purification kit (Invitek, Berlin, Germany) and cycle-sequenced in both directions using the primers used for amplification, the ABI Big Dye Sequencing kit v.3.1 (following manufacturer’s instructions (Applied Biosystems)), and a ABI Genetic Analyzer 3500 (Applied Biosystems).

Sequence trace files were assembled and edited using Sequencher 3.0 (DNA sequence analysis software, Gene Code Corporations, Ann Arbor, MI USA, http://www.genecodes.com). The sequences were then aligned in MEGA v.6 (Tamura et al. 2013) using the ClustalW algorithm and subsequently each non-synonymous mutation was manually cross-checked in the original trace files.

**Phylogenetic analyses**

Each gene was tested for substitution saturation using plots of transitions and transversions against F84 distance in DAMBE v7.0.58 (Xia et al. 2003, Xia and Lemey 2009, Xia 2017).

Congruence between the COI and 16S datasets was assessed using the partition homogeneity test (PHT) in PAUP* (Swofford 2002) with 1000 replicates to verify that the gene sections could be combined for analysis.

Bayesian Inference (BI) analyses were conducted with MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001) using the GTR+I+G model since it is the most complex model, allowing the nesting of simpler models that could be estimated through the Bayesian sampling. Each analysis comprised two independent runs with random starting trees and four chains (three heated and one cold) each, sampled every 200 generations for 20 million generations per run. The cumulative sample sizes were plotted against the likelihood scores and tree length using Tracer v1.7.0 (Rambaut et al. 2018), to ascertain when the analysis reached stationarity after the first 10% of the trees were
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discarded as burn-in. The analysis was run on the CIPRES Science Gateway (Miller et al. 2010) using default parameters for variables not mentioned above.

Maximum likelihood (ML) analyses were conducted with 2 000 bootstrap replicates using the GARLI (Genetic Algorithm for Rapid Likelihood Inference) on XSEDE via the CIPRES (Cyberinfrastructure for Phylogenetic Research) Science Gateway v3.3 (Miller et al. 2010), which is supported by the San Diego Supercomputer Center (SDSC) and the University of California (UC San Diego). Models of molecular evolution for each dataset were selected using the Akaike information criteria (AIC) as implemented by jModeltest 2.1.6 (Darriba et al. 2012) (Table 2). The COI and COI+16S ML phylogenies were compared and presented using Phylo.io software (Robinson et al. 2016).

Parsimony analyses were performed in PAUP* version 4.0b10 (Swofford 2002) using the heuristic search option with 100 random addition replicates. A search with TBR (Tree Bisection and Reconnection) branch-swapping was used to find the approximate length of the shortest trees, with one tree kept with each random addition. To investigate nodal support, all of the trees from this search were used as starting trees for a second heuristic search with MAXTREES set to 5 000. The Demoreptus analysis used FASTBOOTSTRAP with 10 000 replicates.

Phylogeographical structure and variation

Molecular diversity was investigated using the COI datasets. The number of variable sites (S), number of haplotypes (Hap) and haplotype diversity (Hd), Nucleotide diversity (p) and neutrality tests (Tajima's D and Fu's F_S) were calculated in DNAsp (Rozas et al. 2017). Population structures within each species were estimated using one-level Analyses of Molecular Variance (AMOVA) in ARLEQUIN ver. 3.5.2 (Excoffier and Lischer 2010). F_ST (fixation index) values were calculated between localities to determine whether putatively conspecific populations differed significantly in their genetic composition. For all AMOVA analyses (listed in Table 4), a priori groups were defined by each site where the insects were collected. Haplotype networks were illustrated with a median-joining network (MJN) algorithm (ε = 0) (Bandelt et al. 1999) using the software PopART v. 1.7 (Leigh and Bryant 2015) to analyse haplotype genealogy.

Results

Data characteristics

COI sequences (649 bp) were obtained from 86 individuals and 16S sequences (542 bp) obtained from 59 individuals of A. sudaficanum. Shorter (528 bp) COI sequences were obtained for 24 Demoreptus individuals (for D. natalensis, N = 12; D. capensis, N = 11; unidentified, N = 1). DNA characteristics for each gene dataset are summarised in Table 2. The Partition Homogeneity Test for incongruency (Swofford 2002)
showed that the combined COI and 16S datasets were not significantly incongruent (P = 0.3890) and could therefore be combined for analysis. The COI+16S molecular dataset consisted of 59 specimens and 1191 nucleotides including the outgroup. No evidence of saturated substitution was found for either gene (data not shown).

**Phylogenetic analyses**

The parsimony analyses’ results are summarised in Table 2. Phylogenetic analyses of the habitat generalist *A. sudafricanum* consistently retrieved six distinct clades and an unresolved grade of specimens (referred to hereafter as the “widespread grade”) from a wide range of sites for all analyses (Fig. 1). The tree comparison shows that the relationships between these clades in the analysis of the CO1 data and the CO1+16S data sets were consistent, with improved support for the combined dataset. The well-supported clades did not conform to the mountain blocks described in Table 1 and included specimens from across these ranges. The clades were roughly separated into overlapping geographic groups: Southern Montane, Stormberg/Barkly East, and Eastern Cape, while more restricted geographic areas included KwaZulu-Natal and Eastern Cape Drakensberg, the latter clade showing a longer stem branch compared to other clades (Fig. 1).

The phylogenetic analysis of the habitat-restricted *D. capensis* and *D. natalensis* clearly indicated strong genetic structure corresponding to geographic location (Fig. 2). Both species had genetically distinct populations with strong support from parsimony, Bayesian and maximum likelihood analyses. The clades found for *D. capensis* and *D. natalensis* were more closely aligned with the mountain ranges described in Table 1 and appear to be site-restricted, apart from one instance where individuals of *D. natalensis* from Rhodes and Barkly Pass fell into the same well-supported clade. For *D. capensis* the Rhodes clade had a long branch and is clearly distinct from the other clades, which is noteworthy considering the close geographic proximity to Barkly East, which formed a separate well-supported clade nested with other clades (Fig. 2). This pattern was not apparent in *D. natalensis*, where the Barkly East specimen was clearly separate from the *D. natalensis* clade; morphological re-examination suggests that it does not belong to any described *Demoreptus* species.

**Population genetics**

MJN analysis collapsed the 86 *A. sudafricanum* COI sequences into 60 haplotypes (Fig. 3, Table 3), 45 of which were singletons or private haplotypes. Haplotype 17 was the most abundant (N = 8) and occurred in three of the 12 study areas (Fig. 4), which included non-Escarpment Grahamstown and two main mountain Escarpment blocks (Sneeuberg and Winterberg–Amathole). Haplotype 20 was next-most-abundant (N = 4) and exclusive to non-Escarpment Makhanda (= Grahamstown). Haplotype 10 (N = 3) was found in one non-Escarpment site (KwaZulu-Natal) and one main moun-
Table 1. Collecting localities (Site and river name) and non-zero sample sizes for each species from each site. The GenBank sequence accession numbers for each sample are listed in Suppl. material 1.

| Locality                     | Longitude/Latitude | A. sudafricanum | D. capensis | D. natalensis | Demoreptus sp. |
|------------------------------|--------------------|-----------------|-------------|---------------|----------------|
| **Escarpment sites**         |                    |                 |             |               |                |
| Eastern Cape Drakensberg     |                    |                 |             |               |                |
| Barkley East 1: Diepspruit   | -30.751, 27.546    | 3               | 1           |               |                |
| Barkley East 2: Diepspruit   | -30.757, 27.552    | 3               |             |               |                |
| Barkley East 3: Diepspruit   | -30.718, 27.54     | 3               |             | 1             |                |
| Barkley Pass 1: Marais Hoek  | -31.215, 27.686    | 3               |             |               |                |
| Barkley Pass 4: Ben Wyvie    | -31.173, 27.971    | 3               |             | 3             |                |
| Barkley Pass 5: Lymore Lodge | -31.172, 27.854    | 2               |             |               |                |
| Rhodes 1: Hawkshead          | -30.676, 27.884    | 3               | 2           |               |                |
| Rhodes 2: Tiffindell         | -30.674, 27.904    | 3               | 1           |               |                |
| Rhodes 3: Tenahead           | -30.696, 28.150    | 3               | 1           |               |                |
| Maclear 1: Vuvu River        | -30.603, 28.216    | 5               |             |               |                |
| **Stomberg**                 |                    |                 |             |               |                |
| Stomberg 3: Lana River       | -31.163, 26.602    | 3               |             |               |                |
| Stomberg 4: Lemonfountain    | -31.416, 26.842    | 3               |             |               |                |
| **Winterberg-Amatole**       |                    |                 |             |               |                |
| Elsensburg 1: Elandsberg     | -32.506, 26.903    | 3               |             |               |                |
| Winterberg 1: Fanella falls  | -32.363, 26.385    | 2               | 3           |               |                |
| Winterberg 2: Fanella falls  | -32.380, 22.967    | 3               |             |               |                |
| Winterberg 3:                |                    |                 |             | 5             |                |
| **Sneeueberg**               |                    |                 |             |               |                |
| Sneeueberg 1: Fish River     | -32.227, 24.954    | 2               |             |               |                |
| Sneeueberg 2: Melkrivier     | -32.243, 24.941    | 2               | 3           | 3             |                |
| Kamdeboorberg 1: Buffelrivier| -32.177, 24.016    | 3               | 2           |               |                |
| Kamdeboorberg 3: Waterkloof  | -32.353, 23.890    | 2               |             | 2             |                |
| **Nuweveldberge**            |                    |                 |             |               |                |
| Nuweveldberge 1: Maijesvlei  | -32.102, 22.636    | 1               |             |               |                |
| **Non-Escarpment sites**     |                    |                 |             |               |                |
| Grahamstown CR: Coleridge River | -33.349, 26.618    | 2               |             |               |                |
| Grahamstown KP: Kap River    | -33.351, 26.858    | 5               |             |               |                |
| Grahamstown KR: Kowie River  | -33.349, 26.560    | 5               |             |               |                |
| Grahamstown PM: Palmiet River| -33.370, 26.476    | 5               |             |               |                |
| **KwaZulu-Natal**            |                    |                 |             |               |                |
| KwaZulu-Natal KK: Karkloof River | -29.338, 30.307  | 5               |             |               |                |
| KwaZulu-Natal LR: Lions River| -29.492, 30.108    | 5               |             |               |                |
| KwaZulu-Natal UM: Umgeni River| -29.477, 30.261   | 1               |             |               |                |

Table 2. Data characteristics and summary of the parsimony analysis. The number of specimens with sequence data (ntax), total number of base pairs (bp), parsimony informative (# Pi), and percent parsimony informative (% Pi) is reported. The results of the parsimony are summarised with the number of trees retained (# trees), tree length (score), Consistence Index (CI) and Retention Index (RI). The summary of the models for the Maximum Likelihood analysis (ML) selected by jModeltest.

| Species         | Dataset | ntax | Characters | Parsimony analysis | Model     |
|-----------------|---------|------|------------|--------------------|-----------|
|                 |         | bp   | #Var       | # Pi               | % Pi      | # trees | Score | CI     | RI   | ML analysis | BI analysis |
| A. sudafricanum | COI     | 88   | 649        | 217                | 192       | 29.6    | 5 000 | 421    | 0.601 | 0.932        | GTR+I+G     | GTR+I+G |
|                 | COI+16S | 88   | 1191       | 380                | 336       | 28.2    | 5 000 | 645    | 0.662 | 0.939        | TIM3+I+G    | GTR+I+G |
| Demoreptus spp. | COI     | 24   | 528        | 164                | 159       | 30.1    | 8     | 302    | 0.745 | 0.922        | TVM+G       | GTR+I+G |
tain Escarpment block (Eastern Cape Drakensberg, in two study areas: Barkly Pass and Maclear; Figs 3, 4). Haplotypes were clustered according to a broad geographical structure, which correspond to clades from the phylogenetic analyses (Fig. 1). The numerous missing mutational steps in the haplotype network (Fig. 3) suggest that more sampling is needed for some clusters, particularly between sites that are separated by long sampling gaps (for example, the non-Escarpment sites). Other clusters that are separated
by numerous missing intermediates could represent cryptic species or relict lineages that have re-joined the metapopulation (Hinojosa et al. 2019) (encircled with dashes in Fig. 3). The divergent Haplotype 27 from the Kamdebooberg did not cluster with the other haplotypes from the same area and may represent such a lineage. The widespread grade showed little geographic structure, and all haplotypes from Stormberg (Hap 51, 52 and 53) grouped together exclusively, otherwise all other sites are mixed.

The MJN analyses for *D. capensis* retrieved eight haplotypes (Hd = 0.9273, S = 101), six of which were singletons and *D. natalensis* retrieved six haplotypes (Hd = 0.8636, S = 21) including three singletons (Fig. 5). Haplotypes were largely site-restricted for both species with the exception of Haplotype 1 (*N* = 3) for *D. capensis* and Haplotype 4 (*N* = 3) for *D. natalensis* (study areas Barkly Pass and Rhodes), which are both found in the Eastern Cape Drakensberg main mountain block in the Escarpment (Fig. 4). Both networks show many missing mutational steps between haplotypes grouped by locality, which could result from undersampling or haplotype filtering.

Nucleotide diversities (*P*) are reported in Table 3 and are not interpreted further because the small sample sizes for *Demoreptus* spp. make the estimates imprecise. Neutrality tests (Tajima’s D and Fu’s F*) were not significant for *A. sudafricanum, D. capensis* or *D. natalensis* indicating that the nucleotide patterns of variation are consistent with the neutral theory of evolution. Fu’s F* statistic for the widespread grade of *A. sudafricanum* was negative (F* = −11.544) and significant (P < 0.02), indicating a recent population expansion (Table 3). The Fu’s F* statistics for *D. capensis* and *D. natalensis* were

**Figure 2.** Bayesian inference phylogram of *Demoreptus* spp for the COI gene marker. Support for major nodes is shown in the order Bayesian Inference / Maximum Parsimony / Maximum Likelihood (BI/MP). Bars next to clades refer to distinct clades that are colour-coded according to the study areas found within that clade (see colour legend). *Baetis rhodani* Pictet was used as the outgroup.
Table 3. Haplotype characteristics and Neutrality tests for *A. sudafricanum*, *D. capensis* and *D. natalensis*.

| Species           | Number of haplotypes (Hap) | Haplotype characteristic | Nucleotide diversity (Pi) | Number of variable sites (S) |
|-------------------|-----------------------------|---------------------------|---------------------------|------------------------------|
| *A. sudafricanum* | 60                          |                            | 0.07508                   | 129                          |
| *A. sudafricanum* (unresolved) | 28                          |                            | 0.01998                   | 67                           |
| *D. capensis*     | 8                           |                            | 0.08592                   | 101                          |
| *D. natalensis*   | 6                           |                            | 0.01881                   | 21                           |

Figure 3. Median-joining network of *A. sudafricanum* based on COI haplotypes generated in this study. The network was estimated using the median-joining algorithm in PoPArt v.1.7 with epsilon = 0. Each circle represents a different haplotype and the size of a circle correlates with the number of individuals assigned to that haplotype. Only haplotypes found in more than one sample are numbered. Colours indicate the geographic origin of sequences; black dots indicate unsampled or extinct haplotypes.
Figure 4. Distribution of *A. subafricanum*, *D. natalensis* and *D. capensis* COI haplotypes across the study area. The map shows the study areas defined in Table 1, and the pie charts indicate the haplotype composition of the population from each area. Each colour represents a shared haplotype found across the study area; private haplotypes (singletons found in the samples from one particular population and are absent in the samples from other populations) are represented as clear sections within the pie charts.
positive, indicating a deficiency of alleles as expected from a population bottleneck, but they were not significant and need a larger sample size to confirm these results.

The AMOVA results for *A. sudafricanum* revealed that over all localities, 52.33% of the total variance was explained by variation among populations (df = 10, Va = 12.073) while 47.67% (df = 75, Vb = 10.998) was explained by variation within populations (Table 4). A similar result was found with the widespread grade of *A. sudafricanum*, with 39.43% of the total variance explained by variation among populations (df = 5, Va = 2.238) and 60.57% (df = 28, Va = 3.438) explained by variation within populations. In contrast, the AMOVAs for the habitat-restricted species, *D. capensis* and *D. natalensis*, indicated a higher proportion of variation among populations: 94.83% (df = 4, Va = 24.950) and 95.39% (df = 4, Va = 5.423), respectively (Table 4). The total variance explained by variation within populations was only 5.17% (df = 6, Vb = 1.361) for *D. capensis* and 4.61% (df = 7, Vb = 0.262) for *D. natalensis*.
Table 4. One-level AMOVA results for *A. sudafricanum, D. capensis* and *D. natalensis* showing percentage variation among and within populations and the fixation index (F<sub>ST</sub>). Significant p-values (< 0.05) are set in bold.

| Species/clade                  | % variation | F<sub>ST</sub> |
|--------------------------------|-------------|---------------|
|                                | Among       | Within        |
| *A. sudafricanum*              | 52.33       | 47.67         | 0.52327 |
| *A. sudafricanum* unresolved group | 39.43     | 60.57         | 0.39426 |
| *D. capensis*                  | 94.83       | 5.17          | 0.94827 |
| *D. natalensis*                | 95.39       | 4.61          | 0.95393 |

The measure of population differentiation due to genetic structure (F<sub>ST</sub>) was much lower for *A. sudafricanum* compared to the *Demoreptus* species (Table 4). The widespread grade for *A. sudafricanum* had a very low F<sub>ST</sub> value of 0.39 while *D. natalensis* and *D. capensis* had very high F<sub>ST</sub> values of over 0.94 (Table 4).

**Discussion**

This study considered evidence of the phylogenetic structure of three species of Baetidae corresponding to two different habitat requirements. Results indicate that habitat-restricted *Demoreptus* species have greater maternal genetic structure than widespread *A. sudafricanum*, showing notable genetic differentiation associated with geographic localities and catchments. This is evident from the haplotype networks in a MJN analysis, F<sub>ST</sub> values from an AMOVA and the phylogeographical structure indicated by phylogenetic trees.

Phylogeographical structure of habitat generalist, *A. sudafricanum* retrieved six distinct, well-supported clades and one widespread grade of individuals from widespread (Escarpment and non-Escarpment) sites across the sampling area. *Afroptilum sudafricanum* was best represented with a haplotype network (Fig. 3), particularly for the widespread grade as the samples have evolved over such a short time that ancestral and descendant haplotypes exist concurrently, and so it remains unresolved in the hierarchical tree. The species occupies a range of habitats from still to flowing rivers. Remarkably, shared haplotypes (Haps 10, 17, and 19) were identified between Escarpment and non-Escarpment sites, some over 300 km apart (Hap 10), across various mountain chains and differing in altitude by over 900 m (Fig. 4). The genetic differentiation within *A. sudafricanum* is not attributed to purely geographic location or catchments. Most clades seen in both the hierarchical trees and haplotype networks include sites that are widely spread across sampled catchments and mountain blocks, with the exception of one clade that occurs only in the Eastern Cape Drakensberg (Rhodes and Barkly East). Even if *A. sudafricanum* is treated as a species complex and assessed for mitochondrial genetic differentiation, results indicate low divergence between populations, suggesting that gene flow is not particularly limited within catchments and across the geographic range. Although mayflies are traditionally thought to have limited dispersal ability due to weak flight and short adult lifespans (Brittain and
some mitochondrial clades within *A. sudafricanum* are remarkably widespread. These results support studies indicating that long-distance dispersal is in fact more prevalent in mayflies than previously thought (Monaghan et al. 2005, Gattolliat and Staniczek 2011, Pereira-da-Conceicoa et al. 2012, Vuataz et al. 2013, Rutschmann et al. 2016).

The habitat specialist species, *D. natalensis* and *D. capensis* are rheophilic and found on rock faces associated with waterfalls and large bedrock sections in shallow but fast-flowing sections of mountain streams. Analyses indicate restricted gene flow over distance and across catchments, a possible consequence of isolation by habitat limitations in mountainous areas. Distinct clades retrieved from phylogenetic analyses show a close association with geographic locality. *Demoreptus natalensis* returned clades and haplotypes exclusive to Sneeuberg and Winterberg areas; the Eastern Cape Drakensberg clade included two study areas (Barkly Pass and Rhodes areas); and Kamdebooberg was unresolved. *Demoreptus capensis* had a similar result, but the Rhodes area returned a separated clade with a well-supported, long branch. Suggestively, the samples of *A. sudafricanum* and *D. capensis* collected at Rhodes both occupy long branches in their respective phylogenetic analyses (Figs 2, 3). These sites are from the highest regions of the study (2600 m.a.s.l.) on the slopes of Ben MacDhui. This may indicate a historical isolation event or an accelerated local rate of molecular evolution (perhaps through faster fixation in smaller populations) responsible for the pattern observed.

Preliminary re-examinations indicate morphological differences between *D. capensis* from Rhodes and *D. capensis* from other localities, and between *D. natalensis* from Barkly East and *D. natalensis* from other localities (HMBJ, pers. obs.); these characters will be documented in a subsequent taxonomic study. Other areas in the Drakensberg range in KwaZulu-Natal and Lesotho should be sampled to investigate the range of this mitochondrial clade and whether it occurs throughout high altitude, mountainous areas. A caveat is that the *Demoreptus* population analyses involve limited sample sizes from few localities, which can produce misleading clustering (Phiri and Daniels 2014, Hinojosa et al. 2019), and that sampling more localities can address this concern (Phiri and Daniels 2014). Furthermore, mitochondrial genes are inherited asexually and maternally, and may represent gene flow differently from sexually-inherited, recombining nuclear genes (Hinojosa et al. 2019), so quantifying nuclear gene diversity is also necessary to clarify this situation.

Previous studies on South African species have found genetic differentiation according to catchments in both animals with limited dispersal ability (Wishart and Hughes 2001, 2003, Daniels et al. 2009, McDonald and Daniels 2012, Tolley et al. 2014, Barber-James and Pereira-da-Conceicoa 2016) and terrestrial insects with high vagility (Price et al. 2007, 2010). The unexpected limited dispersal potential of cicadas was attributed to their habitat philopatry (Price et al. 2010) and host-plant specificity (Price et al. 2007). Similarly, *D. natalensis* and *D. capensis* are restricted by their habitat, and subsequently show high levels of genetic differentiation. Similar limitations to gene flow have been found in various other mountain-restricted
aquatic insects (Hughes et al. 2003, Wishart and Hughes 2003, Finn et al. 2006, Lehrian et al. 2010).

The high support values for some geographically localised clades within *A. sudafricanum* and the two *Demoreptus* species could indicate the presence of cryptic species or local haplotype filtering and mutation due to protracted isolation (Hinojosa et al. 2019). Mountain-dwelling populations are often fragmented and under-sampled (Phiiri and Daniels 2013), and the reported low diversity of Baetidae in most areas of Africa has been attributed to the lack of data and comprehensive analysis of material collected by taxonomists (Gattolliat et al. 2008). Intensive sampling over large geographical ranges usually results in the discovery of numerous new taxa and the extension of distribution ranges (Gattolliat et al. 2008). Cryptic taxa are not uncommon in aquatic insects (Wishart and Hughes 2003, 200, Pereira-da-Conceicoa et al. 2012). Since the 1980s there has been an exponential increase in the number of studies on cryptic species, partly due to the introduction of the PCR, which resulted in the increasing availability of DNA sequences (Bickford et al. 2007). Molecular (DNA) methods are valuable in resolving morphologically cryptic lineages and have been used extensively in discriminating species with few or no morphological differences (Jackson and Resh 1998, Rutschmann et al. 2014, Leys et al. 2016, Tenchini et al. 2018). Within the Ephemeroptera, cryptic lineages have been discovered in numerous families through electrophoretic studies (Sweeney and Funk 1991, Zloty et al. 1993, Funk and Sweeney 1994) and, more recently, DNA sequence data (Williams et al. 2006, Ståhls and Savolainen 2008, Pereira-da-Conceicoa et al. 2012).

The observed deep haplotype divergences in all three species studied and the recent population expansion in *A. sudafricanum* may be explained by possible Quaternary glaciation in the Drakensberg area, where small glaciers formed as low as 2100 m on south-facing slopes (Lewis and Hanvey 1993, Lewis and Illgner 2001, Grab 2002, Mills and Grab 2005, Lewis 2011, Mills et al. 2012). Small remnant populations in non-glaciated areas at high altitude would have been isolated for some time which may explain the long branch patterns seen in *D. capensis* and *A. sudafricanum* for high altitude populations from Rhodes in the Eastern Cape Drakensberg. Glaciation would exacerbate the difficulty of finding suitable habitats more for *Demoreptus* spp. than for *A. sudafricanum*, which can find suitable habitats at lower altitudes. However, the evidence available for this niche glaciation is considered by some as ambiguous and unclear (Osmaston and Harrison 2005). Cyclical climate changes from the Pleistocene to present interglacial (Dingle and Rogers 1972, Siesser and Dingle 1981) could have resulted in historic population fluctuations including expansions, bottlenecks, drift and allele fixation (especially for *A. sudafricanum*).

However, because they are asexually and maternally inherited, strongly divergent haplotypes that originated in relict populations may not reflect contemporary mating pattern if those isolated populations’ ranges subsequently expand to restore potential panmixis (Hinojosa et al. 2019). More samples and an investigation of nuclear genetic diversity are necessary to get any further resolution into the patterns observed.
Perspectives

These results help to illuminate some of the evolutionary processes occurring in mayfly species and highlight the effect of habitat-specificity on haplotype diversity and partitioning within a species. While all three species have qualitatively similar levels of dispersal potential in terms of flight, they show differences in gene flow, suggesting that other processes, such as species-specific habitat requirements, may contribute to genetic population structure. These results have implications for the conservation of riverine organisms, the reintroduction of locally extinct taxa and the rehabilitation of disturbed environments (Jump et al. 2009, Razgour et al. 2019).

In South Africa, it is legislated that catchments are used as management units (Republic of South Africa 1998). Previous studies on the genetic population structure of winged aquatic insects in South Africa have further supported the use of catchments as units for conservation (Wishart and Hughes 2003, Wishart et al. 2003, Price et al. 2010). The results found here for *D. capensis* and *D. natalensis* further highlight the genetic distinctiveness of populations between catchments, further corroborating the value of using catchments in conservation, management and legislative frameworks. These genetically distinct populations form an important component in the evolutionary legacy of a species. Therefore, the development of inter-basin water transfer schemes poses a threat to both *D. capensis* and *D. natalensis* and many other species by potentially connecting historically isolated and genetically distinct populations (Snaddon and Davies 1998, Davies et al. 2000).

In addition, dispersal among adjacent catchments has implications for the recovery of lotic systems following disturbance (Wishart and Davies 2003, Bellingan et al. 2019, Razgour et al. 2019). These factors should be considered in the development of strategies for the conservation of aquatic biodiversity (Wishart 2000, Thieme et al. 2007, Castello et al. 2013, Bellingan et al. 2019), and most particularly for high altitude catchments.

This study highlights the importance for future studies on community structure, biodiversity, and biomonitoring, where the taxonomic accuracy of species identification is crucial (Hajibabaei et al. 2016). The identification of possible cryptic species in *A. sudafricanum* and new species of *Demoreptus* affect the field of aquatic research in South Africa. Mayflies form a very important component of applied aquatic biology, particularly biomonitoring, the presence of cryptic taxa is being discovered at an increasing rate and poses challenges for some aquatic ecosystem monitoring methods. With bioassessment methods gaining increasing popularity, a detailed understanding of commonly collected species will aid in further development of assessment methods and clarify species identification (Delić et al. 2017, Suh et al. 2019). In addition, a deeper understanding of evolutionary processes and gene flow with regard to commonly occurring mayfly taxa contributes to broader research on ecosystem functioning and environmental processes. The utility of DNA barcoding for elucidating such phenomena is already proven (Jackson and Resh 1998, Plaisance et al. 2009, Raupach and Radulovici 2015) and widely used, with new technologies allowing for the rapid assessment of bio-
diversity using DNA metabarcoding (Pavan-Kumar et al. 2015, Elbrecht et al. 2017, Daravath et al. 2018, Alvarez-Yela et al. 2019). This approach to rapid biodiversity assessment has the potential to revolutionise and streamline management and conservation practices by providing detailed data for informed decision- and policy-making.

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**References**

Alvarez-Yela A, Mosquera J, Noreña-P A, Cristancho M, Lopez-Alvarez D (2019) Microbial diversity exploration of marine hosts at Serrana Bank, a coral atoll of the Seaflower Biosphere. Frontiers in Marine Science 6. https://doi.org/10.3389/fmars.2019.00338

Avise JC (2009) Phylogeography: retrospect and prospect. Journal of Biogeography 36: 3–15. https://doi.org/10.1111/j.1365-2699.2008.02032.x

Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution: 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Barber-James HM, Lugo-Ortiz CR (2003) Ephemeroptera. In: de Moor I, Day J, de Moor F (Eds) Guides to the freshwater invertebrates of southern Africa. WRC Report. Water Research Commission, Pretoria, South Africa.
Barber-James HM, Pereira-da-Conceicooa LL (2016) Efficacy and deficiencies of rapid biomonitoring in biodiversity conservation: a case study in South Africa. African Journal of Aquatic Science 41: 337–343. https://doi.org/10.2989/16085914.2016.1192019

Bellingan TA, Hugo S, Woodford D, Gouws G, Villet MH, Weyl O (2019) Macroinvertebrates in a vulnerable South African Cape Fold Ecoregion stream show minimal impacts of a rotenone treatment. Hydrobiologia 834: 1–11. https://doi.org/10.1007/s10750-019-3885-z

Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2012) GenBank. Nucleic Acids Research 41: D36–D42. https://doi.org/10.1093/nar/gks1195

Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, Ingram KK, Das I (2007) Cryptic species as a window on diversity and conservation. Trends in Ecology & Evolution 22: 148–155. https://doi.org/10.1016/j.tree.2006.11.004

Bohonak AJ (1999) Dispersal, gene flow, and population structure. Quarterly Review of Biology 74: 1–45. https://doi.org/10.1086/392950

Brittain JE, Sartori M (2003) Ephemeroptera. In: Resh VH, Carde RT (Eds) Encyclopedia of Insects. Academic Press, Amsterdam, 373–380.

Bunn SE, Hughes JM (1997) Dispersal and recruitment in streams: evidence from genetic studies. Journal of the North American Benthological Society 16: 338–346. https://doi.org/10.2307/1468022

Castello L, McGrath D, Hess L, Coe M, Lefebvre P, Petry P, Macedo M, Renó V, Arqantes C (2013) The vulnerability of Amazon freshwater ecosystems. Conservation Letters 6: 217–229. https://doi.org/10.1111/conl.12008

Daniels SR, Picker MD, Cowling RM, Hamer ML (2009) Unravelling evolutionary lineages among South African velvet worms (Onychophora: Peripatopsis) provides evidence for widespread cryptic speciation. Journal of the Linnean Society of London, Zoology 97: 200–216. https://doi.org/10.1111/j.1095-8312.2009.01205.x

Daravath S, Naik Bannoth R, Tamil Selvi M, Ankanagari S (2018) DNA barcoding significance and utilities. In: Trivedi S, Rehman H, Saggu S, Panneerselvam C, Ghosh S (Eds) DNA barcoding and molecular phylogeny. Springer International, Heidelberg. https://doi.org/10.1007/978-3-319-90680-5_1

Davies BR, Snaddon CD, Wishart MJ, Thomas M, Meador M (2000) A biogeographical approach to inter-basin water transfers: implications for river conservation. In: Boon PJ, Davies BR, Petts GE (Eds) Global perspectives on river conservation: science, policy and practice. John Wiley and Sons, London, 431–444. https://www.tandfonline.com/doi/pdf/10.2989/160859100780177749

Delić T, Trontelj P, Rendoš M, Fišer C (2017) The importance of naming cryptic species and the conservation of endemic subterranean amphipods. Scientific Reports 7: 3391. https://doi.org/10.1038/s41598-017-02938-z

Dingle RV, Rogers J (1972) Pleistocene palaeogeography of the Agulhas Bank. Transactions of the Royal Society of South Africa 40: 155–165. https://doi.org/10.1080/00359197209519415

Elbrecht V, Vamos EE, Meissner K, Arovita J, Leese F (2017) Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. Methods in Ecology and Evolution 8: 1265–1275. https://doi.org/10.1111/2041-210X.12789
Genetic variation in eurytopic and stenotopic mayflies

Excoffier L, Lischer HEL (2010) ARLEQUIN suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Finn DS, Theobald DM, Black WC, Poff NL (2006) Spatial population genetic structure and limited dispersal in a Rocky Mountain alpine stream insect. Molecular Ecology 15: 3553–3566. https://doi.org/10.1111/j.1365-294X.2006.03034.x

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Funk DH, Sweeney BW (1994) The larvae of eastern North American Eurylophella Tiensuu (Ephemeroptera: Ephemerrillidae). Transactions of the American Entomological Society 120: 209–286.

Gattolliat JL, Staniczek AH (2011) New larvae of Baetidae (Insecta: Ephemeroptera) from Espiritu Santo, Vanuatu. Stuttgart Beiträge zur Naturkunde A, Neue Serie 4: 75–82.

Gattolliat JL, Kondratieff BC, Kaltenbach T, Al Dhafer HM (2018) Labiobaetis from the Kingdom of Saudi Arabia (Insecta: Ephemeroptera: Baetidae). ZooKeys 774: 77–104. https://doi.org/10.3897/zookeys.774.25273

Gattolliat JL, Monaghan MT, Sartori M, Elourd JM, Barber-James HM, Derleth P, Glaiot O, de Moor F, Vogler AP (2008) A molecular analysis of the Afrotropical Baetidae. In: Hauer FR, Welch A (Eds) International advances in the ecology, zoogeography and systematics of mayflies and stoneflies. Entomology. University of California Publications, California. https://doi.org/10.1525/california/9780520098688.001.0001

Grab S (2002) Characteristics and palaeoenvironmental significance of relict sorted patterned ground, Drakensberg plateau, southern Africa. Quaternary Science Reviews 21: 1729–1744. https://doi.org/10.1016/S0277-3791(01)00149-4

Hajibabaei M, Baird DJ, Fahner NA, Beiko R, Golding GB (2016) A new way to contemplate Darwin’s tangled bank: how DNA barcodes are reconnecting biodiversity science and biomonitoring. Philosophical Transactions of the Royal Society B: Biological Sciences 371: 20150330. https://doi.org/10.1098/rstb.2015.0330

Hanski I, Gaggiotti OE (2004) Ecology, genetics and evolution of metapopulations. Elsevier Academic Press, Burlington, 696 pp. https://doi.org/10.1016/B978-0-12-323448-3.X5000-4

Hinojosa JC, Koubinová D, Szenteczki MA, Pettold C, Dincă V, Alvarez N, Vila R (2019) A mirage of cryptic species: Genomics uncover striking mitonuclear discordance in the butterfly Thymelicus sylvestris. Molecular Ecology 28: 3857–3868. https://doi.org/10.1111/mec.15153

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Hughes JM, Mather PB (1995) Estimating patterns of dispersal in aquatic animals using allozyme markers. In: Holmes RS, Lim HA (Eds) Gene families: structure, function, genetics and evolution: Proceedings of The VIII International Congress on Isozymes. World Scientific, Brisbane, 71–92.

Hughes JM, Hillyer M, Bunn SE (2003) Small-scale patterns of genetic variation in the mayfly Bungona narilla (Ephemeroptera: Baetidae) in rainforest streams, south-east Queensland. Freshwater Biology 48: 709–717. https://doi.org/10.1046/j.1365-2427.2003.01044.x
Hughes JM, Bunn SE, Hurwood DA, Cleary C (1998) Dispersal and recruitment of *Tasiagma ciliata* (Trichoptera: Tasmidae) in rainforest streams, south-east Australia. Freshwater Biology 30: 117–127. https://doi.org/10.1046/j.1365-2427.1998.00268.x

Hughes JM, Mather PB, Sheldon AL, Allendorf FW (1999) Genetic structure of the stonefly, *Yoraspera brevis*, populations: the extent of the gene flow among adjacent montane streams. Freshwater Biology 41: 63–72. https://doi.org/10.1046/j.1365-2427.1999.00385.x

Hughes JM, Bunn SE, Cleary C, Hurwood DA (2000) A hierarchical analysis of the genetic structure of an aquatic insect *Bungona* (Baetidae: Ephemeroptera). Heredity 85: 561–570. https://doi.org/10.1046/j.1365-2540.2000.00782.x

Jackson JK, Resh VH (1998) Morphologically cryptic species confound ecological studies of the caddisfly genus *Gumaga* (Trichoptera: Sericostomatidae) in Northern California. Aquatic Insects 20: 69–84. https://doi.org/10.1076/aqin.20.2.69.4503

Jump SA, Marchant R, Peñuelas J (2009) Environmental change and the option value of genetic diversity. Trends in Plant Science 14: 51–58. https://doi.org/10.1016/j.tplants.2008.10.002

Kaltenbach T, Gattolliat JL (2018) The incredible diversity of *Labiobaetis* Novikova and Kluge in New Guinea revealed by integrative taxonomy (Ephemeroptera, Baetidae). ZooKeys 804: 1–136. https://doi.org/10.3897/zookeys.804.28988

Lehrian S, Bálint M, Haase P, Pauls SU (2010) Genetic population structure of an autumn-emerging caddisfly with inherently low dispersal capacity and insights into its phylogeography. Journal of the North American Benthological Society 29: 1100–1118. https://doi.org/10.1899/09-100.1

Leigh JW, Bryant D (2015) POPART: full-feature software for haplotype network construction. Methods in Ecology and Evolution 6: 1110–1116. https://doi.org/10.1111/2041-210X.12410

Lewis CA (2011) Late Quaternary environmental phases in the Eastern Cape and adjacent Plettenberg Bay-Knysna region and Little Karoo, South Africa. Proceedings of the Geologists’ Association 122: 187–200. https://doi.org/10.1016/j.pgeola.2010.06.005

Lewis CA, Hanvey PM (1993) The remains of rock glaciers in Bottelnek, East Cape Drakensberg, South Africa. Transactions of the Royal Society of South Africa 48: 265–289. https://doi.org/10.1080/00359199309520275

Lewis CA, Illgner PM (2001) Late Quaternary glaciation in southern Africa: moraine ridges and glacial deposits at Mount Enterprise in the Drakensberg of the Eastern Cape Province, South Africa. Journal of Quaternary Science 16: 365–374. https://doi.org/10.1002/jqs.610

Leys M, Keller I, Räsänen K, Gattolliat JL, Robinson CT (2016) Distribution and population genetic variation of cryptic species of the Alpine mayfly *Baetis alpinus* (Ephemeroptera: Baetidae) in the Central Alps. BMC Evolutionary Biology 16: 77. https://doi.org/10.1186/s12862-016-0643-y

McDonald DE, Daniels SR (2012) Phylogeography of the Cape velvet worm (*Onychophora: Peripatopsis capensis*) reveals the impact of Pliocene/Pleistocene climatic oscillations on Afromontane forest in the Western Cape, South Africa. Journal of Evolutionary Biology 25: 824–835. https://doi.org/10.1111/j.1420-9101.2012.02482.x

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. 2010 Gateway Computing Environments Workshop, GCE: 1–8. https://doi.org/10.1109/GCE.2010.5676129
Genetic variation in eurytopic and stenotopic mayflies

Miller MP, Blinn DW, Keim P (2002) Correlations between observed patterns of genetic differentiation in populations of four aquatic insect species in the Arizona White Mountains, USA. Freshwater Biology 48: 1660–1673. https://doi.org/10.1046/j.1365-2427.2002.00911.x

Mills SC, Grab SW (2005) Debris ridges along the southern Drakensberg escarpment as evidence for Quaternary glaciation in southern Africa. Quaternary International 129: 61–73. https://doi.org/10.1016/j.quaint.2004.04.007

Mills SC, Grab SW, Rea BR, Carr SJ, Farrow A (2012) Shifting westerlies and precipitation patterns during the Late Pleistocene in southern Africa determined using glacier reconstruction and mass balance modelling. Quaternary Science Reviews 55: 145–159. https://doi.org/10.1016/j.quascirev.2012.08.012

Monaghan MT, Spaak P, Robinson CT, Ward JV (2002) Population structure of three subalpine stream insects: influences of gene flow, demographics and habitat fragmentation. Journal of the North American Benthological Society 21: 114–131. https://doi.org/10.2307/1468304

Monaghan MT, Gattolliat JL, Sartori M, Elouard JM, James H, Derleth P, Glaizot O, de Moor F, Vogler AP (2005) Trans-oceanic and endemic origins of the small minnow mayflies (Ephemeroptera, Baetidae) of Madagascar. Proceedings of the Royal Society B: Biological Sciences 272: 1829–1836. https://doi.org/10.1098/rspb.2005.3139

Osmaston HA, Harrison SP (2005) The Late Quaternary glaciation of Africa: a regional synthesis. Quaternary International 138–139: 32–54. https://doi.org/10.1016/j.quaint.2005.02.005

Palumbi SR (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Moritz C, Mable BK (Eds) Molecular systematics. Sinauer and Associates, Sunderland, Massachusetts, 205–247. https://www.scienceopen.com/document?-1.IListener-header-action~bar-public-button-container-bookmark-button-bookmark-button&vid=4ad0dc5a-3a0a-44e9-a424-1814c15b17e9

Pavan-Kumar A, Gireesh-Babu P, Lakra W (2015) DNA metabarcoding: a new approach for rapid biodiversity assessment. Journal of Cell Science and Molecular Biology 2: 111.

Pereira-da-Conceicoa LL, Price BW, Barber-James HM, Barker NP, de Moor FC, Villet MH (2012) Cryptic variation in an ecological indicator organism: mitochondrial and nuclear DNA sequence data confirm distinct lineages of Baetis harrisoni Barnard (Ephemeroptera: Baetidae) in southern Africa. BMC Evolutionary Biology 12: 26. https://doi.org/10.1186/1471-2148-12-26

Phiri EE, Daniels SR (2013) Hidden in the highlands: the description and phylogenetic position of a novel endemic freshwater crab species (Potamonautidae: Potamonautes) from Zimbabwe. Invertebrate Systematics 27: 530–539. https://doi.org/10.1071/IS13012

Phiri EE, Daniels SR (2014) Disentangling the divergence and cladogenesis in the freshwater crab species (Potamonautidae: Potamonautes perlatus sensu lato) in the Cape Fold Mountains, South Africa, with the description of two novel cryptic lineages. Zoological Journal of the Linnean Society 170: 310–332. https://doi.org/10.1111/zoj.12103

Plaisance L, Knowlton N, Paulay G, Meyer C (2009) Reef-associated crustacean fauna: biodiversity estimates using semiquantitative sampling and DNA barcoding. Coral Reefs 28: 977–986. https://doi.org/10.1007/s00338-009-0543-3

Price BW, Barker NP, Villet MH (2007) Patterns and processes underlying evolutionary significant units in the Platylepura striadula L. species complex (Hemiptera: Cicadidae) in the Cape Floristic Region, South Africa. Molecular Ecology 16: 2574–2588. https://doi.org/10.1111/j.1365-294X.2007.03328.x
Price BW, Barker NP, Villet MH (2010) A watershed study on genetic diversity: phylogenetic analysis of the *Platypleura plumosa* (Hemiptera: Cicadidae) complex reveals catchment-specific lineages. Molecular Phylogenetics and Evolution 54: 617–626. https://doi.org/10.1016/j.ympev.2009.10.011

Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. Systematic Biology 67: 901–904. https://doi.org/10.1093/sysbio/sys032

Razgour O, Forester B, Taggart JB, Bekaert M, Juste J, Ibáñez C, Puechmaille SJ, Novella-Fernández R, Alberdi A, Manel S (2019) Considering adaptive genetic variation in climate change vulnerability assessment reduces species range loss projections. Proceedings of the National Academy of Sciences 116: 10418–10423. https://doi.org/10.1073/pnas.1820663116

Republic of South Africa (1998) South African National Water Act 36. Government Gazette 398: No. 19182.

Robinson O, Dylus D, Dessimoz C (2016) Phylo.io: interactive viewing and comparison of large phylogenetic trees on the web. Molecular Biology and Evolution 33: 2163–2166. https://doi.org/10.1093/molbev/msw080

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A (2017) DnaSP 6: DNA sequence polymorphism analysis of large datasets. Molecular Biology and Evolution 34: 3299–3302. https://doi.org/10.1093/molbev/msx248

Rutschmann S, Detering H, Simon S, Gattolliat JL, Delgada P (2016) Colonization and diversification of aquatic insects on three Macaronesian archipelagos using 59 nuclear loci derived from a draft genome. Molecular Phylogenetics and Evolution 107: 27–38. https://doi.org/10.1016/j.ympev.2016.10.007

Rutschmann S, Gattolliat JL, Hughes SJ, Báez M, Sartori M, Monaghan MT (2014) Evolution and island endemism of morphologically cryptic *Baetis* and *Cloeon* species (Ephemeroptera, Baetidae) on the Canary Islands and Madeira. Freshwater Biology 59: 2516–2527. https://doi.org/10.1111/fwb.12450

Schmidt SK, Hughes JM, Bunn SE (1995) Gene flow among conspecific populations of *Baetis* sp. (Ephemeroptera): adult flight and larval drift. Journal of the North American Benthological Society 14: 147–157. https://doi.org/10.2307/1467730

Siesser WG, Dingle RV (1981) Tertiary sea-level movements around southern Africa. The Journal of Geology 89: 523–536. https://doi.org/10.1086/628618

Slatkin M (1985) Gene flow in natural populations. Annual Review of Ecology and Systematics 16: 393–430. https://doi.org/10.1146/annurev.es.16.110185.002141

Slatkin M (1993) Isolation by distance in equilibrium and non-equilibrium populations. Evolution 47: 264–279. https://doi.org/10.1111/j.1558-5646.1993.tb01215.x

Snaddon CD, Davies BR (1998) A preliminary assessment of the effects of a small South African inter-basin water transfer on discharge and invertebrate community structure. River Research and Applications 14: 421–441. https://doi.org/10.1002/(SICI)1099-1646(199809)14:5%3C421::AID-RRA1099%3E3.0.CO;2-L
Ståhls G, Savolainen V (2008) MtDNA COI barcodes reveal cryptic diversity in the *Baetis ver- nus* group (Ephemeroptera, Baetidae). Molecular Phylogenetics and Evolution 46: 82–87. https://doi.org/10.1016/j.ympev.2007.09.009

Suh K, Hwang J, Jae Bae Y, Kang JH (2019) Comprehensive DNA barcodes for species identification and discovery of cryptic diversity in mayfly larvae from South Korea: Implications for freshwater ecosystem biomonitoring. Entomological Research 49: 46–54. https://doi.org/10.1111/1748-5967.12334

Sweeney BW, Funk DH (1991) Population genetics of the burrowing mayfly *Dolania americana*: geographic variation and the presence of a cryptic species. Aquatic Insects 13. https://doi.org/10.1080/01650429109361419

Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods), version 4.0b10. Sinauer and Associates, Sunderland, Massachusetts. https://doi.org/10.1111/j.0014-3820.2002.tb00191.x

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30: 2725–2729. https://doi.org/10.1093/molbev/mst197

Tenchini R, Cardoni S, Piredda R, Simeone MC, Belfiore C (2018) DNA barcoding and faunistic criteria for a revised taxonomy of Italian Ephemeroptera. European Zoological Journal 85: 253–266. https://doi.org/10.1080/24750263.2018.1480732

Thieme M, Lehner B, Abell R, Hamilton S, Kellndorfer J, Powell G, Riverso JC (2007) Freshwater conservation planning in data-poor areas: an example from a remote Amazon basin (Madre de Dios River, Peru and Bolivia). Biological Conservation 135: 484–501. https://doi.org/10.1016/j.biocon.2006.10.054

Tolley KA, Bowie R, Measey GJ, Price BW, Forest F (2014) The shifting landscape of genes since the Pliocene: terrestrial phylogeography in the Greater Cape Floristic Region. In: Allsopp N, Colville J, Verboom GA (Eds) Fynbos: ecology, evolution and conservation of a megadiverse region. Oxford. https://doi.org/10.1093/acprof:oso/9780199679584.003.0007

Toussaint EFA, Sagata K, Surbakti S, Hendrich L, Balke M (2013) Australasian sky islands act as a diversity pump facilitating peripheral speciation and complex reversal from narrow endemic to widespread ecological supertramp. Ecology and Evolution 3: 1031–1049. https://doi.org/10.1002/ece3.517

Toussaint EFA, Hall R, Monaghan MT, Sagata K, Ibalim S, Shaverdo HV, Vogler AP, Pons J, Balke M (2014) The towering orogeny of New Guinea as a trigger for arthropod megadiversity. Nature Communications 5: 4001. https://doi.org/10.1038/ncomms5001

Vuataz L, Sartori M, Gattolliat J-L, Monaghan MT (2013) Endemism and diversification in freshwater insects of Madagascar revealed by coalescent and phylogenetic analysis of museum and field collections. Molecular Phylogenetics and Evolution 66: 979–991. https://doi.org/10.1016/j.ympev.2012.12.003

Wallace JB (1990) Recovery of lotic macroinvertebrate communities from disturbance. Environmental Management 14: 605–620. https://doi.org/10.1007/BF02394712

Williams HC, Ormerod SJ, Bruford MW (2006) Molecular systematics and phylogeography of the cryptic species complex *Baetis rhodani* (Ephemeroptera, Baetidae). Molecular Phylogenetics and Evolution 40: 370–382. https://doi.org/10.1016/j.ympev.2006.03.004
Wishart MJ (2000) Catchments as conservation units for riverine biodiversity. African Journal of Aquatic Science 25: 169–174. https://doi.org/10.2989/160859100780177749

Wishart MJ, Hughes JM (2001) Exploring patterns of population subdivision in the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae), in mountain streams of the south-western Cape, South Africa. Freshwater Biology 46: 479–490. https://doi.org/10.1046/j.1365-2427.2001.00691.x

Wishart MJ, Davies BR (2003) Beyond catchment considerations in the conservation of lotic biodiversity. Aquatic Conservation: Marine and Freshwater Ecosystems 13: 429–437. https://doi.org/10.1002/aqc.600

Wishart MJ, Hughes JM (2003) Genetic population structure of the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae) in streams of the south-western Cape, South Africa: implications for dispersal. Freshwater Biology 48: 28–38. https://doi.org/10.1046/j.1365-2427.2003.00958.x

Wishart MJ, Davies BR, Stewart BA, Hughes JM (2003) Examining catchments as functional units for the conservation of riverine biota and maintenance of biodiversity. Water Research Commission, Pretoria, South Africa. http://www.fwr.org/wrcsa/975102.htm [July 13, 2019]

Xia X (2017) DAMBE6: new tools for microbial genomics, phylogenetics, and molecular evolution. The Journal of Heredity 108: 431–437. https://doi.org/10.1093/jhered/esx033

Xia X, Lemey P (2009) Assessing substitution saturation with DAMBE. In: Lemey P, Salemi M, Vandamme AM (Eds) The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing. Cambridge University Press, Cambridge, 611–626. https://doi.org/10.1017/CBO9780511819049.022

Xia X, Xie Z, Salemi M, Chen L, Wang Y (2003) An index of substitution saturation and its application. Molecular Phylogenetics and Evolution 26: 1–7. https://doi.org/10.1016/S1055-7903(02)00326-3

Yount JD, Niemi GJ (1990) Recovery of lotic communities and ecosystems from disturbance: a narrative review of case studies. Environmental Management 14: 547–569. https://doi.org/10.1007/BF02394709

Zloty J, Pritchard G, Krishnaraj R (1993) Larval insect identification by cellulose acetate gel electrophoresis and its application to life history evaluation and cohort analysis. Journal of the North American Benthological Society 12: 270–278. https://doi.org/10.2307/1467462

**Supplementary material I**

**List of GenBank sequence accession numbers for each sample**
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