Phylogeography of *Aphanicercella cassida* (Plecoptera: Notonemourida): cryptic speciation?

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Abstract

Stoneflies (Plecoptera) in South Africa are limited to cool pristine mountain streams. This provides them with very limited habitat in which to survive. The species of stonefly studied in this project (*Aphanicercella cassida*) has a very wide distribution unlike most other species which have a limited range. Of particular interest is a population in Mpumalanga (north of South Africa) which is very disjunct from the other populations of *A. cassida* found widely in the southwestern Cape. Despite this large geographic distance the insects appear to be morphologically identical to one another. It is believed that there is limited or no gene flow between the northern and southern populations as stoneflies are poor fliers. Molecular analysis of the cytochrome oxidase I gene in the mitochondrial DNA was analysed to see if any genetic differentiation was occurring inspite of the morphological homogeneity. A 557 base pair region was compared across three populations of *A. cassida* one in Mpumalanga and two in the southwestern Cape. Two congeneric species, *A. bullata* and *A. scutata,* were used as outgroups. The findings of this study were quite dramatic. The northern population is extremely different from the southern populations at the molecular level. There is more variation between the two populations of *A. cassida* than between the two outgroup species and the split between the two disjunct populations displays evidence of an ancient vicariance event. This finding definitely warrants further investigation into whether the population of *A. cassida* in Mpumalanga is a cryptic species. More sampling has to be done within the studied populations and more populations should be added to the analysis before any definite decision can be made regarding this fascinating emergence of possible cryptic species in South African stoneflies.

Introduction

Plecoptera (stoneflies) in southern Africa are represented by two families, the Neoperlidae which have a tropical distribution, and the Notonemouridae which are restricted to cold mountain streams (Picker 1985; Stevens & Picker 1995). The Notonemourid stoneflies are believed to have a Northern Hemisphere origin and are included in the suborder Arctoperlaria (Stevens & Picker 1999) even though they display a southern distribution (Picker & Stevens 1997). They are found in Australia, Tasmania, New Zealand, South America, Madagascar and southern Africa, and are considered typical elements of the palaeogenic fauna (Stevens & Picker 1995). The Notonemouridae in southern Africa occur in the mountains of the southern Cape, through to the KwaZulu/Natal and Mpumalanga Drakensberg and Lesotho (Stevens & Picker 1995). The monophyly of the family is doubtful (Stevens & Picker 1999) and is probably a polyphyletic assemblage derived from various 'old pre-nemourid' lines of the northern Arctoperlaria group (Stevens & Picker 1995).

There are six genera in the family Notonemouridae, with four of them: *Desmonemoura* Tillyard, *Aphanicerca* Tillyard, *Aphanicercella* Tillyard and *Aphanicercopsis* Barnard being restricted to the fold mountains of the southwestern Cape (Stevens & Picker 1995). *Balinskycercella* Stevens & Picker, has a discrete distribution in the Lesotho-Drakensberg highlands. *Afronemoura* has a wider geographical distribution with scattered localities in KwaZulu/Natal and a northern population in the Mpumalanga Drakensberg (Stevens & Picker 1995).

The genus *Aphanicercella* is restricted to South Africa with the majority of the 11 known species concentrated in the southwestern Western Cape Province (Stevens & Picker 1999). *Aphanicercella cassida* Barnard has a distribution that contrasts with this and is

one of a few species occurring beyond the fynbos biome (southwestern Western Cape Province) (Stevens & Picker 1999). In fact, *A. cassida* has the widest distribution of all known southern African Notonemouridae with a disjunct distribution from the southern Cape to the Limpopo Province in the north (*Fig* 1) (Stevens & Picker 1995). Despite this very wide geographical range there is no apparent morphological divergence of populations (Stevens & Picker 1999).

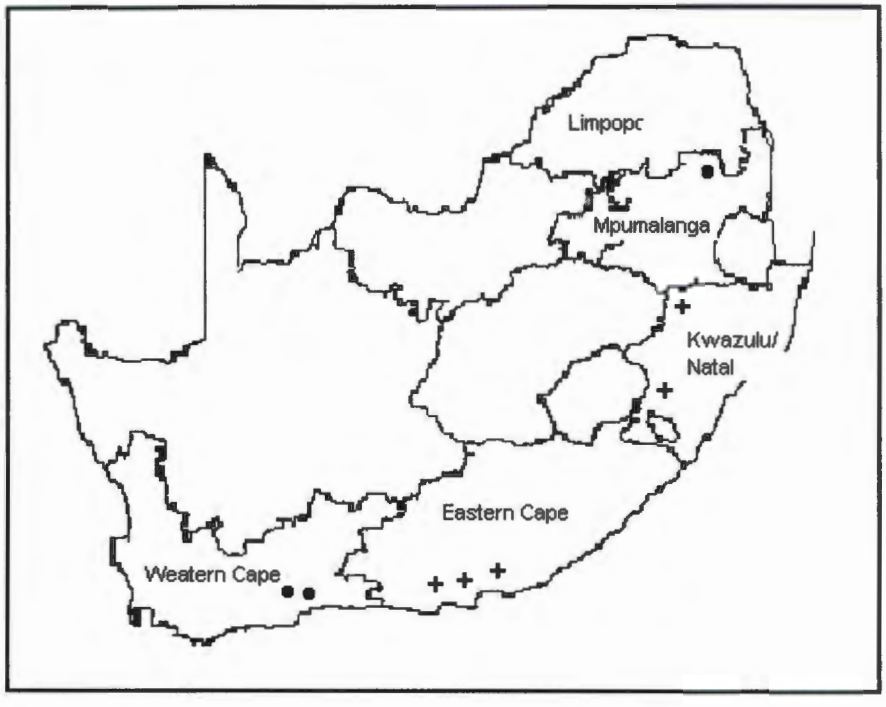

Fig. 1. Distribution of *Aphanicercella cassida* in South Africa. •, Study sites; +, Distribution not sampled.

Notonemourid taxonomy has been based primarily on external genitalia of the male (Stevens & Picker 1999). A recent example of this has been the resolution of the species complex *Aphanicercella barnardi* (Tillyard) into six separate species (Stevens &

Picker 1999) based on morphologically minor but discontinuous and discrete differences in genitalia, believed to indicate a lack of gene flow between the various forms. The localised geographical distributions support the argument that relatively recent speciation has occurred without habitat expansion (Stevens & Picker 1999).

The increasing availability of DNA sequence data for most major insect groups has allowed us to test the correlations between rates of molecular evolution and morphological diversification (McDaniel & Shaw 2002). Analysis of DNA sequences allows the estimation of evolutionary relationships among the different alleles of one locus. This historical information can then be combined with the observed geographical distribution of haplotypes to investigate the demographic factors that have produced the observed patterns (Mardulyn 2001). This approach allows one to discriminate between gene flow and historical events to explain the current distribution of genetic diversity in a population or species (Templeton 1998). This field of study concerned with the principles and processes governing the geographic distributions of genealogical lineages is Phylogeography (Avise 2000).

Genetic divergence unmarked by morphological change is known as cryptic speciation and is relatively rare, as there is a generally strong positive relationship between molecular and morphological evolution (McDaniel & Shaw 2002).

The present study examines phylogeographic structure among populations of the stonefly, *Aphanicercella cassida*, which is disjunct between the southern Cape and the Limpopo Province. The purpose of this study was to determine if cryptic speciation was occurring in the northern population of *A. cassida* as a result of ancient vicariance or an

isolated long-distance dispersal event, as opposed to ongoing migration. If there were cryptic speciation then we would expect to find reciprocal monophyly between northern and southern populations, whereas migration would result in little or no congruence between geographic isolation and phylogenetic structure.

Morphological studies (Stevens & Picker 1999) have found no apparent divergence in populations even though they are geographically isolated. The adult stoneflies are present for a brief period compared to the aquatic larvae and are able to scuttle rapidly over rocks but only occasionally resort to weak fluttering flight to elude capture (Picker 1985). This suggests that they are poorly adapted for long-distance dispersal.

To test if any of the *A. cassida* populations harboured cryptic species the mitochondrial DNA sequence variation in the cytochrome *c* oxidase subunit I gene (*COI*) was examined. Mitochondrial DNA has a number of features which make it the most widely used phylogenetic marker for insects as well as for animals in general: maternal inheritance, typically without intermolecular genetic recombination; rapid evolution at the nucleotide sequence level; and extensive intraspecific polymorphism the great majority of which is apportioned among rather than within individuals (Avise 2000). The *COI* gene is among the most conservative protein-coding genes in the mitochondrial genome of animals (Folmer *et al.* 1994).

The objective of this study was to determine if there was any gene flow between the isolated northern populations of *Aphanicercella cassida* and the southern populations. If no gene flow is found, then has sufficient differentiation taken place in the northern population to warrant recognition as a cryptic species.

Materials and Methods

Species identification

The genera of southern African Notonemouridae are readily separable, both as adults (Stevens & Picker 1995) and larvae (Picker & Stevens 1997). Although the species identification of larvae can be difficult (Picker & Stevens 1999) the nymphs of *Aphanicercella cassida* are easily distinguishable from other *Aphanicercella* species (Picker & Stevens 1997). D. Stevens (University of Cape Town, Department of Zoology) identified larvae collected from the Mpumalanga Province in June 2002. Although examination of adult male genitalia is required for definite identification, *A. cassida* is the only known species of *Aphanicercella* to occur in the Mpumalanga Province (Stevens & Picker 1995) along with having discernible features (Picker & Stevens 1997).

Sample collection

A total of 21 individuals from three populations of *Aphanicercella cassida* were used for the molecular analysis and samples of the congeners *Aphanicercella bullata* and *Aphanicercella scutata* were collected as outgroups (Table 1). All samples were placed in 100% ethanol directly after collection.

Table 1 Collection locations and sample sizes

Species/Collection location	Collector	Date	Lattitude	Longitude	Individuals sequenced
Aphanicercella cassida					
(A) Lisbon River, Mpumalanga Drakensberg, Mpumalanga	JAS	22/06/02	24 53'S	30 51'E	9
(C) Seweweekspoort, Klein Swartberge, Western Cape	DMS	08/07/02	33 24'S	21 24'E	7
(D) Prince Alfred's Pass, Outeniqua Mountains, Western Cape	DMS	19/08/02	33 52'S	23 11'E	5
Aphanicercella bullata					
(E) Cloetes Pass, Langeberg Mountains, Western Cape	DMS	05/08/02	33 55'S	21 45'E	1
(F) Grootswartberge, Western Cape	DMS	09/07/02	33 25'S	22 23'E	1
Aphanicercella scutata					
(G) Bains Kloof Pass, Limietberge, Western Cape	DMS	04/09/02	33 33'S	19 09'E	1
(H) Cederberg, Western Cape	DMS	03/08/01	32 27'S	19 10'E	1

JAS = J. D. van Alphen-Stahl
DMS = D. M. Stevens

DNA extraction, polymerase chain reaction and sequencing

DNA was extracted using a modified CTAB DNA extraction protocol (Villesen 1999). Modifications: 350-µl of 2xCTAB buffer was used and incubation in the water bath was at 60 °C for two hours. The supernatant was precipitated with 1xvolume freezer cold isopropanol.

The polymerase chain reaction (PCR) was used to amplify a 557-base pair (bp) fragment of the mitochondrial gene cytochrome oxidase I (COI) from all individuals sampled (Table 1) using primers LCO1490 and HCO2198 (Folmer *et al.* 1994). 3-µl of the DNA extract was used as template for a 30-µl PCR reaction, using 0.75 units of Taq polymerase per reaction. Each 30-µl reaction consisted of 3-µl 10x NH_4 buffer, 3-µl of 25mM $MgCl_2$, 1 µl of each of the two primer stock solutions (10 µM), 1.2 µl of dNTPs (25mM each), and 17.65 µl sterile distilled water. Reactions were amplified through 35

cycles at the following parameters: one minute at 95 C, one minute at 40 C, and one and a half at 72 C, followed by a final extension step at 72 C for seven minutes. Amplifications were visualised by running 3 µl on a 1% agarose gel stained with ethidium bromide. PCR products were purified using QIAquick spin columns (Quiagen) and prepared for direct sequencing. All PCR products were sequenced in both directions using ABI's Big Dye Terminator kit (Applied Biosystems). Each 10 µl sequencing reaction contained 2µl dye terminator reaction mix, 0.16 µl primer (10 µM), 2 µl 2.5x Buffer, 1.84 µl ddH2O, and 4 µl DNA template. Template dilution ranged from 1:1 to 4:0 depending upon PCR product yield. Cycle sequencing consisted of 30 cycles of 15s at 96 C, 15s at 50 C and 4 min at 60 C. Sequences were run on an ABI 3900 automated sequencer.

Sequence alignment

The forward and reverse sequences were assembled using Seqman (LaserGene systems software, DNAstar inc.) and aligned visually using MegAlign (LaserGene systems software, DNAstar inc.)

Phylogenetic and phylogeographic analyses

Phylogenetic analyses were conducted using PAUP* 4.0b8 (Swofford unpublished) on a Macintosh. A distance tree was generated using the Tamura-Nei distance measure and using a heuristic search procedure. The tree was rooted on two congeneric species, *Aphanicercella bullata* and *Aphanicercella scutata.* A haplotype network was constructed by TCS version 1.13 (Clement *et al.* 2000) estimating relationships between haplotypes at the 0.95 cumulative probability level. Analyses of within- and among-region genetic parameters were conducted using the program Arlequin 2.0 (Schneider *et*

al. 2001). AMOVA (Analysis of MOlecular VAriance) was performed to test the percentage of variation in haplotypes among populations and within populations. Inter-population analyses consisted of population pairwise F_{ST}s (fixation index) and Tamura-Nei corrected average pairwise difference. Analyses at the intra-population level were tested with Mean number of pairwise differences and nucleotide diversity.

Results

A 557-bp portion of the mtDNA COI gene was sequenced for 25 individuals sampled from seven populations.

Phylogenetic analysis

The tree resulting from analysis of the nucleotide data (*Fig. 2*) shows the northern and southern populations of *Aphanicercella cassida* as being reciprocally monophyletic. The COI region of stonefly mtDNA shows a great deal of variation within populations. Only three pairs of individuals showed the same haplotype among the nine individuals sampled from the Mpumalanga (northern) population with six different haplotypes in total. The two southern populations showed 12 discrete haplotypes with no sharing within or between the two. This tree shows the northern and southern populations as being monophyletic sister groups with strong bootstrap support (*Fig 2*) for the monophyly of the northern population. Within the southern clade there is no resolution between the two localities. The two outgroups, *A. bullata* and *A. scutata*, form monophyletic groups with no shared haplotypes.

The genetic distance between the northern population of stoneflies and the southern populations of the same species, *A. cassida*, is greater than the distance between the two outgroup species, *A. bullata* and *A. scutata*.

A haplotype network constructed by TCS (*Fig 3a & 3b*) to suggest mechanisms for genetic variation within and between populations. The haplotypes of the southern populations showed links between the two different localities ('C'+'D', *see Table 1*) but the northern haplotypes fail to link with the southern ones at the 0.95 cumulative probability level, and the northern individuals. Some of the 'D' haplotypes share an ancestor with 'C' haplotypes as can be seen in *Fig 3a*.

The AMOVA analysis showed that 70.6% of molecular variation was due to haplotype differences among populations and 29.4% within populations. Northern and southern populations were separated by a minimum Tamura-Nei's corrected distance (d_A) of 11.7 (Mpumalanga vs Klein Swartberge) and a maximum of 12.9 (Mpumalanga vs Outeniqua Mts) (*Table 2*) while there was only 0.83 difference between the two southern populations. The F_{ST} values between the northern population and the southern populations were greater than 0.77 and were significant at the $P<0.05$ level with the two southern populations having an F_{ST} value of 0.14 ($P<0.05$) (*Table 2*).

Table 2. Between population demographic statistics based on *COI* sequences

Tamura-Nei corrected distance (d_A) above diagonal F_{ST} values below diagonal

	1	2	3
1. Mpumalanga	*	11.7	12.9
2. Klein Swartberge	0.78	*	0.8
3. Outeniqua Mts	0.77	0.14	*

All values significant at $P<0.05$

Analysis of intra-population level variation was expressed by the mean number of pairwise differences and nucleotide diversity (*Table 3*). The southern populations have higher variation amongst individuals than the northern population with at least twice as much pairwise difference and nucleotide diversity.

Table 3. Analysis of intra-population variation

Population	**Mpumalanga**	**Klein Swartberge**	**Outeniqua Mts**
Mean no. of pairwise differences:	2.1+/- 1.3	4.5 +/- 2.5	7.0 +/- 3.9
Nucleotide diversity:	0.004 +/- 0.003	0.008 +/- 0.005	0.013 +/- 0.008

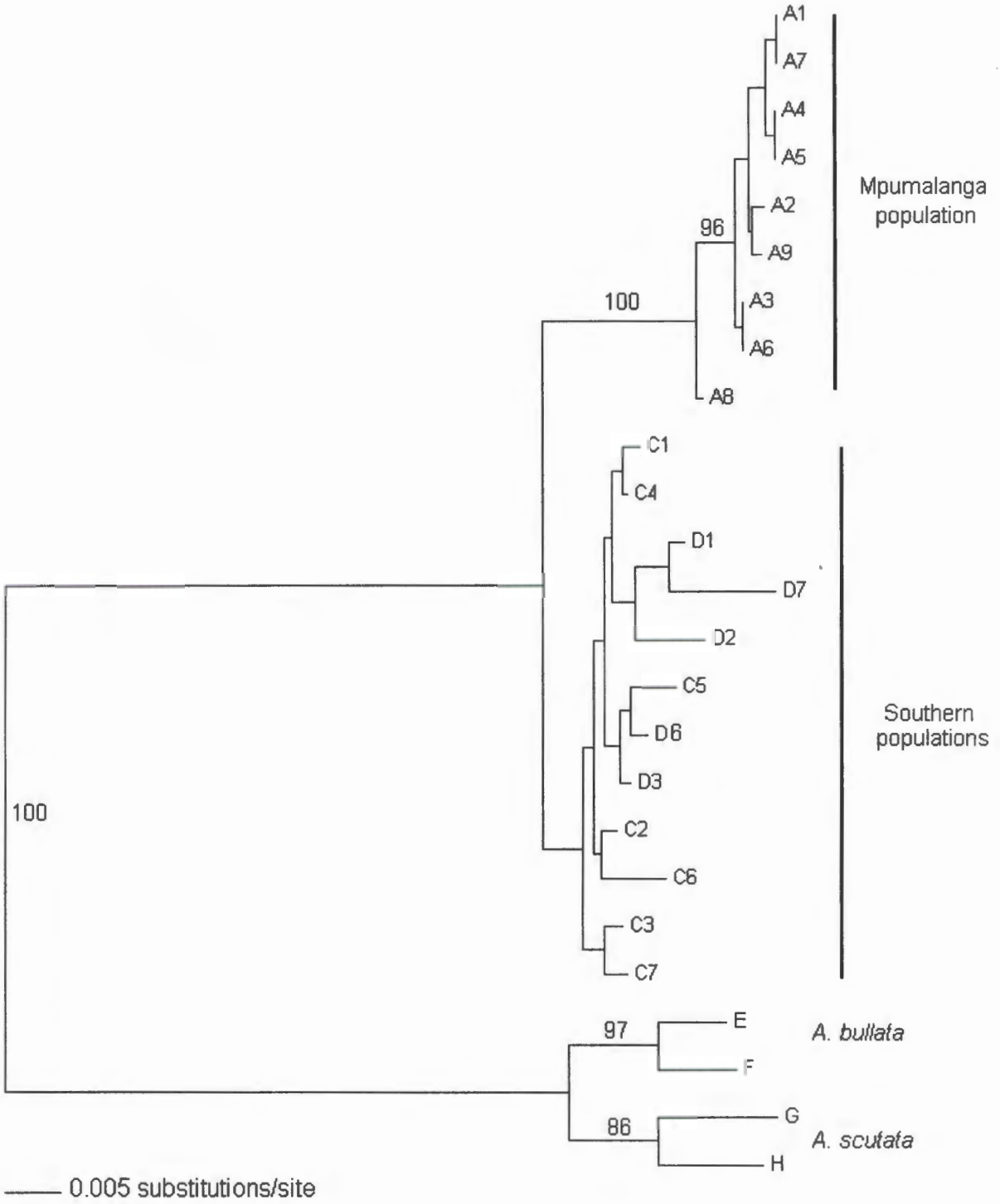

Fig. 2. Distance tree indicating genetic differentiation among individuals. Numbers at nodes indicate bootstrap values. The haplotype names on the terminals correspond to collections in *Table 1*.

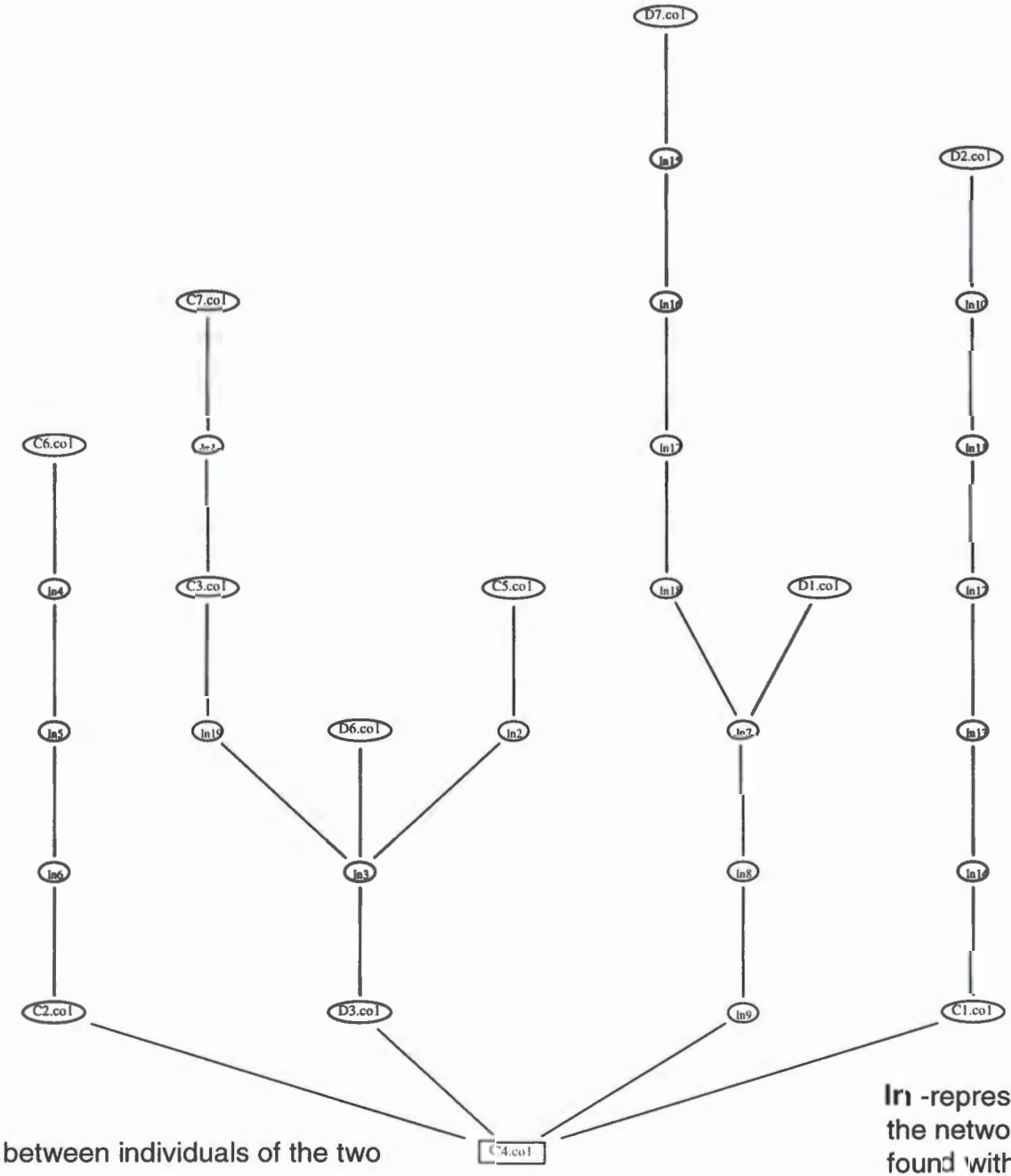

Fig 3a. Haplotype network between individuals of the two southern localities

In -represents missing links in the network which may be found with greater sampling

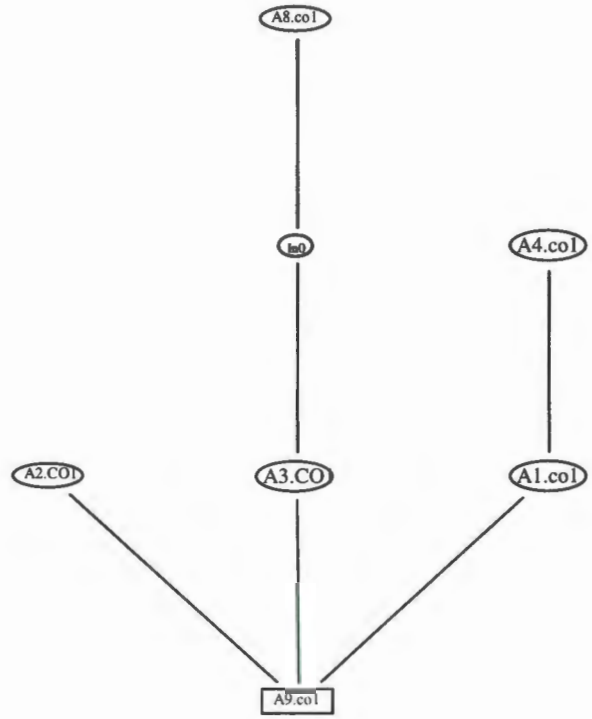

Fig 3b. Haplotype network of Mpumalanga population of *Aphanicercella cassida*

Discussion

The reciprocal monophyly of the northern and southern haplotypes of *A. cassida* strongly supports either an ancient vicariance or long-distance dispersal event, rather than ongoing migration. This finding, combined with the degree of molecular divergence between northern and southern populations, makes the morphological uniformity between disjunct populations even more remarkable. Stevens and Picker (1999) report that there is no apparent morphological difference between populations, despite the fact that genetic distance between the populations in this study exceed that between two other species in the same genus *A. bullata* and *A. scutata.*

Although there is a large amount of haplotype variation within populations, the fact that the northern population is very separate from the southern populations in a haplotype network (they do not join) reinforces the idea that there was an ancient separation. These findings are most consistent with ancient vicariance followed by molecular differentiation, particularly combined with the knowledge that stoneflies are weak fliers and long-distance dispersal is highly unlikely. It is evident from the tree that there is no gene flow between the northern and southern populations and there hasn't been for a long time.

The haplotype network (*Fig 3a*) shows some 'D' haplotypes sharing an ancestor with 'C' haplotypes. This implies that there is migration between the two localities and as a result gene flow. There is no link at the 0.95 cumulative probability level between the northern population and the southern populations implying lack of migration. This is hardly surprising as there is a vast geographical distance between the two.

Inter- and intra-population variance is tested by Arlequin (*Table 2 & 3*) and shows how much genetic variation there is between populations and how much variation there is within populations. The Tamura-Nei corrected distance and F_{ST} values (*Table 2*) show clearly that there is very little difference between the two southern populations and a large differentiation between southern and northern populations. This is an indication of how isolated these populations are in terms of gene flow. The very high nucleotide diversity in the population from the Outeniqua mountains (*Table 3*) indicates a prominent amount of gene flow within that population. This may be due to a complex local environment with a diverse array of aquatic niches.

Even though there is a clear distinction between the northern and southern populations, no categorical statements can be made as to the mode of differentiation. This is due to the small sample size of both populations and individuals within populations. There are populations of *A. cassida* in KwaZulu/Natal that may provide a link between the southern and northern populations. To get a true idea of the phylogeography of *A. cassida* it is necessary to sample a number of populations in each of the Mpumalanga Drakensberg, KwaZulu/Natal Drakensberg, Western Cape and Eastern Cape.

Indications from this study are that the populations of *Aphanicercella cassida* in Mpumalanga have undergone cryptic speciation and are very different at the molecular level from those in the Western Cape. Further research will have to be done to determine if the northern variety warrants species status. This will have implications for its conservation, as the preservation of the watershed in which they occur is essential for their survival.

The species *Afronemoura amatolae* shows a similar disjunct distribution, occurring in the Amatola Mountains in the south and the Mpumalanga Drakensberg in the north (Picker & Stevens 1999). A phylogeographic study of this species may show similar results to that of *Aphanicercella cassida* and add further evidence to the means of genetic divergence.

Stoneflies are used as bioindicators in limnological studies because they are restricted to pristine mountain streams. The advent of molecular systematics has multiplied the potential applications of aquatic insects as bioindicators. In the past, identification of insects was restricted by the stage in the life cycle and the sex of the insect; for example stonefly identification was based on the genitalia of adult males (Stevens & Picker 1999). Molecular markers, particularly mitochondrial DNA, can be used to identify adult or larvae and male or female stoneflies. This is particularly useful since adults are only present for a brief period compared to the aquatic larvae, and are frequently scarcer and more difficult to collect. In addition, limnological studies invariably sample a larval stage and not the adult in species where the latter is terrestrial. The determination of inter-basin water transfers is a potential use for molecular studies as very little is known about the effects that dams have on the distribution of biota in a watershed.

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