# Phylogenetic relationships of *Archaeochlus* Brundin, *Austrochlus* Cranston and *Afrochlus* Freeman (Diptera: Chironomidae), basal genera with a Gondwanan connection

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In this paper the phylogenetic relationships among the podonomine genera *Archaeochlus* Brundin, *Austrochlus* Cranston and *Afrochlus* Freeman are re-examined using DNA sequence data from two mitochondrial (mt) genes, *Cytochrome b* and *Cytochrome oxidase subunit II*, and the nuclear 18S ribosomal RNA gene. The results confirm that the African species of *Archaeochlus* and the Australian *Austrochlus* species are not monophyletic. *Afrochlus* is, however, monophyletic within *Archaeochlus* and there is some doubt as to whether generic separation is justified. Although *Afrochlus* is a monotypic genus, larvae of two species, form A and form B, were present in the studied sample from the type locality. These two species appear differentiated in their mt sequences, although more closely related on the basis of their 18S sequences. Form A appears more closely related to the species of *Archaeochlus* than to form B. Some of the African *Archaeochlus* species show a similar amount of sequence divergence to the populations of the Australian species. They appear to have diverged only within the last 10 my, while the Australian species are at least 18 my in age.

## INTRODUCTION

Although originally described from the Northern Hemisphere, Brundin (1966) clearly showed that the subfamily Podonominae had a higher diversity in the Southern Hemisphere, consistent with a Gondwanan origin. He considered the genus *Archaeochlus* Brundin, 1966, to be the most plesiomorphic podonomine, with *Afrochlus* Freeman, 1964, its probable sister-group. The plesiomorphic status of *Archaeochlus* was confirmed when adults from Western Australia were found to possess biting mouthparts (Cranston *et al.* 1987; Downes & Colless 1967). The presence of apparently congeneric species in Australia strengthened the case for *Archaeochlus* being of Gondwanan origin. Since Africa had separated from Antarctica and Australia by 120 mya, the African and Australian species are assumed to have been isolated from each other for at least this length of time (Cranston *et al.* 1987). Edward (1989) used enzyme electrophoresis to show that there was little genetic similarity between African and Australian species, consistent with a long period of isolation.

The larvae of this group live in the episodic streams associated with rock outcrops (Figure 1), with the exception of one *Archaeochlus* species from Namibia (Cranston *et al*. 2002). Suitable habitats are, therefore, disjunct in occurrence, raising questions as to how the species have achieved their present distributions, as the adults appear disinclined to fly great distances (Martin *et al*. 2002). Martin *et al.* (2002) used molecular data to investigate the

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Figure **1**. Episodic watercourse at the type locality of *Afrochlus harrisoni* Freeman (Chironomidae) near the summit of a granite outcrop, Ngoma Kuriru, Chindomora Reserve, near Harare, Zimbabwe. This is also typical of the larval habitat of most species of *Archaeochlus* Brundin and *Austrochlus* Cranston (Photograph courtesy of P.S. Cranston).

relationships between the Australian *Austrochlus* species. One unexpected finding from that study was that the populations of these species were of relatively recent origin, indicating that, under certain circumstances, species have the potential to cross the normally unsuitable terrain between granite outcrops. The mitochondrial (mt) gene used was, however, unable to clearly resolve the relationships between the species, which are considerably older. A similar lack of resolution of the most basal bran-ches was encountered in the molecular phylogeny of the chironomid genus *Chironomus* Meigen, 1803, based on mt genes (Guryev *et al*. 2001). A more slowly evolving gene was, however, required if the branching order at lower levels was to be clarified. As the unresolved branches in both *Archaeochlus* and *Chironomus* date to 100 mya or more, the nuclear 18S rDNA gene was selected for analysis, as it has been shown to work well for this time interval (Whiting *et al*. 1997). Relationships within and between the African and Australian members of the mandibulate podonomines are here compared using two mt genes and the nuclear gene.

## MATERIAL & METHODS

The species and populations used in this study, as well as the codes for localities used in the text, are listed in Table 1. Some of the species have not been described and are given identifying names relating to the locality from which they were collected.

Total DNA, used for mtDNA amplification from some populations, was initially isolated from ten or more larvae as described in Guryev *et al*. (2001). For subsequent samples, including all those used for 18S rDNA, total DNA was isolated from individual larvae using the technique of Sunnucks & Hales (1996). Six hundred and ninety-one base pairs (bp) from the mt cytochrome b (*cob*) gene were amplified and sequenced using the primers as in Guryev *et al*. (2001), while 519 bp from the mt cytochrome oxidase II (*cox2*) and approximately 1023 bp of the nuclear 18S rDNA (18S) gene were amplified and sequenced using the primers listed in Table 2. PCR and sequencing of mtDNA followed Guryev *et al*. (2001). PCR conditions for 18S were essentially as in Whiting *et al*. (1997), but maintaining the denaturation time constant through all cycles. Initially primers 18Sai and 18Sbi were used for 18S, but amplification proved to be very sensitive to PCR conditions and solutions. The combination of 18Sb.05 with 18Sai was found to give superior results and so was used for later runs. The 18Sai / b0.5 combination produces a fragment with about 200 less bases at the 3' end (Whiting *et al*. 1997). The DNA fragments were sequenced in both forward and reverse directions using BigDye terminator chemistry (Perkin-Elmer, USA) on automated sequencers at the Australian Genome Research Facility in Brisbane, Queensland, Australia.

Nucleotide sequence accession numbers in GenBank for these sequences are: *cox2* - AY261430 AY261455; *cob* - AF425719 - AF425732, AY263798-AY263806; and 18S - AY257511-AY257524, AY259178-AY259179. The *cob* sequences, AF425719-AF425732, are from Martin *et al*. (2002)

Mitochondrial nucleotide sequences were translated using the mt code to ensure that there were no stop codons or other anomalies, and were aligned

**Table 1.** List of Podonominae and Tanypodinae species (Diptera: Chironomidae), plus Diptera: Ceratopgonidae populations used in the study. For author names and dates of publication *vide* text. Letter and numerical codes for localities as used in the text are provided in column three.

| Species | Population | Coding |
|---|---|---|
| **Podonominae** | | |
| *Austrochlus brundini* | Baladjie Rock, Western Australia | BaR |
| | Tandegin Rock, SE Merredin, Western Australia | TR |
| | Yeerakine Rock, Western Australia | YeR |
| | Yorkrakine Rock, N Tammin, Western Australia | YoR |
| *Austrochlus parabrundini* | Boorabbin Rock, Western Australia | BoR |
| | Dead Horse Rock, N Menzies, Western Australia | DHR |
| | Emu Hill, SW Narembeen, Western Australia | EH |
| *Austrochlus centralaustralis* | John Hayes Waterhole, East MacDonnell Ranges, Northern Territory. | JHW |
| *Archaeochlus drakensbergensis* | Qachas Neck, Drakensberg Mts., South Africa | - |
| *Archaeochlus* sp. 'Amieb' | Bulls Party, Farm Amieb, Erongo Mts., Namibia | - |
| *Archaeochlus* sp. 'Matchless' (1 pupa, 1 adult) | Matchless River, Namibia. | - |
| *Afrochlus harrisoni* | Ngoma Kurira, Harare, Zimbabwe | A & B |
| **Tanypodinae** | | |
| *Procladius villosimanus* | 'The Barcoo' Litzows Rd., Tarampa, Queensland, Australia | |
| **Ceratopogonidae** | | |
| *Dasyhelea* sp. | North Balwyn, Victoria, Australia | |

manually without ambiguity. The 18S sequences were aligned using SeqEd v.1.0.1 because the presence of indels precluded manual alignment. It was found necessary, however, to apply a small number of manual corrections, where the automatic alignment between certain species was not the most logical alignment when compared to the sequences and alignments of other, more closely related, species. When the regions associated with gaps (*vide supra*) and the unclear regions close to the primers were removed, 787 bp of the 18S gene were included in the analysis. The alignments were used for construction of trees based on the Neighbor-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods implemented in the PAUP* v.4.0b10 (Swofford 1998) package. Statistical support for the NJ and MP trees was evaluated by bootstrapping (bs) (1000 replications) (Felsenstein 1985). The models to be used in the ML analyses were determined using Modeltest v.6.1.0 (Posada & Crandall 1998). Consensus between trees generated by different genes was tested using the Templeton non-parametric (TNP) (Templeton 1983), the Kishino-Hasegawa (KH) (Kishino & Hasegawa 1989) and the Shimodaira-Hasegawa (SH) (Shimodaira & Hasegawa 1999) tests, as appropriate, as implemented in PAUP*. Values indicated by an asterisk are significant at the 0.05 level. Tamura-Nei distances (Tamura & Nei 1993) were calculated using PAUP* (Swofford 1998), and the standard errors using MEGA2 (Kumar *et al.* 2001).

## RESULTS

The writers sampled the three Australian species of *Austrochlus* Cranston, 2002, *Au. brundini* (Cranston, Edward & Colless, 1987) (four populations), *Au. parabrundini* Cranston, Edward & Cook, 2002 (three populations), and *Au. centralaustralis* Cranston, Edward & Cook, 2002 (one population), as well as a single population of each of four southern African species: *Afrochlus harrisoni* Freeman, 1964, *Archaeochlus drakensbergensis* Brundin, 1966 and two undescribed species of *Archaeochlus* from Namibia. One of these is *Archaeochlus* sp. 'Amieb' used as an outgroup by Martin *et al.* (2002), and corresponds to the *Archaeochlus* 'Amieb' of

**Table 2**. Nucleotide sequences of oligonucleotide primers used in this study. Abbreviations: ***cox2*** = cytochrome oxidase II; **18S** = 18S ribosomalDNA

| Gene | Primers | Length of Amplified Fragment | References |
|---|---|---|---|
| ***cox2*** | C2-J-3138m 5'- AGAGCTTCTCCTTTAATAGAACA -3'<br>C2-N-3661 5'-CCACAAATTTCTGAACATTGACCA –3' | 617 bp | modified after Simon *et al.* (1994) |
| **18S** | 18Sai 5'- CCTGAGAAACGGCTACCACATC -3'<br>18Sbi 5'- GAGTCTCGTTCGTTATCGGA -3'<br>18Sb0.5 5'- GTTTCAGCTTTGCAACCAT –3' | abt 1023 bp<br>abt 824 bp | Whiting *et al.* (1997) |

Cranston *et al*. (2002). The other species, *Archaeochlus* sp. 'Matchless' does not correspond to either of the species 'Namibia 1' and 'Namibia 2' of Cranston *et al*. (2002), which are a further new species and *Archaeochlus biko* Cranston, Edward & Colless, 1987 (P.S. Cranston pers. comm.). It is, however, the Namibian species with a different ecology referred to in Cranston *et al*. (2002: 358). A tanypodine chironomid, *Procladius villosimanus* Kieffer, 1917, and a species of *Dasyhelea* Kieffer, 1911 (Diptera: Ceratopogonidae), were used as outgroups. The *Dasyhelea* species used in the analysis is closely related, but not identical, with that used by Cranston *et al*. (2002), differing by 6% at the *cox2* locus (L.G. Cook pers. comm.). A number of other species from *Aedes* Meigen, 1818 (Diptera: Culicidae), *Drosophila* Fallén, 1923 (Diptera: Drosophilidae) and *Chironomus*, were tested as outgroups, but were found to make no difference to the topology of the trees obtained (data not shown). An unexpected finding was the presence of two taxa in the population of *Af. harrisoni*, which are referred to as *Af. harrisoni* A and *Af. harrisoni* B, respectively. The form B, as used here, corresponds to the '*Af. harrisoni*' used by Cranston *et al*. (2002) (based on *cox2* comparisons (J. Martin & L.G. Cook unpubl.).

None of the data sets was found to differ significantly in the base ratios between different samples (Homogeneity Chi-squared probability values about 0.96 or higher) (Table 3). There was no marked difference in the base ratios of the two mt genes (Homogeneity Chi-squared = 2.329, $df$=3, $p$=0.49), and their Adenine plus Thymine (AT) frequency was higher than in 18S, a well-documented characteristic of insect mt genes (Simon *et al*. 1994).

The level of base pair differences between different taxonomic levels, from within populations to between ingroups and outgroups, for both the mt and the 18S genes are provided in Table 4. This differs between the mt and nuclear genes, as would be expected from their different substitution rates. There are a number of points that can be made about these data. The low values within popu-lations may be a reflection that those for which this was checked were small populations that are likely to have been established from the

**Table 3.** Mean base composition of the three genes studied. Abbreviations: **A** = Adenine; **C** = Cytosine; **G** = Guanine; **T** = Thymine; ***cox*2** = mitochondrial cytochrome oxidase II; ***cob*** = mitochondrial cytochrome b; **comb mt** = combined mitochondrial sequences; **18S** = 18S ribosomalDNA.

| Gene | % A | % C | % G | % T | Chi square |
|---|---|---|---|---|---|
| ***cox*2** | 34.4 | 14.2 | 12.1 | 39.2 | 17.34 (*df*=33), *p*=0.989 |
| ***cob*** | 30.9 | 16.8 | 11.5 | 40.6 | 16.39 (*df*=42), *p* =0.999 |
| **comb. mt** | 32.5 | 15.6 | 11.8 | 40.2 | 20.16 (*df*=33), *p* =0.961 |
| **18S** | 30.5 | 17.9 | 24.1 | 27.3 | 7.21 (*df*=42), *p* =1.0 |

**Table 4.** Mean base pair differences between different taxonomic levels for the mitochondrial and 18S ribosomalDNA genes.

| Gene | Within pops* (*Austrochus*) | Between pops | Between species | Between ingroup genera | Between ingroups and outgroups |
|---|---|---|---|---|---|
| **mt** | 0.5-1.0 | 1-17 | 8-17 | 10-19 | 17-24 |
| **18S** | 0 | 1-6 | 1-9 | 2-10 | 5-10, 20-27 |

*Excluding the two forms (A & B) of *Afrochlus harrisoni*.

offspring of one or a few females. The lack of variation within populations for the 18S gene would however be expected because of its slow mutation rate (Whiting *et al*. 1997). The mt differentiation between populations of *Austrochlus* is mostly in the order of 1-6%, with the high values being attributable to those comparisons that involved *Au. brundini* BaR, as previously noted by Martin *et al*. (2002). This was not seen in the 18S comparisons, where those involving the BaR population were at the low end of the range. The broad overlap in the de-gree of differentiation between species and genera for the mt sequences is also in accord with the findings of Martin *et al*. (2002). While a similar overlap is seen in the 18S data, the cause is slightly different, being due to generally lower levels of differentiation between the species and genera within southern Africa, and is influenced by the two forms of *Af. harrisoni*. Form A differs from *Archaeochlus* only by 2-5%, while form B differs by 5-7%. The two forms differ from each other by 4%. In the mt data, there is 12% difference between the two forms of *Af. harrisoni*, at the high end of the differentiation between populations or species, and in the low range of differentiation between genera. This figure is intermediate between the *Af. harrisoni* A / *Archaeochlus* comparison (10-11%) and the *Af. harrisoni* B / *Archaeochlus* comparison (13-15%). A final point to note is the markedly bimodal range for comparisons between the ingroups and outgroups. This is easily explained, since the low values are those involving *P. villosimanus*, another chironomid, and so more closely related to the ingroup species than the *Dasyhelea* sp. The same effect is seen in the mt data where the equivalent figures are 17-22% between ingroups and *P. villosimanus*, and 21-24% between the chironomids and *Dasyhelea* sp.

It was determined from Modeltest (Posada & Crandall 1998) that HKV+Gamma was the most appropriate model for use in ML analyses. The MP analyses utilised the ASIS branch-swapping model, and the NJ analyses the TBR model, as implemented in PAUP* (Swofford 1998). Initially, the two mt genes were analysed separately. Only twelve sequences were available for the *cox2* gene, as no PCR product was obtained from three of the Australian samples, despite several attempts. This may indicate sequence changes in the region where primer binding needs to occur. Previous analyses have indicated saturation at the third base position of codons in the mt genes beyond the species level in this group (Martin *et al*. 2002). Therefore weighting the third base position at one fifth of the value of first and second base positions has been suggested (L.G. Cook, pers. comm.), and was tested against the unweighted

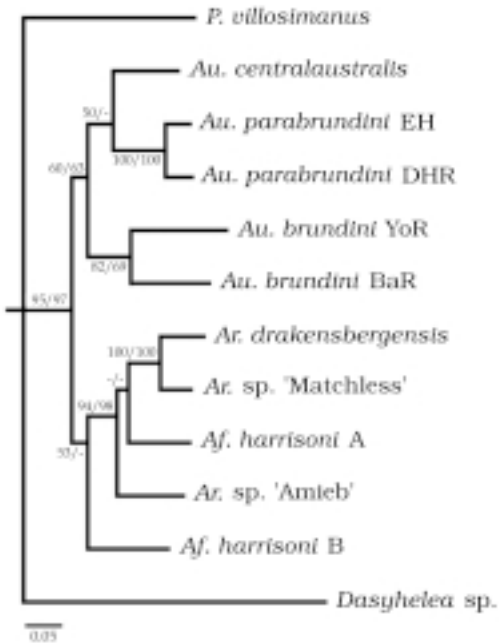


Figure **2**. ML tree inferred from the combined *cob* and *cox2* nucleotide sequence data, upon which is included the bootstrap values (based on 1000 replications) for the equivalent branches from the MP and NJ consensus trees. Scale represents 0.05 substitutions per site. Generic abbreviations: *Af.* = *Afrochlus*; *Ar.* = *Archaeochlus*; *Au.* = *Austrochlus*; *P.* = *Procladius*. The letter codes for the populations are as listed in Table 1.

data. The weighted data showed the same tree topology, but shorter branch lengths (results not shown). The similarity of the tree topology was confirmed by the KH test ($t$=1.1770) and Templeton's non-parametric (TNP) test ($n$=2, $z$=-1.3416), which gave respective probabilities of 0.24 and 0.18, indicating no significant difference between the trees. Consequently, the unweighted values were used for further analyses. It was also found that there was no significant difference between the ML and the consensus MP trees obtained from the *cox2* and the *cob* sequences (trees not shown). The SH (Diff –Ln $L$=19.57) and TNP ($n$=14, $z$=-0.5345) tests gave respective probabilities of 0.11 and 0.59. The NJ trees were also compared and found to be similar, although no significance test could be performed. The two sets of mt sequences were therefore concatenated and the ML tree, with the bootstrap values for the MP and NJ trees included, is shown in Figure 2. All methods produced essentially similar trees, which separate the Australian *Austrochlus* species from the African *Archaeochlus* and *Afrochlus* species. In the consensus MP and NJ trees there is high bs support for the African clade (excluding *Af. harrisoni* B) but lower support for an Australian clade because of low bs resolution of the *Af. harrisoni* B separation from the Australian species. The relationship of *Au. centralaustralis* in the Australian clade is unresolved in these trees. There is some indication that it may be more closely related to *Au. parabrundini* in the ML tree (Figure 2) and there is bs support of just 50% for that relationship in the MP tree.

Since 18S is a non-protein encoding gene, the DNA sequence is prone to the insertion or deletion of bases (indels) in regions not constrained by the secondary structure (Whiting *et al*. 1997). In this study, the majority of such indels occurred between the outgroup species, relative to the ingroup species. As such they had no phylogenetic significance, since they generally occurred, or were absent, in one species only. There were only four indels that were phylogenetically informative, all contrasting the *Austrochlus* species and the *Archaeochlus*/*Afrochlus* species (Table 5). These indels were not included in the analyses of the 18S sequences, since it was found that the small number of informative sites made no difference to the trees obtained. A region of about 45 bp near the 5' end of the *Ar.* sp. 'Amieb' sequence, which shows at least 11 changes compared to the other African sequences, was considered most likely to be a single mutation, i.e. an insertion that replaced the sequence seen in the other species. This region was, therefore, treated as an indel in the analyses. The analysed sequence, once the indels were removed, was 787 bp. Initial analyses used all 15 samples in order to see whether the 18S data supported the greater separation of the BaR population from other populations of *Au. brundini*, seen in the *cob*-derived phylogeny of Martin *et al*. (2002), and reproduced with good bs support in analyses of *cob* sequences for the present 15 samples (tree not shown). The 18S trees did not indicate that the BaR population was more differentiated than the other populations of *Au. brundini*, rather that the YoR population was more differentiated and clus-

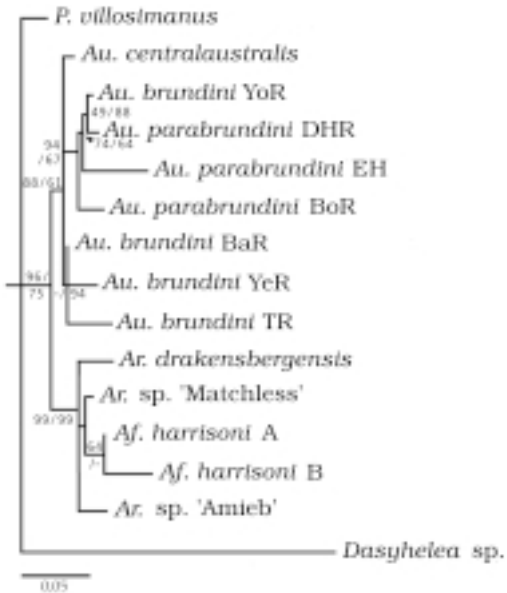


Figure **3**. ML tree inferred from the nucleotide sequence data of the nuclear 18S gene, upon which is included the bootstrap values (based on 1000 replications) for the equivalent branches from the MP and NJ consensus trees. Scale represents 0.05 substitutions per site. Generic abbreviations: *Af.* = *Afrochlus*; *Ar.* = *Archaeochlus*; *Au.* = *Austrochlus*; *P.* = *Procladius*. The letter codes for the populations are as listed in Table 1.

tered with the *Au. parabrundini* populations (BoR, DHR and EH). This relationship of the YoR population to the *Au. parabrundini* populations, as well as the other features of the tree for the 15 samples, is seen in the 18S trees of the 12 taxa used for the mt analyses (Figure 3). All trees clearly recognise an African clade, in line with the evidence from the indels (Table 5) and, with relatively low bs support in the NJ tree, an Australian clade. Within the African clade, the relationships of the various species are not well resolved. The two forms of *Af. harrisoni* cluster separately from the *Archaeochlus* species in the ML and MP trees, but not in the NJ tree. In the NJ tree *Ar. drakensbergensis* and *Ar.* sp. 'Matchless' form a separate cluster (bs=89), to *Ar.* sp. 'Amieb' and *Af. harrisoni* A, with *Af. harrisoni* B at the base of the group. However bs support for the separation of the last three mentioned taxa is only about 50%. The African clade in this NJ tree is similar to the trees obtained when the 45 bp region, assumed to be an indel in *Ar.* sp. 'Amieb', is included in the analyses (trees not shown).

Inspection of Figures 2 & 3 shows that the mt and 18S sequences tend to resolve different levels of branching in the respective trees. Thus, the faster evolving mt sequences better resolve the branches at the tip of the tree, while the slower evolving 18S sequences better resolve the basal branches. Combining the data may therefore give a better overall picture of the true phylogenetic relationships in this group. For the ML analysis, the mt and 18S trees were homogeneous (SH test: *Diff -Ln L*=5.41951, $p$=0.554), so this tree was used as the basis for a composite tree (Figure 4). Although it might be expected that the combined tree would be dominated by the greater number of informative sites in the mt data, there is a significant

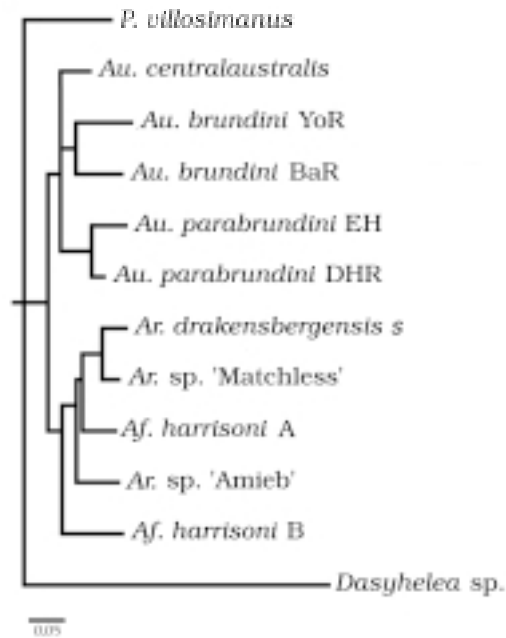


Figure **4**. Composite tree, based on the ML tree, inferred from the combined nucleotide sequence data of the mt and 18S genes. Scale represents 0.05 substitutions per site. Generic abbreviations: *Af.* = *Afrochlus*; *Ar.* = *Archaeochlus*; *Au.* = *Austrochlus*; *P.* = *Procladius*. The letter codes for the populations are as listed in Table 1.

**Table 5**. Regions of the four parsimony informative indels in the nucleotide sequence of the 18S rDNA gene. Letter codes listed after specific names refer to populations used in the study (*vide* Table 1 for full details).

| | 2222222222 3333 44444<br>7777777788 4445 99999<br>2345678901 7890 12345 |
|---|---|
| *Dasyhelea* sp. | CTAGGACTAT TKGG ATATA |
| *Procladius villosimanus* | -TA-----AT TTCG ATATA |
| *Archaeochlus drakensbergensis* | CTA------T C-CG AG--T |
| *Archaeochlus* sp. 'Amieb' | CTA------T C-CG AG--T |
| *Afrochlus harrisoni* A & B | CTA------T C-CG AG--T |
| *Austrochlus brundini* YoR | C-A-----GT T--G AAAAT |
| *Austrochlus brundini* YeR | C-A-----GT T--G AAAAT |
| *Austrochlus brundini* TR | C-A-----GT T--G AAAAT |
| *Austrochlus brundini* BaR | C-A-----GT T--G AAAAT |
| *Austrochlus centralaustralis* | C-A-----GT T--G AAAAT |
| *Austrochlus parabrundini* DHR | C-A-----GT T--G AAAAT |
| *Austrochlus parabrundini* EH | C-A-----GT T--G AAAAT |
| *Austrochlus parabrundini* BoR | C-A-----GT G--G AAAAT |

Note column 2 is base numbers.

difference between the combined and mt trees (SH test: *Diff -Ln L*=47.75015, *p*=0.007*). The composite tree differs from the ML tree only in the placement of *Au. centralaustralis.* The ML tree places *Au. centralaustralis* basal to the other *Austrochlus* species, while the MP and NJ combined trees show a common branch with the other two species, as in the 18S tree (Figure 3). This more conservative placement is therefore used in the composite tree, and is the major difference from the mt tree (Figure 2). The African species in the composite tree are essentially as in Figure 2. It may be noted that the MP and NJ analyses of the combined data provide more bs support for *Af. harrisoni* A being more closely related to *Ar. drakensbergensis* and *Ar.* sp. 'Matchless' than is *Ar.* sp. 'Amieb' (58% and 75% respectively) than did those analyses of the mt data, where this relationship was unresolved or reversed.

Cranston *et al.* (2002) used some different African species to those in this study, and incorporation of their species can provide additional information on the relationships of the African species. L.G. Cook (unpubl.) kindly provided 519 bp of *cox*2 sequences for *Ar. biko* and *Ar. bicirratus* Brundin, 1966. These sequences were included with the equivalent sequences from our *cox2* data and analysed by the MP, ML and NJ methods. The African portion of these trees (Figure 5) shows a grouping of *Ar. drakensbergensis*, *Ar.* sp. 'Matchless' and *Ar. biko*, with *Ar. bicirratus* less closely related to this group. *Ar.* sp. 'Amieb' species was grouped with the forms of *Af. harrisoni* and in the ML and NJ trees, *Af. harrisoni* B was basal to the other species as in Figure 2.

## DISCUSSION

In a previous paper Martin *et al.* (2002) investigated the relationships between populations of the Australian species of *Austrochlus* using sequence data from the mt *cob* gene. The phylogeny obtained gave well supported branching of the populations within each species, but less well resolved relationships between the species (Martin *et al.* 2002). The phylogeny also included only one species of the sister genus *Archaeochlus* from Africa, which was used as an outgroup. In this study, an additional mt gene, *cox2*, and the nuclear 18S gene were used to further resolve the relationships of the Australian species and of the African species, as well as those between the species on the two continents.

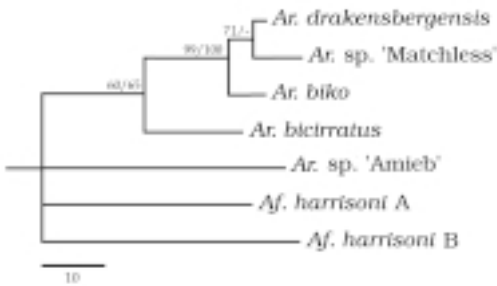


Figure **5**. Partial consensus MP tree for *cox2* sequences with bootstrap values for the MP tree and for the equivalent branches of the NJ tree, showing the relationships of the African species after including additional sequence data provided by L.G. Cook. Scale represents 10 changes. Generic abbreviations: *Af.* = *Afrochlus*; *Ar.* = *Archaeochlus*; *Au.* = *Austrochlus*; *P.* = *Procladius*.

The additional mt gene permitted more resolution in the phylogenetic trees (Figure 2) than was obtained with either gene alone. The relationship of *Af. harrisoni* B to either the Australian or African species was not clarified, however, as it appeared as sister to both groups with low bs support. The 18S trees (Figure 3) resolved this point, showing that *Af. harrisoni* B is clearly in the African clade, but provided relatively poor resolution of the relationships at the tips of the tree, probably as a result of the relatively small number of informative substitutions at this level. Given that the mt and nuclear trees resolve different branch levels of the phylogenies, it was hoped that a combination of the two data sets might better resolve the phylo-geny overall. Combining of data sets from multiple genes, including mt and nuclear genes is now commonly recommended, although the effect of differing substitution rates, Ti/Tv ratios and gamma distributions is not yet clarified (Pupko *et al.* (2002). Although a more complex model may give a better result, the combined ML tree gave a result compatible with the features best supported by the separate trees (Figures 2 & 3), other than in the placement of *Au. centralaustralis* basal to the other *Austrochlus* species. In Figure 4 the Austra-lian clade was adjusted to show the three *Austro-chlus* species arising from a common branch, as seen in Figure 3 and supported by the consensus MP and NJ analyses of the combined data. This differs from the relationships of these Australian species shown by Cranston *et al.* (2002) and Martin *et al.* (2002), where *Au. centralaustralis* was linked to *Au. brundini*. In both cases, however, this branch was supported by only a low bs value and the arrangement shown in Figure 4 is compatible with those data. Considering all results, it is clear that the Australian and African clades are distinct groups and that each group is monophyletic.

The relationships of the Australian species and populations have been investigated previously (Martin *et al.* 2002), but there has been limited consideration of the African species. Cranston *et al.* (2002) included four *Archaeochlus* species and *Afrochlus harrisoni* in their molecular phylogeny, but noted only the close relationship of *Af. harrisoni* to the *Archaeochlus* species. One interesting aspect is that, although sampling in Africa has been relatively limited, more species have been identified than in the more extensively sampled Australia. This speciation also appears to be of more recent origin than that in Australia. For example, *Archaeochlus drakensbergensis* and *Ar.* sp. 'Matchless' are very closely related, being less differentiated at the molecular level than many of the populations within the Australian species. Martin *et al.* (2002) estimated the divergence time of *Au. parabrundini* populations Dead Horse Rock and Gorge Rock at 9.3 my. Estimated on the same basis, the divergence time of *Ar. drakensbergensis* and *Ar.* sp. 'Matchless' would be 9 my. By contrast, Martin *et al.* (2002) estimated the divergence times for the Australian species as at least 18 my.

The presence of two forms of *Af. harrisoni* also requires further comment. The molecular evidence clearly suggests that these are different species, and that they are not very closely related. This latter point is not unexpected because of their sympatric occurrence at the Harare locality. For example, *Ar. drakensbergensis* and *Ar. bicirratus* were both originally collected from the same site in the Drakensberg Mountains (Cranston *et al.* 1987), and these species are not the most closely related of the studied *Archaeochlus* species (Figure 5). Similarly, Martin *et al.* (2002) found that the sympatric

populations of *Au. brundini* and *Au. parabrundini* at Gorge Rock were amongst the most distantly related populations of these species. This suggests that sympatric occurrence at the present time is the result of secondary contact rather than sympatric speciation. From the phylogenies, it would appear that form B of *Af. harrisoni* is basal to the African clade, while form A is either sister to form B, or to *Ar.* sp. 'Amieb', or even to both these species. This raises the question of the status of the two forms of *Af. harrisoni*. They could both be species of *Afrochlus*, but these data are also consistent with form A being a, presumably new, species of *Archaeochlus*. A definitive answer would require further morphological examination and this would probably require the collection of additional material from the type locality, since the existing larvae apparently lack the critical features required to separate *Afrochlus* and *Archaeochlus* (P.S. Cranston pers. comm.). The status of the two forms of *Af. harrisoni* is not a trivial matter. Cranston *et al.* (2002) noted that it might be necessary to broaden the generic concept for the African species so that *Afrochlus* was included in *Archaeochlus*. If form A of *Af. harrisoni* is truly a species *Afrochlus*, merging of the two genera would be necessary. It might be noted that this broadened genus would be *Afrochlus*, and not *Archaeochlus* as implied by Cranston *et al.* (2002), since the former name has precedence. If form A is a species of *Archaeochlus*, retention of the two genera would still be possible.

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