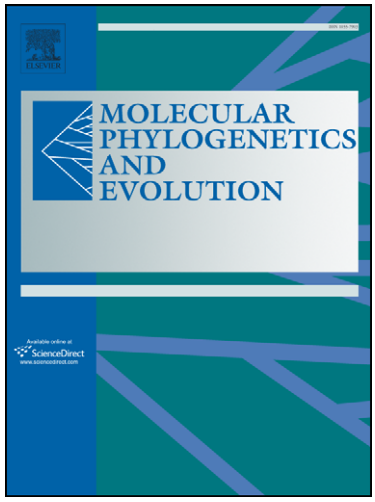
Accepted Manuscript



Molecular phylogenetics of the spider family Micropholcommatidae (Arachnida: Araneae) using nuclear rRNA genes (18S and 28S)

Michael G. Rix, Mark S. Harvey, J. Dale Roberts

PII: S1055-7903(07)00386-7
DOI: 10.1016/j.ympev.2007.11.001
Reference: YMPEV 2688

To appear in: *Molecular Phylogenetics and Evolution*

Received Date: 10 July 2007
Revised Date: 24 October 2007
Accepted Date: 9 November 2007

Please cite this article as: Rix, M.G., Harvey, M.S., Roberts, J.D., Molecular phylogenetics of the spider family Micropholcommatidae (Arachnida: Araneae) using nuclear rRNA genes (18S and 28S), *Molecular Phylogenetics and Evolution* (2007), doi: 10.1016/j.ympev.2007.11.001

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Molecular phylogenetics of the spider family Micropholcommatidae (Arachnida: Araneae) using nuclear rRNA genes (18S and 28S)

Michael G. Rix[1,2*], Mark S. Harvey[2], J. Dale Roberts[1]

[1]*The University of Western Australia, School of Animal Biology,*

35 Stirling Highway, Crawley, Perth, WA 6009, Australia.

E-mail: rixm01@student.uwa.edu.au

E-mail: droberts@cyllene.uwa.edu.au

[2]*Western Australian Museum, Department of Terrestrial Zoology,*

Locked Bag 49, Welshpool D.C., Perth, WA 6986, Australia.

E-mail: mark.harvey@museum.wa.gov.au

*Corresponding author. Phone +61 8 6488 1493

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abstract

The spider family Micropholcommatidae is an enigmatic taxon of uncertain limits and uncertain affinities. Various phylogenetic hypotheses have been proposed for the family, but these hypotheses have never been tested with a robust phylogenetic analysis. The existence of similar Australasian and New World taxa, the possibility of morphological convergence associated with extreme 'smallness', and the apparent paucity of synapomorphic morphological characters, have all clouded generic relationships in this group. We used fragments from two nuclear ribosomal RNA genes (18S and 28S) to test the monophyly and phylogenetic position of the Micropholcommatidae. The analyses incorporated 50 ingroup spider species, including 23 micropholcommatid species and representatives from 14 other spider families. Ribosomal RNA secondary structures were inferred for the V3-V5 region of the 18S rRNA gene, and Domain II of the 28S rRNA gene of *Hickmania troglodytes* (Higgins & Petterd, 1883). These secondary structures were used to guide multiple sequence alignments, and determine the position and nature of indels in different taxa. Secondary structure information was also incorporated into a structurally partitioned rRNA analysis in MrBayes Version 3.1.2, using a doublet model of nucleotide substitution. This structurally partitioned rRNA analysis provided a less resolved but more conservative and informative estimate of phylogeny than an otherwise identical, unpartitioned rDNA analysis. With the exception of the Chilean species *Teutoniella cekalovici* Platnick & Forster, 1986, the family Micropholcommatidae was found to be monophyletic with three monophyletic sub-lineages – congruent with the Micropholcommatinae, Textricellinae,

ACCEPTED MANUSCRIPT

and a group of 'taphiassine' species. *Teutoniella cekalovici* never grouped with the other micropholcommatid taxa, and could not be assigned to any family group with confidence.

Keywords: Araneoidea, Symphytognathoidea, Palpimanoidea, Anapidae, Malkaridae, *Teutoniella*, combined analysis, autocorrelation, secondary structures, Bayesian analysis, doublet model.

1. Introduction

The Micropholcommatidae (Fig. 1) are a family of tiny, leaf litter- and moss-dwelling spiders, with 33 species in eight genera currently described from Australia, New Zealand, Papua New Guinea, Chile and Brazil (Platnick, 2007). Micropholcommatids are among the smallest of spiders (usually 0.5 – 2 mm in body length), and possess a number of morphological traits that are associated with extreme 'smallness' (Forster and Platnick, 1977; Platnick and Forster, 1986). Although abundant in some forest systems in Australia and New Zealand, very little is known of their general biology, methods of food acquisition, web architecture, or trophic importance in terrestrial ground-level habitats. Their distribution on the southern continents, in combination with what little is known about their dispersal biology, is indicative of a potentially vicariant Gondwanan distribution.

Since the first micropholcommatid species were described in the early twentieth century (see Crosby and Bishop, 1927; Butler, 1932), and the family was erected by Hickman (1943), the limits, familial status and phylogenetic placement of the

ACCEPTED MANUSCRIPT

Micropholcommatidae have been in flux. The small size of individual spiders, and their similarity to a number of different taxa at the base of the Entelegynae (see Griswold et al., 2005), have obscured the phylogenetic placement and affinities of the micropholcommatid genera. Forster (1959) synonymized the Micropholcommatidae and Textricellidae with the Symphytognathidae, which resulted in the then described micropholcommatid and textricellid species, along with large numbers of other taxa, being merged into a single family group within the Araneoidea (the superfamily including the orb-weaving spiders; see Griswold et al., 1998). This union was based on a number of characters that seem to be associated with 'smallness', including the relative elongation of the leg tarsi relative to the metatarsi, the reduction in the size of the female pedipalps in many species, the reduction and conversion of the anterior booklungs into anterior tracheae, the loss of individual eyes in some taxa, and the elevation of the anterior carapace in several genera.

The 'enlarged Symphytognathidae' concept of Forster (1959) came under scrutiny in later decades, and was criticised – even by Forster himself – for being phenetically convenient and possibly highly polyphyletic (Forster and Platnick, 1977); a "dump heap of minute Araneoidea" (Lehtinen, 1975 *in* Forster and Platnick, 1977: 2). The Symphytognathidae was thereafter limited by Forster and Platnick (1977) to include only five closely-related genera (*Symphytognatha*, *Globignatha*, *Patu*, *Anapistula* and *Curimagua*), and the Micropholcommatidae and Textricellidae were resurrected for the genera *Micropholcomma*, *Parapua*, *Pua* and *Textricella*. At the time it was considered premature to address the issue of interfamilial relationships, and thus the newly-delimited

ACCEPTED MANUSCRIPT

Symphytognathidae, Micropholcommatidae and Textricellidae, along with the other resurrected familial taxa, remained in the Araneoidea.

After a revision of the archaeid spiders and their relatives by Forster and Platnick (1984), the Micropholcommatidae and Textricellidae were moved to the newly-delimited superfamily Palpimanoidea (Fig. 2A). Their position in the Palpimanoidea, along with the Palpimanidae, Stenochilidae, Huttoniidae, Mimetidae (plus Malkaridae; see Wunderlich, 1986, 2004) and the four 'archaeoid' families, is contentious. Indeed, this 'expanded Palpimanoidea' hypothesis was challenged by Schütt (2000, 2003), who suggested that the Micropholcommatidae, Mimetidae and Pararchaeidae should all be returned to the superfamily Araneoidea. A cladistic analysis of exemplar micropholcommatid genera (*Micropholcomma*, *Textricella*, *Parapua*) plus araneoid taxa in the families Anapidae, Synaphridae, Symphytognathidae, Mysmenidae, Theridiosomatidae, Theridiidae and Linyphiidae (see Schütt, 2003 and Fig. 2B), concluded that the Anapidae were paraphyletic with respect to the Micropholcommatidae, and that the latter should be synonymised. This hypothesis was, however, based on analysis of very few taxa, and was dismissed by Platnick (2007).

The status of the Micropholcommatidae is further complicated by the issue of what genera actually belong in this family. The Micropholcommatidae and Textricellidae were both described separately by Hickman (1943, 1945), but were later synonymised with the Symphytognathidae by Forster (1959). Forster and Platnick (1977) resurrected both families, before the Textricellidae were then synonymised with the Micropholcommatidae by Platnick and Forster (1986). The latter synonymy was based on a revision of the Chilean genus *Teutoniella*, and the subsequent complications this

ACCEPTED MANUSCRIPT

unusual taxon brought to the issue of micropholcommatid versus textricellid monophyly. The authors lamented the lack of appropriate synapomorphic characters for the two families, and noted the similarities of *Teutoniella* to both. Given "this rather unsatisfactory situation" (Platnick and Forster, 1986: 7), the only options were to describe *Teutoniella* in its own family, or place it, along with all of the other micropholcommatid and textricellid genera, into a single family. The latter option was chosen, and the Micropholcommatidae Hickman, 1943 took precedence over the Textricellidae Hickman, 1945 (see Platnick and Forster, 1986).

The Micropholcommatidae currently stands, therefore, as a family of uncertain limits and uncertain affinities. Apart from the work of Schütt (2003), there has been no quantitative phylogenetic analysis of micropholcommatid taxa, nor has there been a robust test of the superfamily Palpimanoidea using a wide taxon sample and a diverse selection of araneoid reference taxa. The current study aims to address these gaps using a detailed molecular phylogenetic approach, avoiding any problems resulting from morphological homoplasy and small body size. The monophyly and phylogeny of the Micropholcommatidae (*sensu* Platnick and Forster, 1986), and the relationships among Australian, New Zealand and Chilean micropholcommatid taxa were tested using two nuclear gene fragments – 18S rDNA and 28S rDNA – and 23 ingroup micropholcommatid species in five described genera. In addition, the monophyly of the Palpimanoidea was tested using representative taxa from all of the major palpimanoid lineages, along with a wide selection of araneoid, and especially anapid, exemplar taxa.

ACCEPTED MANUSCRIPT

2. Materials and methods

2.1 Taxon sample

The outgroup taxon in all analyses was the liphistiid genus *Liphistius*, with 18S rDNA and 28S rDNA sequence data acquired from GenBank (see Hedin and Bond, 2006; Table 1). Ingroup taxa were chosen to most effectively test the monophyly and phylogenetic position of the Micropholcommatidae, given the current suite of published hypotheses regarding this family. Three ingroup species from non-entelegyne lineages were chosen as reference taxa, to help root the internal nodes and provide maximum information on plesiomorphic character states. These taxa were the two hexathelid mygalomorph genera *Atrax* and *Hadronyche* (both with sequences obtained from GenBank; see Hedin and Bond, 2006; Table 1), and the Tasmanian austrochilid *Hickmania troglodytes* (Higgins & Petterd, 1883).

The monophyly of the superfamily Palpimanoidea of Forster and Platnick (1984) (Fig. 2A) was tested with the inclusion of exemplar species of Palpimanidae, Mimetidae, Malkaridae, Pararchaeidae, Holarchaeidae and Mecysmaucheniidae, in addition to the 23 micropholcommatid taxa. The Archaeidae, although sequenced for this study, were not included in the analyses due to extremely divergent and unusual sequences obtained for both 18S rDNA and 28S rDNA. Given the probable sister-group relationship between the Mecysmaucheniidae and the Archaeidae (Forster and Platnick, 1984; Platnick et al., 1991), and the inclusion of a mecysmaucheniid ingroup species, we saw no compelling reason to also include an archaeid exemplar.

ACCEPTED MANUSCRIPT

The monophyly of the Micropholcommatidae of Platnick and Forster (1986) was tested with the inclusion of the Chilean species *Teutoniella cekalovici* Platnick & Forster, 1986, four species of *Parapua* from Australia and New Zealand including *P. punctata* Forster, 1959, five species of *Micropholcomma* (Fig. 1A) from Australia including *M. caeligenum* Crosby & Bishop, 1927 and *M. bryophilum* (Butler, 1932), three species of '*Plectochetos*-like' *Micropholcomma* (see Butler, 1932) from Australia including *M. longissimum* (Butler, 1932), the Tasmanian species *Olgania excavata* Hickman, 1979 (Fig. 1C), nine species of *Textricella* from Australia and New Zealand including *T. vulgaris* Forster, 1959, *T. fulva* Hickman, 1945, *T. parva* Hickman, 1945 (Fig. 1B), *T. luteola* Hickman, 1945 and *T. hickmani* Forster, 1959, and a relatively large, enigmatic species from Victoria (coded in this analysis as 'Gen. nov. sp. VICBig'; Fig. 1D).

Representative taxa were also included from the superfamily Araneoidea, and the araneoid superfamily Symphytognathoidea (see Griswold et al., 1998), to test the hypotheses of Schütt (2000, 2003) (Fig. 2B). These taxa included exemplar species of Araneidae, Theridiidae, Cyatholipidae, Symphytognathidae, Anapidae and Mysmenidae, along with two Australian species of the mysmenid (although distinctly *Parapua*-like) genus *Taphiassa*.

In total, 48 species representing 14 spider families were newly sequenced for the 18S rDNA and 28S rDNA gene fragments. Taxon names, accession numbers, museum voucher registration numbers and collection information for all taxa included in the analyses are listed in Table 1.

ACCEPTED MANUSCRIPT

2.2 *Gene choice*

The nuclear ribosomal RNA genes 18S rRNA and 28S rRNA were chosen due to their widespread use and utility in phylogenetic studies examining relationships among orders or families of arachnids and other invertebrates (Wheeler et al., 2001; Maddison and Hedin, 2003; Prendini et al., 2003; Arnedo et al., 2004; Hedin and Bond, 2006). Despite the ubiquitous alignment problems caused by rRNA sequence length variation, the ability to incorporate detailed secondary structure information into multiple sequence alignments and phylogenetic priors is one of the powerful benefits associated with modern rRNA studies.

2.3 *Specimen collection and preservation*

Most eastern Australian and New Zealand specimens were collected by M. Rix in April 2006, and preserved in 95% monopropylene glycol before being transferred after five weeks to 95% ethanol (Vink et al., 2005). Western Australian taxa were also collected during 2006, and preserved directly in 95% ethanol. Specimens of the Chilean genera *Teutoniella*, *Crassanapis* and *Minanapis* were acquired for the Western Australian Museum by Tomás Cekalovic in late 2006 and early 2007, and specimens of *Otiothops birabeni* Mello-Leitão, 1945 were loaned from the Museo Argentino de Ciencias Naturales (Buenos Aires) by Martín Ramírez. All South American taxa were preserved directly in 95% ethanol.

ACCEPTED MANUSCRIPT

After initial or subsequent preservation in 95% ethanol, the distal segments of single legs (for larger taxa), the posterior left legs (for ethanol-preserved taxa) or whole abdomens (for monopropylene glycol-preserved taxa) of specimens were removed for use in DNA extractions, using adult male specimens wherever possible. The remaining abdomen and/or cephalothorax of each specimen was then retained as a voucher, and lodged in the Western Australian Museum. All original sequences in this paper can, therefore, be linked back to a single, registered museum specimen (Table 1).

2.4 Molecular and laboratory methods

Whole genomic DNA was extracted from spider tissue samples using the QIAGEN DNeasy Blood and Tissue Kit protocol for animal tissues. Polymerase chain reaction (PCR) amplification of target gene regions was achieved using Promega PCR Master Mix chemistry, in an MJ Research PTC-200 or Eppendorf Mastercycler ep thermal cycler. Between 1 and 8.5 µl of template DNA, 12.5 µl of PCR Master Mix, and 1-2 µl of each primer (at 10 µM concentration) were used in every 25 µl PCR reaction.

The V3-V5 region of the 18S rRNA gene (~720 bp; see Wuyts et al., 2000) was amplified using the primers 18S-ai (5'-CCTGAGAAACGGCTACCACATC-3') and 18S-b0.5 (5'-GTTTCAGCTTTGCAACCAT-3') (Tautz et al., 1988) (primer combination from Hong-Chun et al., 2004). Domain II of the 28S rRNA gene (~850 bp; see Schnare et al., 1996) was amplified using the primers 28Sa (5'-GACCCGTCTTGAAACACGGA-3') (Nunn et al., 1996) and LSUR (5'-GCTACTACCACCAAGATCTGCA-3') (designed in this study from Schnare et al., 1996). The PCR protocol used for amplifying both gene

ACCEPTED MANUSCRIPT

fragments was as follows: 95ºC for 2.5 minutes; 40 cycles of (95ºC for 30 seconds; 50ºC for 30 seconds; 72ºC for 1.5 minutes); and a final 72ºC for 10 minutes. For every set of PCR reactions prepared using a particular PCR master mix, a single negative control was included to test for contamination. This negative control used nuclease-free water instead of template DNA.

The presence of PCR products in PCR reactions was confirmed using standard agarose gel electrophoresis. If PCR products were detected, PCR reactions were then purified using the MoBio UltraClean PCR Clean-up Kit. Bi-directional sequencing of purified PCR products was performed by Macrogen Corporation (South Korea), using supplied PCR primers.

2.5 Sequence annotation, inference of rRNA secondary structures and sequence alignments

Sequence (.abi) files for the coding and non-coding strands were assembled automatically as anti-parallel contigs, and then visualised as text sequences and chromatograms using Sequencher 4.7 (Demonstration Version; Gene Codes Corporation, Madison, WI). Nucleotide sites exhibiting double chromatogram peaks were scored as ambiguous (?) for those taxa in which they occurred. Annotated sequences were saved as text files, and imported into ClustalX Version 1.83 (Thompson et al., 1997) for assistance with alignment and secondary structure analysis.

Hickmania troglodytes was chosen as the reference araneomorph taxon for secondary structure analysis, given the basal position of this species relative to the other

ACCEPTED MANUSCRIPT

ingroup taxa, and the absence of marked indels relative to the outgroup. For the V3-V5 region of the 18S rRNA gene, the nucleotide sequence of *H. troglodytes* (GenBank accession number **TBA**) was manually folded according to the eukaryotic secondary structure model of Wuyts et al. (2000), incorporating the V4 region eukaryotic pseudoknot. For Domain II of the 28S rRNA gene, the nucleotide sequence (GenBank accession number **TBA**) was manually folded according to the eukaryotic secondary structure model of Schnare et al. (1996). For both secondary structure models, only G-C, A-U and G-U bonds were considered in stem helix regions.

For the phylogenetic analyses, the rRNA secondary structures of *H. troglodytes* were used to guide multiple sequence alignments, and to help clarify the position of insertion-deletion events (indels) in regions of varying sequence length. Automatic alignments of all nucleotide data were compared to *H. troglodytes* and its associated secondary structure models using default alignment settings in ClustalX. Single nucleotide indels, or autapomorphic multiple nucleotide indels in different taxa (Figs 3-4, Table 2) were assessed in relation to their surrounding secondary structure, and aligned accordingly. Highly length-variable loop and linking regions, and variable stem/loop regions in 28S rRNA (variable regions 1-3; Fig. 4), were folded manually for all taxa. Within these variable regions, smaller hypervariable regions were discarded from the multiple sequence alignments by manual editing (as shown in Figs 3-4).

ACCEPTED MANUSCRIPT

2.6 Phylogenetic analyses

Two Bayesian analyses were performed using the program MrBayes Version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), with a combined 18S rDNA plus 28S rDNA matrix. Before analysis, MrModeltest Version 2.2 (Nylander, 2004; see also Posada and Crandall, 1998) was executed to choose the appropriate model of nucleotide substitution for each of the 18S rDNA, 28S rDNA and combined matrices. All three models incorporated a General Time Reversible (GTR) model of nucleotide substitution with invariants and gamma, coded as the MrBayes likelihood settings <Lset nst=6 rates=invgamma>. The combined 18S rDNA plus 28S rDNA multiple sequence alignment – annotated as a nexus file with an appropriate MrBayes command block – was then analysed twice, using different data partitioning strategies as incorporated by MrBayes. The data were first partitioned by gene and were analysed using a standard prior model of nucleotide substitution (<Lset nucmodel=4by4>). The same combined matrix was then further partitioned according to gene and rRNA secondary structure. In this second analysis, the paired nucleotide sites involved in double-stranded base-pairing in *Hickmania troglodytes* were listed under the MrBayes command <Pairs>, and all nucleotide sites were partitioned according to their presence in either stem helix (<Charset stems>) or single-stranded (<Charset loops>) regions. For these structural partitions, the doublet prior model of nucleotide substitution (<Lset nucmodel=doublet>) was used for stem partitions, while the standard (4by4) model was used for single-stranded partitions. For both analyses parameters were estimated independently (<Unlink statefreq=(all) revmat=(all) shape=(all) pinvar=(all)>),

ACCEPTED MANUSCRIPT

rates were allowed to vary across partitions (<Prset applyto=(all) ratepr=variable>), and four Markov Chain Monte Carlo (MCMC) chains were run for 2,000,000 generations, sampling every 1000 generations, with the first 200,000 sampled trees discarded as 'burn-in'. Burn-in times were assessed by first running shorter analyses, and graphing the Bayesian log likelihoods (LnL); these burn-in times were subsequently confirmed by comparison to the complete log likelihood graphs of all analyses after 2,000,000 generations. For each analysis, posterior probabilities were calculated and reported on a 50% majority-rule consensus tree of the post-burn-in sample.

A single parsimony analysis was performed using PAUP* Version 4.0b10 (Swofford, 2002). The combined 18S rDNA plus 28S rDNA matrix was analysed using a heuristic tree-bisection-reconnection (TBR) search algorithm, with 10,000 replicates and 10 trees held at each step during random stepwise addition. Gaps were treated as missing data and all characters were equally weighted. Clade support values were estimated using non-parametric bootstrapping (Felsenstein, 1985) in PAUP*, with 1000 pseudoreplicates of a heuristic (TBR) search algorithm incorporating 10 replicates of random stepwise addition of taxa, and 10 trees held at each step.

To explore the possible contribution of an individual gene to the combined analyses, and as an example of how analysis of an individual partition may affect results, the 28S rRNA data were also analysed independently in MrBayes. These data were partitioned according to secondary structure using the doublet model of nucleotide substitution, and otherwise analysed similarly to the combined Bayesian analyses, with the exception that the MCMC chains were run for 1,000,000 generations, with the first 100,000 sampled trees discarded as 'burn-in'.

ACCEPTED MANUSCRIPT

3. Results

3.1 Data characteristics and rRNA secondary structures

The 18S rDNA data partition consisted of 51 taxa, and was 722 nucleotides in length. Of these nucleotide sites, 465 were invariant across all taxa, and 169 (23%) were parsimony-informative. The 28S rDNA data partition consisted of the same 51 taxa and was 763 nucleotides in length, of which 467 sites were invariant across all taxa and 193 (25%) were parsimony-informative. The combined 18S rDNA plus 28S rDNA matrix was 1485 nucleotides in length, with a mean base composition across the entire matrix as follows: A = 0.25; T = 0.20; G = 0.30; C = 0.25.

The secondary structure model of the 18S rRNA V3-V5 region of *Hickmania troglodytes* (Fig. 3) was consistent with that proposed for eukaryotes by Wuyts et al. (2000, fig. 1). Sequence length variation was minimal, with only four regions for which length variation was widespread and variable enough across taxa to make alignment ambiguous; these were always single-stranded loop or linking regions. Short (1-6 bp) indels were present at 17 sites, and all except two of these indels were autapomorphies for single taxa (Table 2).

The secondary structure model of the 28S rRNA Domain II region of *Hickmania troglodytes* (Fig. 4) was consistent with that proposed for eukaryotes by Schnare et al. (1996). Three regions of significant length variation were found, and these corresponded to similarly length-variable regions identified among other eukaryotes by Schnare et al. (1996, fig. 1a). Variable Region 1 in the current study corresponds to the variable '650 Region' of Schnare et al. (1996, fig. 5). This region usually consists of three branching

ACCEPTED MANUSCRIPT

'arms' in Metazoa, and these three arms could be modelled in all of the spiders sequenced except *Textricella fulva*, in which Arm 1 was completely lost. Variable Region 2 in the current study corresponds to variable 'Positions 929 to 932' of Schnare et al. (1996). This region consisted of a single stem/loop hairpin structure in all of the spiders sequenced, with terminal loop length variation of 8-13 bp. Variable Region 3 in the current study corresponds to the variable 'Positions 1164 to 1185' of Schnare et al. (1996). This region consisted of a single stem/loop hairpin structure in all of the spiders sequenced, with terminal stem and loop length variation of 13-33 bp. Outside of the three variable regions identified above, short (1-2 bp) indels were present at only 6 sites, and all except two of these were autapomorphies for single taxa (Table 2).

3.2 Phylogenetic analyses

The four phylogenetic analyses – the Bayesian combined rRNA analysis with structural partitions (Fig. 5), the Bayesian combined rDNA analysis (Fig. 6), the parsimony combined rDNA analysis (Fig. 7) and the Bayesian 28S rRNA analysis with structural partitions (Fig. 8) – produced phylogenies that usually recovered twelve identical clades, labelled 1-12 in Figures 5-8 and Table 3. Of these clades, relationships between lineages in Clade 4 were the least resolved, with very different internal topologies suggested by different analyses. The most important and consistent result was the universally-recovered monophyly of Clades 6, 7 and 8, and the inclusion of these three clades in a monophyletic Micropholcommatidae (Clade 5), to the exclusion of *Teutoniella cekalovici*. Clade 5 did, however, include additional species in two of the four

ACCEPTED MANUSCRIPT

analyses (see Clades 5' and 5" in Figs 7, 8, Table 3). There was no evidence for a monophyletic Palpimanoidea as hypothesised by Forster and Platnick (1984) (Fig. 2A, 5).

The results of the three combined analyses (each executed with identical matrices) showed fundamental similarities but also striking differences in detail. The structurally-partitioned Bayesian rRNA analysis (Fig. 5) differed from the standard Bayesian rDNA analysis (Fig. 6) in an overall reduction in clade support, and in forming a largely unresolved polytomy at Clade 4. Two novel relationships were also inferred by the combined rRNA analysis, but neither of these clades were supported by a posterior probability of greater than 75%. Similarly, the combined parsimony rDNA analysis (Fig. 7) differed markedly from the Bayesian analyses in the detail of Clade 4, but these relationships showed no significant bootstrap support.

The 28S rRNA analysis (Fig. 8) was similar to the combined analyses in recovering 11 of the 12 labelled clades, and in providing limited resolution within Clade 4. This gene did, however, recover two novel, and biologically informative, relationships: (1) the enigmatic taxon Gen. nov. sp. VICBig (Fig. 1D) grouped very strongly with the rest of the Micropholcommatidae (Clade 5" in Fig. 8); and (2) the Chilean anapid *Minanapis talinay* Platnick & Forster, 1989 grouped for the first time with the other Chilean anapid species *Crassanapis cekalovici* Platnick & Forster, 1989, along with the Australasian Anapidae in Clade 10.

ACCEPTED MANUSCRIPT

4.0 Discussion

4.1 Micropholcommatid systematics

The Micropholcommatidae – as a monophyletic family of araneoid spiders – was recovered by all four phylogenetic analyses in this study (see Clades 5, 5' and 5" in Figs 5-8, Table 3). The family does, however, appear to be slightly different in its constitution compared to that proposed by Platnick and Forster (1986). There is strong evidence for at least three monophyletic subfamilies – the Micropholcommatinae, Textricellinae and a subfamily of 'taphiassine' species – rather than a single family with a potentially paraphyletic Textricellinae, as suggested by Platnick and Forster (1986). Gen. nov. sp. VICBig (Fig. 1D), which did group with the Micropholcommatidae in the 28S rRNA analysis but never within any of the other three subfamilies, may also belong in its own subfamily, representing a separate lineage of micropholcommatid spiders. Gen. nov. sp. VICBig is found in Antarctic Beech (*Nothofagus*) forest in Victoria and Tasmania, and another undescribed species in the same genus (which was not sequenced as part of this study) occurs in the southern Chilean provinces of Chiloé, Palena, Valdivia (Region de los Lagos) and Aisén (Region de Aisén) (specimens examined from the American Museum of Natural History collection, courtesy of Norman I. Platnick).

The genus *Teutoniella*, hypothesised as being an unusual micropholcommatid by Platnick and Forster (1986), was never found to group with any of the other Micropholcommatidae, nor indeed did it ever group explicitly with any other one araneoid taxon. The concept of *Teutoniella* belonging in its own family is not new (see Introduction; Platnick and Forster, 1986). *Teutoniella* apparently lacks a labral spur

ACCEPTED MANUSCRIPT

(Platnick and Forster, 1986), whereas a labral spur was reported by Schütt (2000, 2003) in at least *Micropholcomma* and *Parapua* (see also discussion on Anapidae, 4.4, below). Similarly, the book lungs of *Teutoniella* were described by Platnick and Forster (1986) as only partially reduced, and regarded as either reduced lamellae or incipient tracheae. In *Micropholcomma*, *Textricella*, *Parapua* and *Olgania* the booklungs are completely transformed into anterior tracheal tubes, although the morphology of these tracheae varies (Forster, 1959; Hickman, 1979). Platnick and Forster (1986) briefly discussed the resemblance of *Teutoniella* to the family Cyatholipidae, although this resemblance was superficial and quickly rejected; a conclusion supported by the results of the current study. While a discussion on the possible relationships of *Teutoniella* to any other families is speculative, there appear to be no obvious morphological characters linking this genus to other araneoid or palpimanoid taxa.

The results of the current study provide evidence for a micropholcommatid subfamily of 'taphiassine' species, uniting the genus *Taphiassa* with *Parapua* and *Olgania*. *Taphiassa impressa* Simon, 1880 was originally described from a single female specimen from Noumea, New Caledonia (holotype examined, in the Muséum National d'Histoire Naturelle, Paris), although numerous other New Caledonian and Australian congeneric species have since been collected. *Taphiassa*, *Parapua* and *Olgania* are all distinctly long-legged Micropholcommatidae, sharing a heavily punctured carapace morphology (see Schütt, 2000, fig. 3C), a spherical or globose and mostly unsclerotised abdomen without a dorsal scute, and a simple pedipalpal bulb with an exposed, curved or looped embolus.

ACCEPTED MANUSCRIPT

The issue of the sister-taxon to the Micropholcommatidae was unresolved in the current study. While clearly belonging to the superfamily Araneoidea, the Micropholcommatidae were never found to have a consistent sister-lineage. Indeed, only in the combined parsimony analysis was the Sternoidinae found to be weakly embedded within a paraphyletic Micropholcommatidae (Clade 5' in Fig. 7); in other analyses, this sternoidine lineage invariably grouped elsewhere.

4.2 Monophyly of the superfamilies Palpimanoidea and Araneoidea

The superfamily Palpimanoidea, as proposed by Forster and Platnick (1984) (Fig. 2), was not recovered or supported by the results of this study. The Palpimanidae only rarely grouped with the Mecysmaucheniidae, and both families were consistent outgroups to the diverse array of Clade 4 taxa in all analyses. While an alternative 'expanded Araneoidea' hypothesis (Schütt 2000, 2003) was not explicitly tested due to the absence of non-araneoid, non-palpimanoid Entelegynae, all four analyses nonetheless strongly supported a monophyletic and diverse Araneoidea, as represented by Clade 4. This Araneoidea included the Micropholcommatidae, Pararchaeidae, Holarchaeidae, Mimetidae, *Malkara* and the Sternoidinae among former palpimanoids, in addition to the araneoid taxa already recognised. The superfamily Palpimanoidea, therefore, should eventually be relimited to include only the Palpimanidae and its closest haplogyne relatives, the Stenochilidae and Huttoniidae, as originally suggested by Schütt (2000). It is equivocal whether the family Mecysmaucheniidae is a sister-lineage to the true palpimanoids, but there is little evidence to suggest that these remarkable haplogyne

ACCEPTED MANUSCRIPT

spiders are derived members of the Entelegynae. Certainly, this result is entirely consistent with the morphological analysis of entelegyne spiders by Griswold et al. (2005, fig. 217), who also inferred a polyphyletic Palpimanoidea, with the Huttoniidae and Archaeidae extremely basal.

4.3 The Malkaridae

While interrelationships among the Clade 4 araneoid taxa were largely unresolved in the current study, several results are worthy of special mention. The sternoidine malkarid taxa *Carathea* and *Perissopmeros* (Clade 9) were particularly unstable with respect to their phylogenetic position in the different analyses, but never once grouped with the nominate genus *Malkara*. The Malkaridae currently contains four genera of sclerotised, slow-moving hunting spiders, including the sternoidine taxa *Carathea*, *Perissopmeros* and *Chilenodes*, from Tasmania, Victoria and South America, respectively, along with *Malkara loricata* Davies, 1980 from eastern Australia. The monophyly of this family is thought to rest upon the presence of deep alveolations on the carapace and a large flange on the male pedipalpal conductor (Platnick and Forster, 1987). *Malkara* is, however, an unusual genus of uncertain affinity, which was thought to represent a highly divergent lineage of the Araneidae when first described by Davies (1980). There was no evidence in the current study to suggest it is related to the otherwise monophyletic and distinctive Sternoidinae, although a relationship with the Araneidae could not be rejected (see Figs. 6-8). Agnarsson (2004) discussed the similarities of *Chilenodes* and *Perissopmeros* (formerly *Sternodes*) to certain theridiid taxa

ACCEPTED MANUSCRIPT

(Hadrotarsinae and *Phoroncidia*), and this observation was at least weakly supported by the results of the Bayesian combined rRNA analysis (Fig. 5). Further phylogenetic research is needed to clarify the phylogenetic position of both *Malkara* and the Sternoidinae.

4.4 The Anapidae

The monophyly of the Anapidae of Forster and Platnick (1989) was tested with seven species from seven genera, including both Australasian and Chilean taxa. Unlike the Micropholcommatidae, anapid monophyly was not supported by the results of the current study, although two clades within the Anapidae were monophyletic and strongly supported by all analyses. Clade 10 consisted of the Australasian genera *Risdonius* and *Zealanapis*, which share a similar triangular abdominal morphology, respiratory system and cephalothoracic pore-pit morphology (Platnick and Forster, 1989). Clade 11 consisted of the other Australasian genera *Octanapis*, *Novanapis* and *Chasmocephalon*. The Chilean taxa *Crassanapis* and *Minanapis* only grouped with each other (and with Clade 10) in the Bayesian 28S rRNA analysis.

While the lack of molecular support for anapid monophyly was unexpected, the issue of anapid monophyly may have been underestimated and overlooked in the literature. The family is currently large (34 genera, 144 described species; Platnick, 2007), worldwide in its distribution, and includes a diverse array of tiny spiders. Only two morphological characters have been proposed in the taxonomic literature as anapid autapomorphies: the presence of a pore-bearing depression on the antero-lateral margins

ACCEPTED MANUSCRIPT

of the carapace (Platnick and Forster, 1989) and a sclerotised spur on the labrum (Platnick and Shadab, 1978; Platnick and Forster, 1989). Pore-bearing depressions are not found in all anapid taxa, and are absent in the South American species of *Pecanapis* and *Minanapis* (Forster & Platnick, 1989), South African species of *Dippenaaria* and circum-tropical species of *Pseudanapis* (Schütt, 2000). When such depressions do occur, they can be in different forms: some are exposed on the corners of the main carapace sclerite itself, as in Chilean *Crassanapis*, *Sheranapis* and *Sofanapis*, Australian *Queenslanapis*, *Risdonius*, *Tasmanapis* and *Victanapis*, New Zealand *Zealanapis* (see Forster and Platnick, 1989, figs 5-6), Central and South American *Anapisona* (see Platnick and Forster, 1986, figs 1-4) and apparently also Neotropical *Anapis* (see Platnick and Shadab, 1978, fig. 1); whereas others are concealed on a separate sclerite adjacent to the pedipalp, as in New Caledonian *Caledanapis*, *Mandanapis* and *Montanapis*, Australian *Chasmocephalon*, *Hickmanapis*, *Maxanapis*, *Nortanapis*, *Octanapis* and *Spinanapis*, and New Zealand *Novanapis* and *Paranapis* (see Forster and Platnick, 1989, figs. 271-272). In the current study, the supported sister-group relationships between *Risdonius* and *Zealanapis* (Clade 10), and between *Chasmocephalon*, *Novanapis* and *Octanapis* (Clade 11), were consistent with the morphology of the pore-bearing depressions on these taxa. However, *Crassanapis* did not group strongly with the similarly-modified taxa *Risdonius* and *Zealanapis*.

Most of the characters listed as unambiguous synapomorphies for the 'expanded Anapidae' (Micropholcommatidae plus Anapidae) of Schütt (2003) – the presence of a "more or less armoured prosoma", at least one labral sclerite, and dorsal plus ventral abdominal scutes on males – are also found in some other araneoid taxa. Pleural sclerites

ACCEPTED MANUSCRIPT

joining the carapace to the sternum, along with dorsal and ventral abdominal scutes in males, are also found in the Pararchaeidae and Malkaridae, and can be variably developed in different taxa. In contrast, the 'labral spur', 'labral sclerite' or 'labral clasp' (see Schütt, 2000, 2003) is an unusual character, apparently found only in the Micropholcommatidae and the Anapidae (Schütt, 2003). This character may indeed be synapomorphic for a larger clade including the Micropholcommatidae and one or more anapid clades (as broadly suggested by Schütt, 2003), but this hypothesis requires further testing. The results of the current study suggest that the Anapidae may be polyphyletic at worst, or at best paraphyletic or monophyletic with deeply-divergent tribes or subfamilies. A more rigorous sampling regime across the Anapidae is required to assess the phylogeny of this group, particularly by including clades from outside southern-temperate regions.

4.5 The effects of rRNA structural partitioning in MrBayes

Methodological comparisons are central to systematic biology, and have traditionally generated much debate and little consensus. Parsimony, likelihood and Bayesian methods each bring a set of assumptions, benefits and recognised problems to the issue of phylogenetic inference. For each method there is a suite of accepted – and sometimes not so accepted – 'best practice' implementations, which may or may not greatly influence the results of phylogenetic analyses. While not wanting to enter into a methodological debate concerning the validity or otherwise of Bayesian methods, a direct

ACCEPTED MANUSCRIPT

comparison of the effect of structurally partitioning rRNA data in MrBayes was considered useful, given its potential utility to phylogenetic studies.

Secondary structure inference is an important (see Kjer et al., 2007), well-recognised, yet extremely time-consuming method for improving sequence alignments. Despite the utility of secondary structure inference to phylogenetic studies, alignments for large matrices incorporating rRNA data are almost exclusively generated using automatic alignment methods, and analysed as unpartitioned DNA nucleotides. If secondary structure information is available, however, MrBayes Version 3.1.2 has the ability to implement a doublet model of nucleotide substitution (see Schoniger and Haesler, 1994) to account for the stem regions of ribosomal DNA, and the pairwise way in which the nucleotides of such regions evolve (Ronquist et al., 2005). Nucleotide pairing in double-stranded stem helices results in the autocorrelation of substitutions across sites, such that when a substitution occurs at one site, it is usually accompanied by another, compensatory substitution at the paired site (Ronquist et al., 2005). These compensatory substitutions maintain a particular secondary structure, but often result in nucleotide changes at two sites in the rDNA sequence. Given this correlation, parametric statistical methods used in Bayesian analysis, along with non-parametric methods of bootstrapping, may overestimate the confidence that should be placed in the best trees or the best clades resulting from a phylogenetic analysis (Ronquist et al., 2005).

Despite the potential utility of the rRNA doublet model as applied in MrBayes, very few phylogenetic studies have utilised rRNA structural partitions (Telford et al., 2005; Erpenebeck et al., 2007). Indeed, Erpenbeck et al. (2007: 544) stated that partitioned analyses using a doublet model are still uncommon, and that “their advantages

ACCEPTED MANUSCRIPT

do not yet appear to be fully recognized and acknowledged". This is despite the small but growing number of studies which have used and compared rRNA structural partitioning strategies, and overwhelmingly reported more informative and potentially more accurate phylogenies as a result (see Telford et al., 2005; Dohrmann et al., 2006; Erpenbeck et al, 2007).

In the current study, an identical combined matrix of 18S rDNA and 28S rDNA sequences was executed in MrBayes under standard versus structurally partitioned substitution models. While the same 12 fundamental clades were recovered in both analyses (Figs 5-6), there were striking differences in the detail of the topology within the Araneoidea (Clade 4). Most of the higher araneoid clades recovered by the standard rDNA analysis were not recovered in the partitioned rRNA analysis, 'collapsing' to form a largely polytomic Clade 4. Indeed, in addition to the Micropholcommatidae, the partitioned rRNA analysis only recovered two higher araneoid taxa: one an 'expanded Theridiidae' (see discussion on the Malkaridae, above), and the other a weakly-supported 'expanded Symphytognathoidea', with anapid, pararchaeid, symphytognathid, mysmenid and cyatholipid taxa (Fig. 5). Most importantly, the tree resulting from the structurally partitioned rRNA analysis (Fig. 5) appears to be a relatively conservative estimate of araneoid interrelationships, which, in comparison with the combined rDNA tree (Fig. 6), appears to be indicative of the limited power of nuclear rRNA genes for inferring intermediate, supra-familial relationships in spiders.

The effects of rRNA data partitioning, and of using the doublet model of nucleotide substitution in MrBayes, are therefore significant. The results of this study would suggest that structurally partitioned analyses are useful in assessing the resolution

ACCEPTED MANUSCRIPT

of ribosomal genes at different phylogenetic levels, and in determining the support that can be placed in clades inferred using standard rDNA analyses. Given the rarity of secondary structure inference in large phylogenetic analyses using rRNA genes, and the even scarcer implementation of the doublet model in Bayesian phylogenetic analyses, the overestimation of clade support may be a widespread weakness of automatic multiple sequence alignments. As suggested by this and other studies which have utilised doublet model analyses, there should be scope for another caveat relating to 'Bayesian best practice' – that of rRNA structural partitioning and its comparative effect on Bayesian phylogenetic inference when using rRNA genes.

Acknowledgments

This research contributed to a Doctor of Philosophy (Ph.D.) degree by M. Rix, and was funded by the Australian Biological Resources Study (ABRS) Ph.D. Research Scholarship (2006-2008), and the School of Animal Biology at the University of Western Australia. Invaluable field assistance was provided in New Zealand by Cor Vink, in Queensland and New South Wales by Alan Rix, and in Tasmania by Lisa Boutin and Arthur Clarke. Collections were made under permit in New Zealand (National Permit Number CA-18205-FAU), Queensland (Permit Number WITK01449103), New South Wales (Licence Number S11901), Victoria (Permit Number 10003705), Tasmania (Permit Number TFA 06431) and Western Australia (Licence Number SF005357). Other specimens for sequencing were graciously supplied by Martín J. Ramírez (Museo Argentino de Ciencias Naturales, Buenos Aires), and Tomás Cekalovic (Concepcíon).

ACCEPTED MANUSCRIPT

Norman I. Platnick (American Museum of Natural History, New York) is thanked for alerting us to the possibility that the genus *Taphiassa* was a micropholcommatid, and for the loan of the holotype of *T. impressa* Simon, 1880. Norman I. Platnick and Louis Sorkin are also thanked for the loan of many important Chilean and Australasian micropholcommatid specimens. Special thanks to the members of the Department of Terrestrial Zoology at the Western Australian Museum, and the Roberts Laboratory at the University of Western Australia, for all their discussion and assistance during this study. Thanks also to Stephen Barker of the School of Molecular and Microbial Sciences at the University of Queensland, for his initial tuition and encouragement of M. Rix during previous molecular research conducted in 2004.

References

Agnarsson, I., 2004. Morphological phylogeny of cobweb spiders and their relatives (Araneae, Araneoidea, Theridiidae). Zool. J. Linn. Soc. 141, 447-626.

Arnedo, M.A., Coddington, J., Agnarsson, I., Gillespie, R.G., 2004. From a comb to a tree: phylogenetic relationships of the comb-footed spiders (Araneae, Theridiidae) inferred from nuclear and mitochondrial genes. Mol. Phylogenet. Evol. 31, 225-245.

Butler, L.S.G., 1932. Studies in Australian spiders, No. 2. Proc. R. Soc. Vic. 44, 103-117.

Crosby, C.R., Bishop, S.C., 1927. New species of Erigoneae and Theridiidae. J. N. Y. Entomol. Soc. 35, 147-154.

ACCEPTED MANUSCRIPT

Davies, V.T., 1980. *Malkara loricata*, a new spider (Araneidae: Malkarinae) from Australia. Verh. int. arachnol. Kong. 8, 377-382.

Dohrmann, M., Voigt, O., Erpenbeck, D., Wörheide, G., 2006. Non-monophyly of most supraspecific taxa of calcareous sponges (Porifera, Calcarea) revealed by increased taxon sampling and partitioned Bayesian analysis of ribosomal DNA. Mol. Phylogenet. Evol. 40, 830-843.

Erpenbeck, D., Nichols, S.A., Voigt, O., Dohrmann, M., Degnan, B.M., Hooper, J.N.A., Wörheide, G., 2007. Phylogenetic analyses under secondary structure-specific substitution models outperform traditional approaches: Case studies with Diploblast LSU. J. Mol. Evol. 64, 543-557.

Felsenstein, J., 1985. Confidence limits on phylogenies - an approach using the bootstrap. Evolution 39, 783-791.

Forster, R.R., 1951. New Zealand spiders of the family Symphytognathidae. Rec. Canterbury Mus. 5, 231-244.

Forster, R.R., 1955. Spiders of the family Archaeidae from Australia and New Zealand. Trans. R. Soc. New Zealand 83, 391-403.

Forster, R.R., 1959. The spiders of the family Symphytognathidae. Trans. R. Soc. New Zealand 86, 269-329.

Forster, R.R., Platnick, N.I., 1977. A review of the spider family Symphytognathidae (Arachnida, Araneae). Am. Mus. Novit. 2619, 1-29.

Forster, R.R., Platnick, N.I., 1984. A review of the archaeid spiders and their relatives, with notes on the limits of the superfamily Palpimanoidea (Arachnida, Araneae). Bull. Am. Mus. Nat. Hist. 178, 1-106.

ACCEPTED MANUSCRIPT

Griswold, C.E., Coddington, J.A., Hormiga, G., Scharff, N., 1998. Phylogeny of the orb-web building spiders (Araneae, Orbiculariae: Deinopoidea, Araneoidea). Zool. J. Linn. Soc. 123, 1-99.

Griswold, C.E., Ramírez, M.J., Coddington, J.A., Platnick, N.I., 2005. Atlas of phylogenetic data for Entelegyne spiders (Araneae: Araneomorphae: Entelegynae) with comments on their phylogeny. Proc. Cal. Acad. Sci. 56 (Supp. II), 1-324.

Harvey, M.S., 1992. A new species of *Symphytognatha* Hickman (Araneae: Symphytognathidae) from Western Australia. Rec. West. Aust. Mus. 15, 685-689.

Hedin, M., Bond, J.E., 2006. Molecular phylogenetics of the spider infraorder Mygalomorphae using nuclear rRNA genes (18S and 28S): Conflict and agreement with the current system of classification. Mol. Phylogenet. Evol. 41, 454-471.

Heimer, S., 1986. Notes on the spider family Mimetidae with description of a new genus from Australia (Arachnida, Araneae). Ent. Abh. Mus. Tierk. Dresden 49, 113-137.

Hickman, V.V., 1939, On a dipneumone spider (*Risdonius parvus*, gen. et sp. n.), the female of which has reduced palpi. Proc. Zool. Soc. Lond. (B) 108, 655-660.

Hickman, V.V., 1943. On some new Australian Apneumonomorphae with notes on their respiratory system. Pap. Proc. R. Soc. Tasman. 1943, 179-195.

Hickman, V.V., 1945. A new group of apneumone spiders. Trans. Conn. Acad. Arts Sci. 36, 135-148.

Hickman, V.V., 1979. Some Tasmanian spiders of the families Oonopidae, Anapidae and Mysmenidae. Pap. Proc. R. Soc. Tasman. 113, 53-79.

ACCEPTED MANUSCRIPT

Higgins, E.T., Petterd, W.F., 1883. Description of a new cave-inhabiting spider, together with notes on mammalian remains from a recently discovered cave in the Chudleigh district. Pap. Proc. R. Soc. Tasman. 1882, 191-192.

Hong-Chun, P., Kai-Ya, Z., Da-Xiang, S., Yang, Q., 2004. Phylogenetic placement of the spider genus *Nephila* (Araneae: Araneoidea) inferred from rRNA and *MaSp*1 gene sequences. Zool. Sci. 21, 343-351.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755.

Kjer, K.M., Gillespie, J.J., Ober, K.A., 2007. Opinions on multiple sequence alignment, and an empirical comparison of repeatability and accuracy between POY and structural alignment. Syst. Biol. 56, 133-146.

Lehtinen, P.T., 1975. Notes on the phylogenetic classification of Araneae. Proceedings of the Sixth International Arachnological Congress. Nederlandse Entomologische Vereniging, Amsterdam, pp. 26-29.

Maddison, W.P., Hedin, M.C., 2003. Jumping spider phylogeny (Araneae: Salticidae). Invert. Syst. 17, 529-549.

Mello-Leitão, C.F. de. 1945. Arañas de Misiones, Corrientes y Entre Ríos. Revta. Mus. La Plata (N.S., Zool.) 4, 213-302.

Nunn, G.B., Theisen, B.F., Christensen, B., Arctander, D., 1996. Simplicity-correlated size growth of the nuclear 28S ribosomal RNA D3 expansion segment in the crustacean order Isopoda. J. Mol. Evol. 42, 211-223.

Nylander, J.A.A., 2004. MrModeltest version 2. Program distributed by the author.

ACCEPTED MANUSCRIPT

School of Computational Science, Florida State University (http://www.csit.fsu.edu/~nylander).

Platnick, N.I., 2007. The World Spider Catalog, Version 7.5. American Museum of Natural History, New York, online at http://research.amnh.org/entomology/spiders/catalog/INTRO1.html.

Platnick, N.I., Coddington, J.A., Forster, R.R., Griswold, C.E., 1991. Spinneret morphology and the phylogeny of haplogyne spiders (Araneae, Araneomorphae). Am. Mus. Novit. 3016, 1-73.

Platnick, N.I., Forster, R.R., 1986. On *Teutoniella*, an American genus of the spider family Micropholcommatidae (Araneae, Palpimanoidea). Am. Mus. Novit. 2854, 1-9.

Platnick, N.I., Forster, R.R., 1987. On the first American spiders of the subfamily Sternodinae (Araneae, Malkaridae). Am. Mus. Novit. 2894, 1-12.

Platnick, N.I., Forster, R.R., 1989. A revision of the temperate South American and Australasian spiders of the family Anapidae (Araneae, Araneoidea). Bull. Am. Mus. Nat. Hist. 190, 1-139.

Platnick, N.I., Shadab, M.U., 1978. A review of the spider genus *Anapis* (Araneae, Anapidae), with a dual cladistic analysis. Am. Mus. Novit. 2663, 1-23.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Prendini, L., Crowe, T.M., Wheeler, W.C., 2003. Systematics and biogeography of the family Scorpionidae (Chelicerata: Scorpiones), with a discussion on phylogenetic methods. Invert. Syst. 17, 185-259.

ACCEPTED MANUSCRIPT

Rix, M.G., 2006. Systematics of the Australasian spider family Pararchaeidae (Arachnida: Araneae). Invert. Syst. 20, 203-254.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Ronquist, F., Huelsenbeck, J.P., van der Mark, P., 2005. MrBayes 3.1 Manual. Draft 5/26/2005. Distributed by the authors.

Schnare, M.N., Damberger, S.H., Gray, M.W., Gutell, R.R., 1996. Comprehensive comparison of structural characteristics in eukaryotic cytoplasmic large subunit (23 S-like) ribosomal RNA. J. Mol. Biol. 256, 701-719.

Schoniger, M., Haeseler, A.v., 1994. A stochastic model and the evolution of autocorrelated DNA sequences. Mol. Phylogenet. Evol. 3, 240-247.

Schütt, K., 2000. The limits of the Araneoidea (Arachnida: Araneae). Aust. J. Zool. 48, 135-153.

Schütt, K., 2003. Phylogeny of Symphytognathidae *s.l.* (Araneae, Araneoidea). Zool. Scr. 32, 129-151.

Simon, E., 1880. Matériaux pour servir a une faune arachnologique de la Nouvelle-Calédonie. Ann. Soc. ent. Belg. 23, 164-175.

Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Tautz, D., Hancock, J.M., Webb, D.A., Tautz, C., Dover, G.A., 1988. Complete sequences of the rRNA genes of *Drosophila melanogaster*. Mol. Biol. Evol. 5, 366-376.

Telford, M.J., Wise, M.J., Gowri-Shankar, V., 2005. Consideration of RNA secondary

ACCEPTED MANUSCRIPT

structure significantly improves likelihood-based estimates of phylogeny: Examples from Bilateria. Mol. Biol. Evol. 22, 1129-1136.

Thompson, J.D., Gibson, T.J., plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment. Nucleic Acids Res. 25, 4876-4882.

Vink, C.J., Thomas, S.M., Paquin, P., Hayashi, C., Hedin, M., 2005. The effects of preservatives and temperatures on arachnid DNA. Invert. Syst. 19, 99-104.

Wheeler, W.C., Whiting, M., Wheeler, Q.D., Carpenter, J.M., 2001. The phylogeny of the extant Hexapod orders. Cladistics 17, 113-169.

Wunderlich, J., 1986. Spinnenfauna gestern und heute: Fossile Spinnen in Bernstein und ihre heute lebenden Verwandten. Quelle & Meyer, Wiesbaden.

Wunderlich, J., 2004. Fossil pirate spiders (Araneae: Araneoidea: Mimetidae s. l.) in Baltic and Dominican amber, with notes on intrafamiliar higher taxa. Beitr. Araneol. 3, 1249-1278.

Wuyts, J., De Rijk, P., Van de Peer, Y., Pison, G., Rousseeuw, P., De Wachter, R., 2000. Comparative analysis of more than 3000 sequences reveals the existence of two pseudoknots in area V4 of eukaryotic small subunit ribosomal RNA. Nucleic Acids Res. 28, 4698-4708.

ACCEPTED MANUSCRIPT

Table Captions

Table 1. Taxa sequenced as part of this study, listed according to their current family classification (see Platnick, 2007). Collection localities for voucher specimens, GenBank accession numbers for 18S rDNA and 28S rDNA sequences, and museum registration numbers for voucher specimens are also listed. Highlighted taxa (*) were not sequenced as part of this study. WAM = Western Australian Museum, Perth; MACN = Museo Argentino de Ciencias Naturales, Buenos Aires.

Table 2. List of insertion/deletion events (indels) in different taxa, as numbered in Figures 3-4.

Table 3. Summary of the phylogenetic relationships inferred as part of this study. Taxa and their associated clades are listed, as numbered in the text and in Figures 5-8. Associated records in each column detail whether clades were recovered or not recovered by the four different analyses, and whether clades were strongly- or weakly-supported by Bayesian posterior probability (PP) or parsimony bootstrap (BS) values. Bold highlighted records indicate clades not recovered in a particular analysis or clades recovered in modified form. Note the boxed micropholcommatid clades (Clades 5-8).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure Captions

Fig. 1. Preserved specimens of representative Micropholcommatidae: (A) female *Micropholcomma* sp. from Torndirrup National Park, Western Australia; (B) male *Textricella parva* Hickman, 1945 from Mount Wellington, Tasmania; (C) holotype male *Olgania excavata* Hickman, 1979 from the Gordon River Valley, Tasmania; (D) male Gen. nov. sp. VICBig from the Otway Ranges, Victoria.

Fig. 2. Alternative hypotheses proposed for the phylogenetic position of the Micropholcommatidae. (A) The 'expanded Palpimanoidea' hypothesis of Forster and Platnick, 1984; (B) the 'expanded Anapidae' hypothesis of Schütt, 2003.

Fig. 3. Proposed secondary structure model of the 18S rRNA V3-V5 region of *Hickmania troglodytes* (Higgins & Petterd, 1883). Indels in the different spider taxa studied are numbered (1-17), and further listed in Table 2. Length variable regions excluded from the multiple sequence alignments are shaded, with length variation noted. Primer binding sites are also illustrated at the 5' and 3' ends of the fragment.

Fig. 4. Proposed secondary structure model of the 28S rRNA Domain II region of *Hickmania troglodytes* (Higgins & Petterd, 1883). Indels in the different spider taxa studied are numbered (1-6), and further listed in Table 2. Length variable regions excluded from the multiple sequence alignments are shaded, with length variation noted. Primer binding sites are also illustrated at the 5' and 3' ends of the fragment.

ACCEPTED MANUSCRIPT

Fig. 5. Bayesian majority rule consensus tree resulting from the combined rRNA analysis in MrBayes, with data partitioned according to gene and secondary structure. Clades with posterior probability support > 0.95 are shown on thickened branches, and individual posterior probability values are shown for all other nodes. Clades 1-12 are highlighted, and shaded clades (5-8) represent the Micropholcommatidae. Note the underlined taxa *Teutoniella cekalovici* and Gen. nov. VICBig. Type species are highlighted (*).

Fig. 6. Bayesian majority rule consensus tree resulting from the combined rDNA analysis in MrBayes, with data partitioned only according to gene. Clades with posterior probability support > 0.95 are shown on thickened branches, and individual posterior probability values are shown for all other nodes. Clades 1-12 are highlighted, and shaded clades (5-8) represent the Micropholcommatidae. Note the underlined taxa *Teutoniella cekalovici* and Gen. nov. VICBig. Type species are highlighted (*).

Fig. 7. Parsimony strict consensus tree (of 211 trees) resulting from the combined rDNA analysis in PAUP*. Length = 1767 steps; Ensemble Consistency Index (CI) = 0.463; Ensemble Retention Index (RI) = 0.624; Rescaled Consistency Index (RC) = 0.289. Clades with bootstrap support > 95 are shown on thickened branches, clades with bootstrap support < 50 are shown on thin branches, and individual bootstrap values are shown for all other nodes. Clades 1-12 are highlighted, and shaded clades (5-8) represent the Micropholcommatidae. Note the underlined taxa *Teutoniella cekalovici* and Gen. nov. VICBig. Type species are highlighted (*).

ACCEPTED MANUSCRIPT

Fig. 8. Bayesian majority rule consensus tree resulting from the 28S rRNA analysis in MrBayes, with data partitioned according to secondary structure. Clades with posterior probability support > 0.95 are shown on thickened branches, and individual posterior probability values are shown for all other nodes. Clades 1-12 are highlighted, and shaded clades (5-8) represent the Micropholcommatidae. Note the underlined taxa *Teutoniella cekalovici* and Gen. nov. VICBig. Type species are highlighted (*).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 1
Taxa sequenced as part of this study, listed according to their current family classification (see Platnick, 2007). Collection localities for voucher specimens, GenBank accession numbers for 18S rDNA and 28S rDNA sequences, and museum registration numbers for voucher specimens are also listed. Highlighted taxa (*) were not sequenced as part of this study. WAM = Western Australian Museum, Perth; MACN = Museo Argentino de Ciencias Naturales, Buenos Aires.

Taxon	**Country and Locality**	**18S, 28S, Museum Reg. No.**
LIPHISTIIDAE: Outgroup (1)		
**Liphistius* sp.	MALAYSIA: Gua Tempurong Cave	**DQ639767**, **DQ639851**, NA
HEXATHELIDAE (2)		
**Atrax* sp.	AUSTRALIA: QLD, Lamington National Park	**DQ639787**, **DQ639873**, NA
**Hadronyche* sp.	AUSTRALIA: NSW, Scalloway	**DQ639788**, **DQ639874**, NA
AUSTROCHILIDAE (1)		
Hickmania troglodytes (Higgins & Petterd, 1883)	AUSTRALIA: TAS, Bubs Hill Caves, BH-203	**TBA**, **TBA**, WAM T79989
PALPIMANIDAE (1)		
Otiothops birabeni Mello-Leitão, 1945	ARGENTINA: Parque Nacional El Palmar	**TBA**, **TBA**, MACN-Ar 11491
MECYSMAUCHENIIDAE (1)		
Mecysmaucheniidae sp. NZ	NEW ZEALAND: St James Walkway, Lewis Pass	**TBA**, **TBA**, WAM T79990
HOLARCHAEIDAE (1)		
Holarchaea sp. NZ	NEW ZEALAND: Mt Thomas, near Christchurch	**TBA**, **TBA**, WAM T79991
PARARCHAEIDAE (2)		
Nanarchaea binnaburra (Forster, 1955)	AUSTRALIA: QLD, Lamington National Park	**TBA**, **TBA**, WAM T79992
Westrarchaea spinosa Rix, 2006	AUSTRALIA: WA, Neerabup National Park	**TBA**, **TBA**, WAM T79993
MIMETIDAE (1)		
Australomimetus pseudomaculosus Heimer, 1986	AUSTRALIA: QLD, Lamington National Park	**TBA**, **TBA**, WAM T79994
MALKARIDAE (3)		
Carathea sp. TAS	AUSTRALIA: TAS, Bubs Hill Caves, BH-203	**TBA**, **TBA**, WAM T79995
Malkara sp. QLD	AUSTRALIA: QLD, Mt Tinbeerwah, near Noosa	**TBA**, **TBA**, WAM T79996
Perissopmeros sp. VIC	AUSTRALIA: VIC, The Beeches, near Marysville	**TBA**, **TBA**, WAM T79997
ARANEIDAE (1)		
Eriophora sp. WA	AUSTRALIA: WA, Shenton Park, Perth	**TBA**, **TBA**, WAM T79998
THERIDIIDAE (2)		
Achaearanea sp. WA	AUSTRALIA: WA, Shenton Park, Perth	**TBA**, **TBA**, WAM T79999
Dipoena sp. WA	AUSTRALIA: QLD, Mt Tinbeerwah, near Noosa	**TBA**, **TBA**, WAM T80000
CYATHOLIPIDAE (1)		
Matilda sp. WA	AUSTRALIA: WA, Jarrahdale, near Perth	**TBA**, **TBA**, WAM T80001
SYMPHYTOGNATHIDAE (1)		
Symphytognatha picta Harvey, 1992	AUSTRALIA: WA, near Beedelup National Park	**TBA**, **TBA**, WAM T80002
ANAPIDAE (7)		
Chasmocephalon acheron Platnick & Forster, 1989	AUSTRALIA: VIC, The Beeches, near Marysville	**TBA**, **TBA**, WAM T80003
Crassanapis cekalovici Platnick & Forster, 1989	CHILE: BIO-BIO, Concepción, Cerro Caracol	**TBA**, **TBA**, WAM T80004
Minanapis talinay Platnick & Forster, 1989	CHILE: BIO-BIO, Concepción, Cerro Caracol	**TBA**, **TBA**, WAM T80005
Novanapis spinipes (Forster, 1951)	NEW ZEALAND: Mt Thomas, near Christchurch	**TBA**, **TBA**, WAM T80006
Octanapis octocula (Forster, 1959)	AUSTRALIA: QLD, Redbank Plains, near Goodna	**TBA**, **TBA**, WAM T80007

ACCEPTED MANUSCRIPT

Risdonius parvus Hickman, 1939	AUSTRALIA: VIC, Maits Rest, Otway Ranges	**TBA, TBA**, WAM T80008
Zealanapis montana Platnick & Forster, 1989	NEW ZEALAND: St James Walkway, Lewis Pass	**TBA, TBA**, WAM T80009
MYSMENIDAE (3)		
Trogloneta sp. QLD	AUSTRALIA: QLD, Burbank, Brisbane	**TBA, TBA**, WAM T80010
Taphiassa sp. QLD	AUSTRALIA: QLD, Lamington National Park	**TBA, TBA**, WAM T80011
Taphiassa sp. QLD	AUSTRALIA: TAS, Cuckoo Falls, near Scottsdale	**TBA, TBA**, WAM T80012
MICROPHOLCOMMATIDAE (23)		
Micropholcomma bryophilum (Butler, 1932)	AUSTRALIA: VIC, Cora Lynn Cascades, near Lorne	**TBA, TBA**, WAM T80013
Micropholcomma caeligenum Crosby & Bishop, 1927	AUSTRALIA: VIC, Cardinia Reservoir, near Emerald	**TBA, TBA**, WAM T80014
Micropholcomma sp. QLDBunyas	AUSTRALIA: QLD, Bunya Mountains	**TBA, TBA**, WAM T80015
Micropholcomma sp. QLDTinbeerwah	AUSTRALIA: QLD, Mt Tinbeerwah, near Noosa	**TBA, TBA**, WAM T80016
Micropholcomma sp. WA	AUSTRALIA: WA, Tuart Hill, Perth	**TBA, TBA**, WAM T80017
Micropholcomma '*Plectochetos*' *longissimum* (Butler, 1932)	AUSTRALIA: VIC, Cora Lynn Cascades, near Lorne	**TBA, TBA**, WAM T80018
Micropholcomma '*Plectochetos*' sp. VIC	AUSTRALIA: VIC, Beauchamp Falls, Otway Ranges	**TBA, TBA**, WAM T80019
Micropholcomma '*Plectochetos*' sp. WA	AUSTRALIA: WA, Tuart Hill, Perth	**TBA, TBA**, WAM T80020
Olgania excavata Hickman, 1979	AUSTRALIA: TAS, Bubs Hill Caves, BH-203	**TBA, TBA**, WAM T80021
Parapua punctata Forster, 1959	NEW ZEALAND: St James Walkway, Lewis Pass	**TBA, TBA**, WAM T80022
Parapua sp. QLD	AUSTRALIA: QLD, Mt Tinbeerwah, near Noosa	**TBA, TBA**, WAM T80023
Parapua sp. TAS	AUSTRALIA: TAS, Cuckoo Falls, near Scottsdale	**TBA, TBA**, WAM T80024
Parapua sp. WA	AUSTRALIA: WA, near Beedelup National Park	**TBA, TBA**, WAM T80025
Teutoniella cekalovici Platnick & Forster, 1986	CHILE: BIO-BIO, Concepción, Cerro Caracol	**TBA, TBA**, WAM T80026
Textricella fulva Hickman, 1945	AUSTRALIA: TAS, Mt Wellington, near Hobart	**TBA, TBA**, WAM T80027
Textricella hickmani Forster, 1959	AUSTRALIA: VIC, Beauchamp Falls, Otway Ranges	**TBA, TBA**, WAM T80028
Textricella luteola Hickman, 1945	AUSTRALIA: TAS, Mt Wellington, near Hobart	**TBA, TBA**, WAM T80029
Textricella parva Hickman, 1945	AUSTRALIA: TAS, Mt Wellington, near Hobart	**TBA, TBA**, WAM T80030
Textricella vulgaris Forster, 1959	NEW ZEALAND: St James Walkway, Lewis Pass	**TBA, TBA**, WAM T80031
Textricella sp. NZ	NEW ZEALAND: Mt Thomas, near Christchurch	**TBA, TBA**, WAM T80032
Textricella sp. VICBlack	AUSTRALIA: VIC, Mt Donna Buang	**TBA, TBA**, WAM T80033
Textricella sp. WA	AUSTRALIA: WA, Woodman Point, near Perth	**TBA, TBA**, WAM T80035
Gen. nov. sp. VICBig	AUSTRALIA: VIC, Mt Donna Buang	**TBA, TBA**, WAM T80034

ACCEPTED MANUSCRIPT

Table 2
List of insertion/deletion events (indels) in different taxa, as numbered in Figures 3-4.

Indel No.	Motif	Indel Type	Taxa
18S rRNA INDELS (see Fig. 3)			
1.	C	insertion	*Teutoniella cekalovici*
2.	1 bp	deletion	*Symphytognatha picta*
3.	G	insertion	*Symphytognatha picta*
4.	1 bp	deletion	*Crassanapis cekalovici*
5.	1 bp	deletion	*Micropholcomma* QLDTinbeerwah
6.	GGUC	insertion	*Trogloneta* QLD
7.	C	insertion	*Trogloneta* QLD
8.	C	insertion	*Carathea* TAS, *Perissopmeros* VIC
9.	U	insertion	*Textricella vulgaris*
10.	1 bp	deletion	*Symphytognatha picta*
11.	UU	insertion	*Textricella hickmani*
12.	U	insertion	*Textricella parva*, *T. luteola, T. fulva*, *T.* WA, *Trogloneta* QLD
13.	CU	insertion	*Trogloneta* QLD
14.	A	insertion	*Textricella fulva*
15.	2 bp	deletion	*Nanarchaea binnaburra*
16.	UAUAAA	insertion	*Trogloneta* QLD
17.	2 bp	deletion	*Otiothops birabeni*
28S rRNA INDELS (see Fig. 4)			
1.	U	insertion	*Plectochetos* VIC
2.	G	insertion	*Malkara* QLD
3.	1bp	deletion	*Symphytognatha picta*
4.	A	insertion	*Trogloneta* QLD
5.	A	insertion	*Micropholcomma bryophila*, *M.* QLDTinbeerwah, *Plectochetos longissimum*, *P.* VIC
6.	AA	insertion	*Micropholcomma* WA, *Plectochetos* WA

ACCEPTED MANUSCRIPT

Table 3.
Summary of the phylogenetic relationships inferred as part of this study. Taxa and their associated clades are listed, as numbered in the text and in Figures 5-8. Associated records in each column detail whether clades were recovered or not recovered by the four different analyses, and whether clades were strongly- or weakly-supported by Bayesian posterior probability (PP) or parsimony bootstrap (BS) values. Bold highlighted records indicate clades not recovered in a particular analysis or clades recovered in modified form. Note the boxed microphol commatid clades (Clades 5-8).

Taxon and Clade No.	**Bayesian Combined rRNA (Fig. 5)**	**Bayesian Combined rDNA (Fig. 6)**	**Parsimony Combined rDNA (Fig. 7)**	**Bayesian 28S rRNA (Fig. 8)**
HEXATHELIDAE				
Clade 1	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (BS > 95)	Recovered and strongly-supported (PP > 0.95)
ARANEOMORPHAE				
Clade 2	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and weakly-supported (BS = 75)	**Not recovered**
ENTELEGYNAE + 'ARCHAEOIDEA'				
Clade 3	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (BS > 95)	Recovered and strongly-supported (PP > 0.95)
ARANEOIDEA				
Clade 4	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (BS > 95)	Recovered and strongly-supported (PP > 0.95)
MICROPHOLCOMMATIDAE				
Clade 5 (+ 5' & 5")	Recovered and weakly-supported (PP = 0.70)	Recovered and weakly-supported (PP = 0.72)	**Recovered (as 5' with Clade 9) and weakly-supported (BS < 50)**	**Recovered (as 5" with Gen. nov. VICBig) and strongly-supported (PP > 0.95)**
'TAPHIASSINES'				
Clade 6	Recovered and weakly-supported (PP = 0.55)	Recovered and strongly-supported (PP > 0.95)	Recovered and weakly-supported (BS = 64)	Recovered and strongly-supported (PP > 0.95)
TEXTRICELLINAE				
Clade 7	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and weakly-supported (BS = 78)	Recovered and strongly-supported (PP > 0.95)
MICROPHOLCOMMATINAE				

ACCEPTED MANUSCRIPT

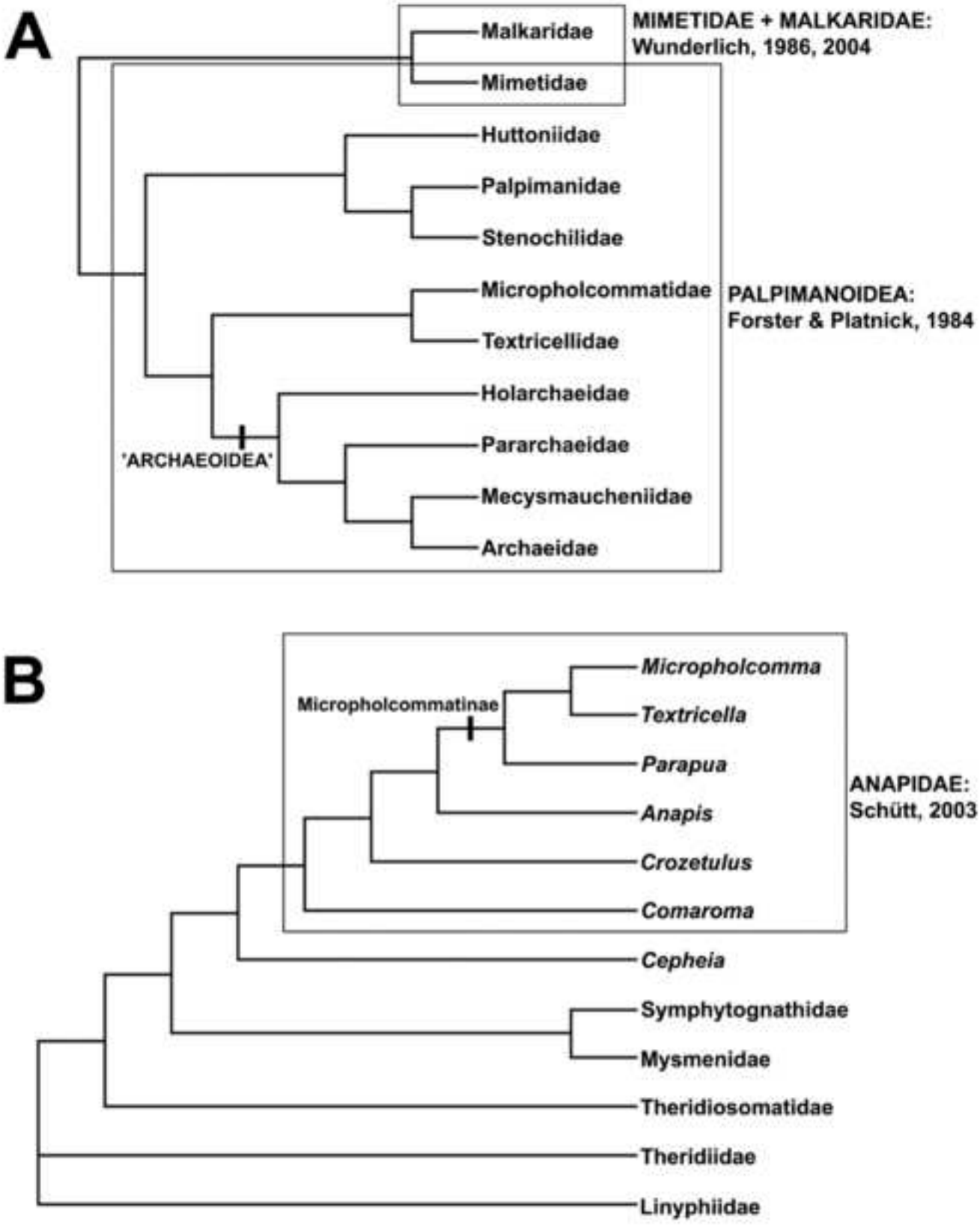
Clade 8	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (BS > 95)	Recovered and strongly-supported (PP > 0.95)
STERNOIDINAE				
Clade 9	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (BS > 95)	Recovered and strongly-supported (PP > 0.95)
ANAPIDAE (*RISDONIUS*-GROUP)				
Clade 10	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (BS > 95)	Recovered and strongly-supported (PP > 0.95)
ANAPIDAE (*CHASMOCEPHALON*-GROUP)				
Clade 11	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (BS > 95)	Recovered and strongly-supported (PP > 0.95)
PARARCHAEIDAE				
Clade 12	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (PP > 0.95)	Recovered and strongly-supported (BS > 95)	Recovered and strongly-supported (PP > 0.95)

ACCEPTED MANUSCRIPT

Figure 1 colour

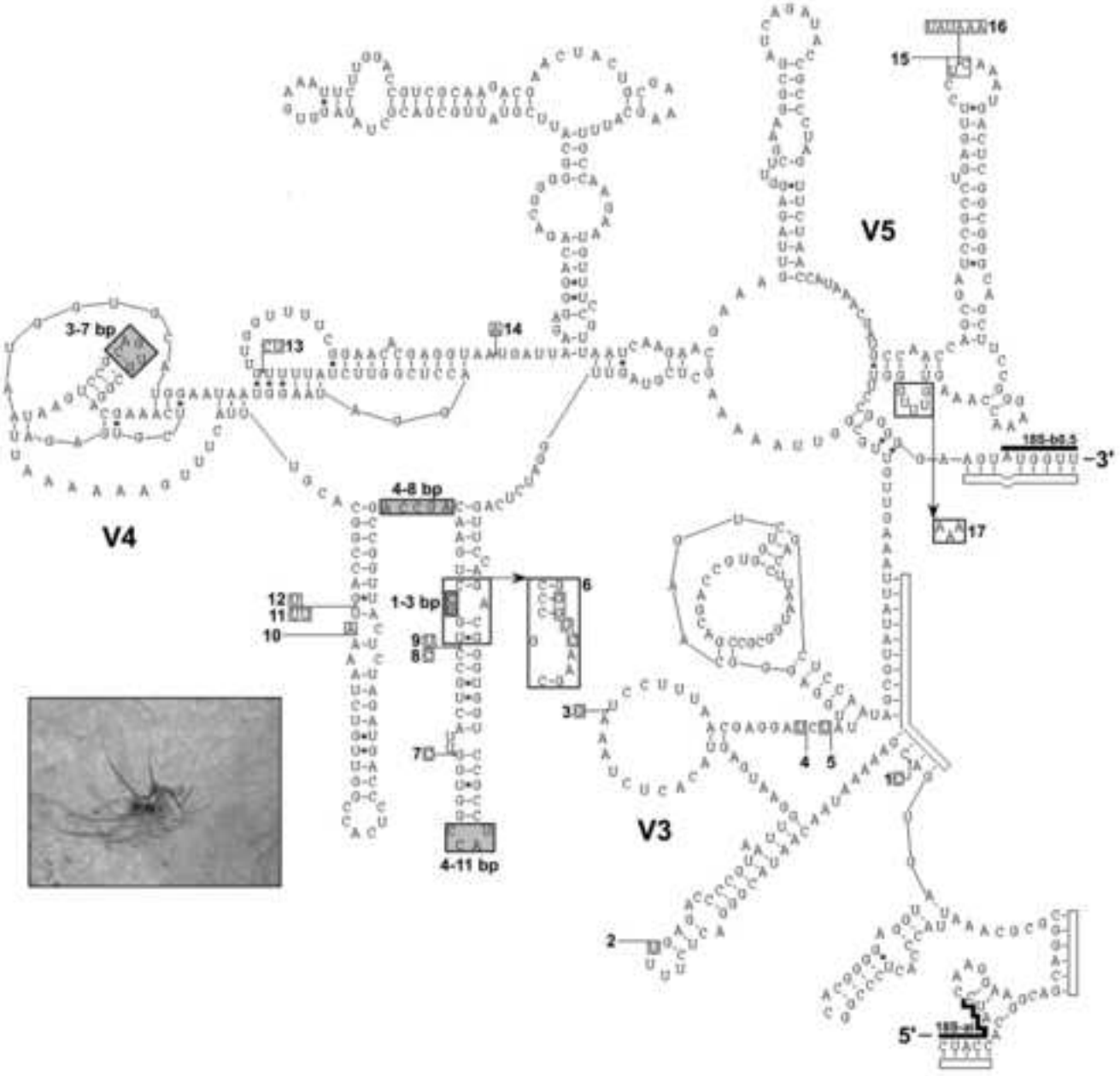
ACCEPTED MANUSCRIPT

Figure 2



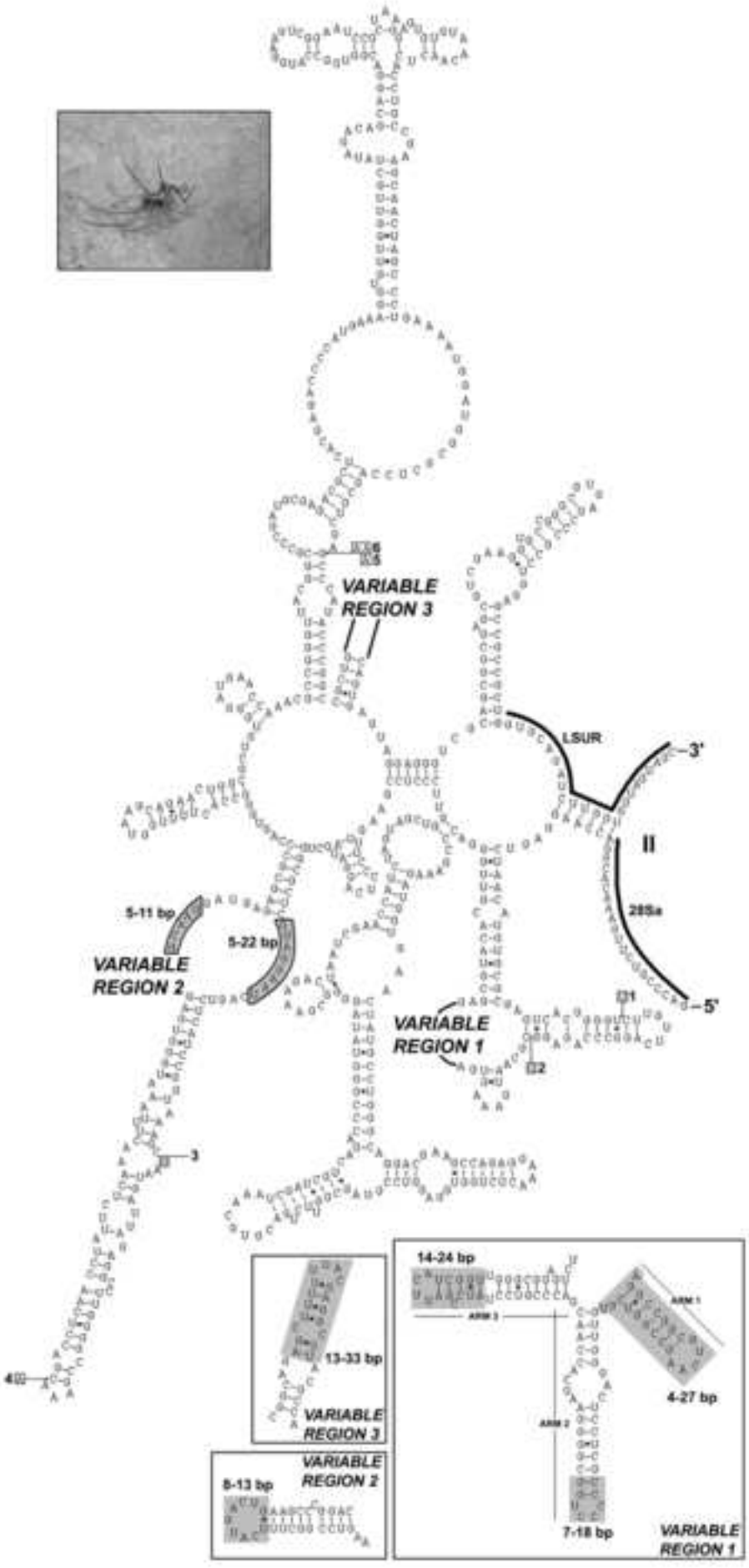
ACCEPTED MANUSCRIPT

Figure 3



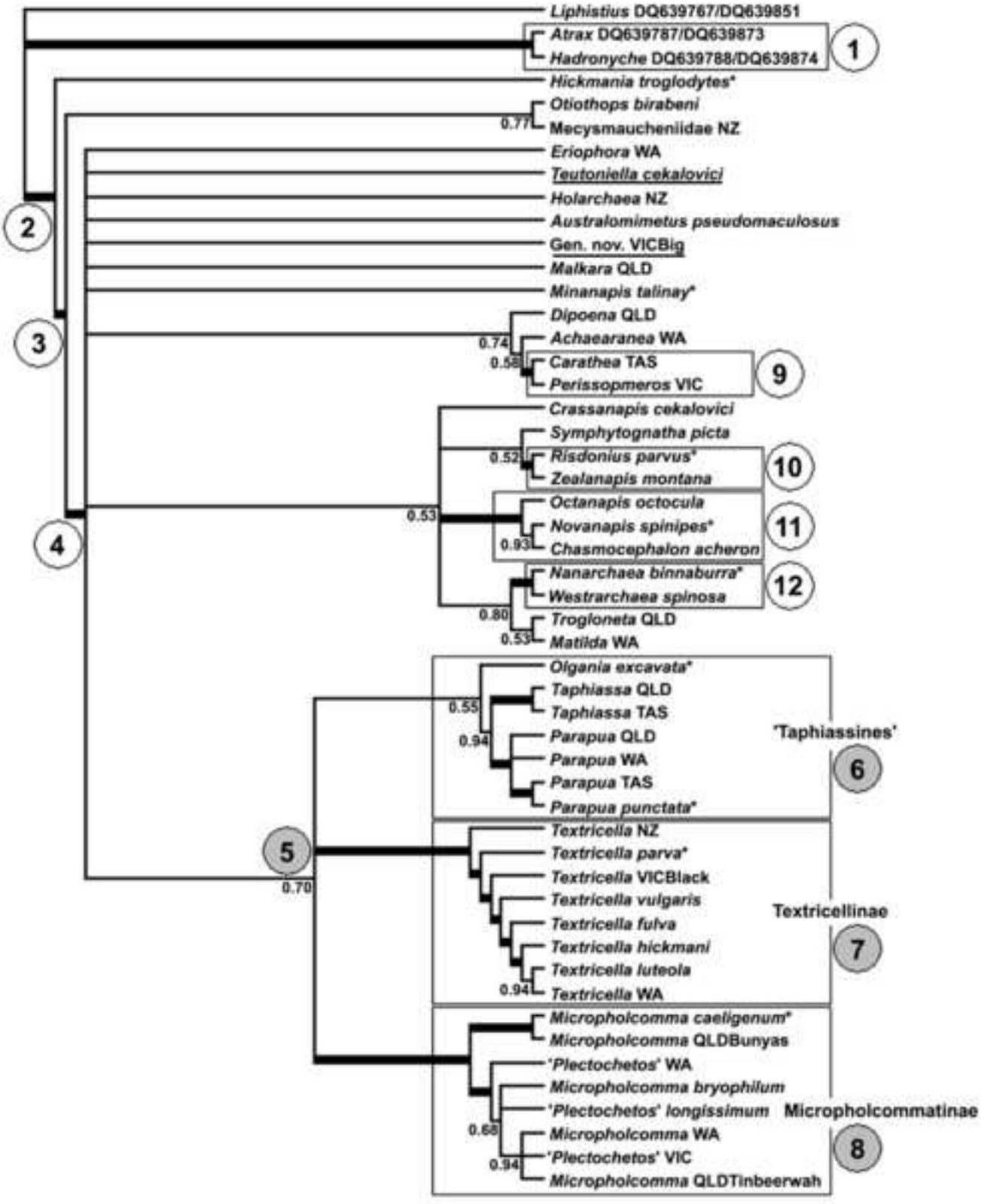
ACCEPTED MANUSCRIPT

Figure 4



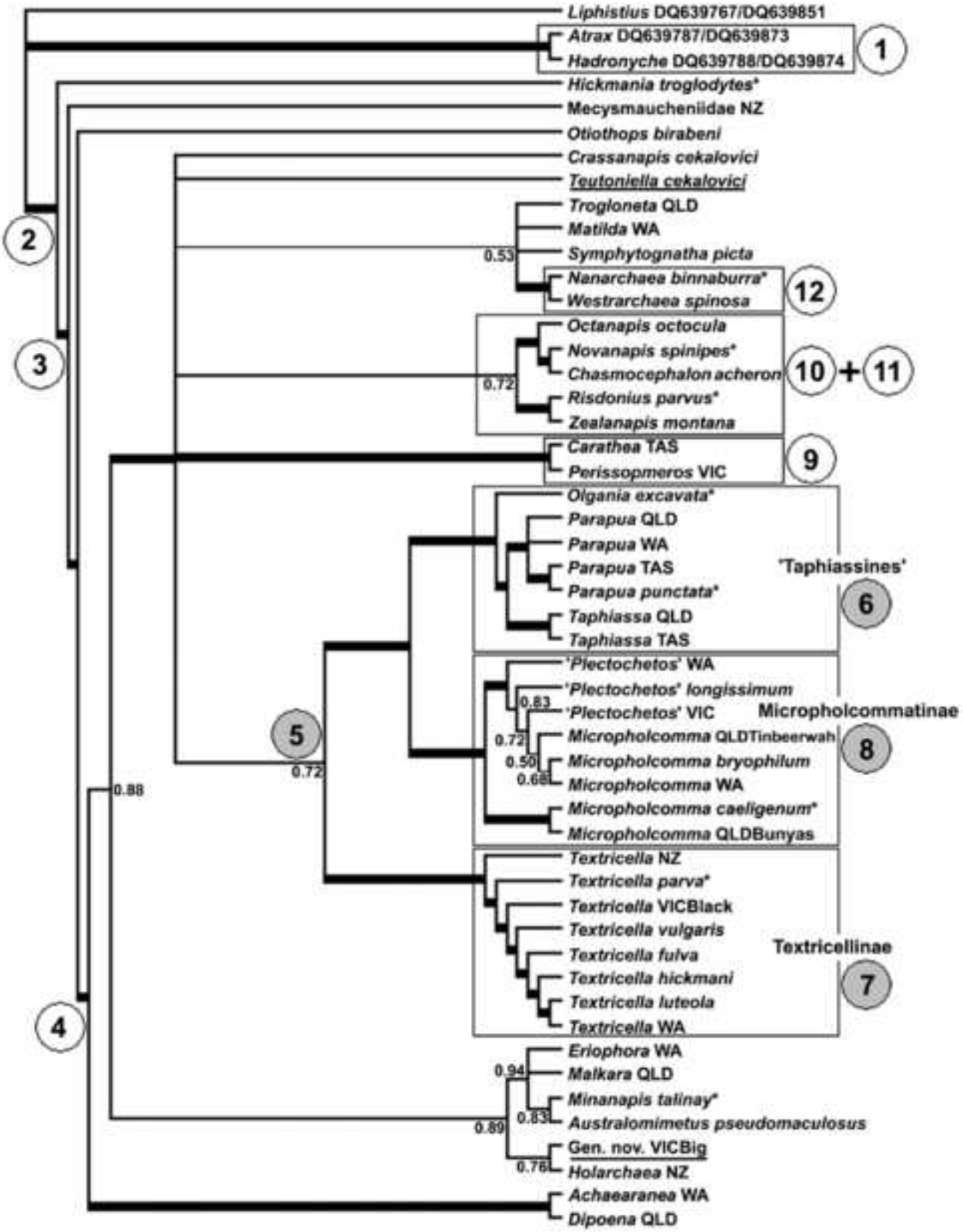
ACCEPTED MANUSCRIPT

Figure 5



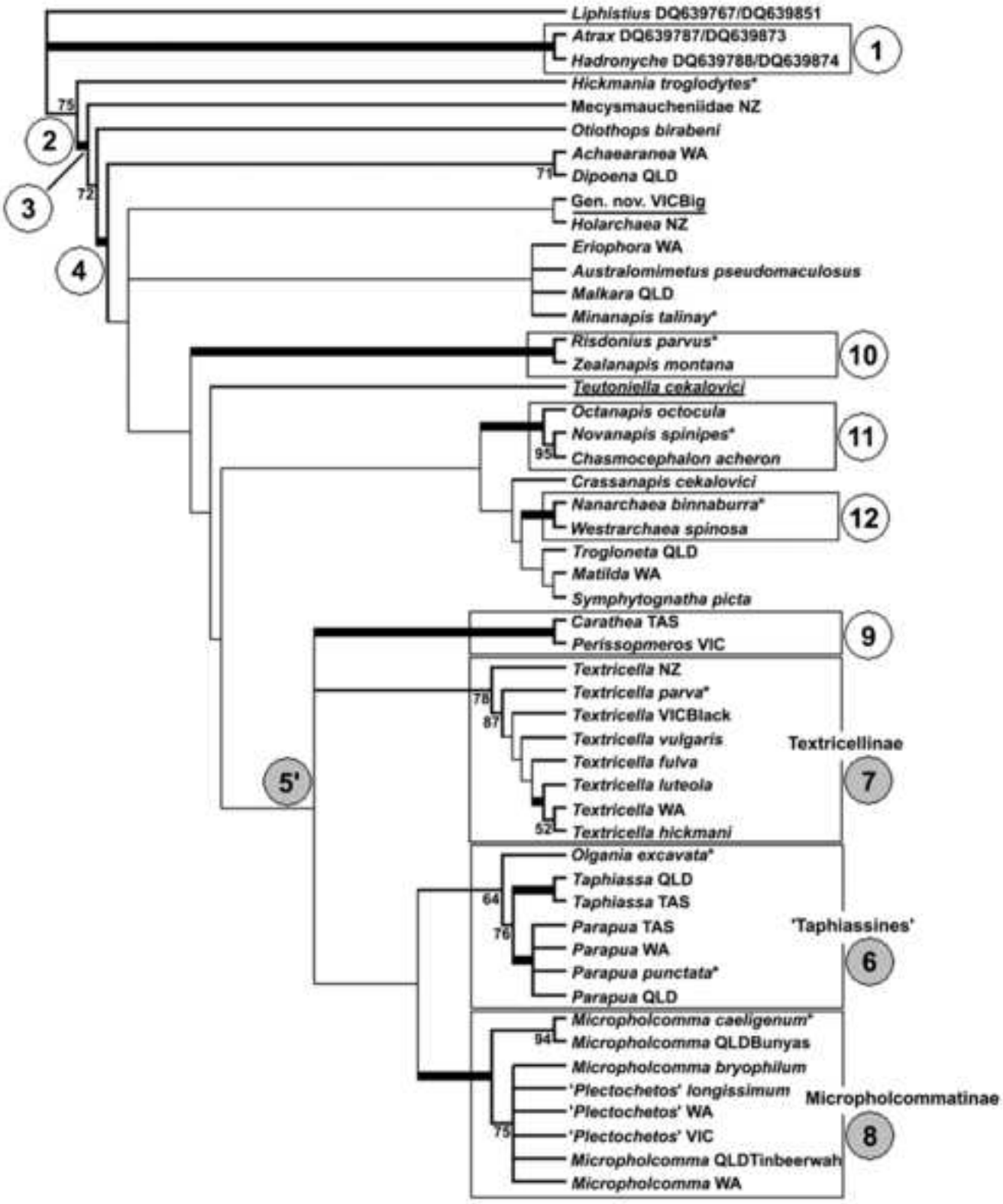
ACCEPTED MANUSCRIPT

Figure 6



ACCEPTED MANUSCRIPT

Figure 7



ACCEPTED MANUSCRIPT

Figure 8