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Phylogenetic Relationships of Basal Hexapods Reconstructed from Nearly Complete 18S and 28S rRNA Gene Sequences

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This study combined nearly complete 28S and 18S rRNA gene sequences (>4100 nt long) to investigate the phylogenetic relationships of basal hexapods (Protura, Collembola, and Diplura). It sequenced more 28S genes, to expand on a previous study from this lab that used 18S plus only a tiny part of the 28S gene. Sixteen species of basal hexapods, five insects, six crustaceans, two myriapods, and two chelicerates were included in the analyses. Trees were constructed with maximum likelihood, Bayesian analysis, and minimum-evolution analysis of LogDet-transformed distances. All methods yielded consistent results: (1) Hexapoda was monophyletic and nested in a paraphyletic Crustacea, and Hexapoda was divided into Entognatha [Collembola+Nonoculata (Protura plus Diplura)] and Insecta (=Ectognatha), but the Nonoculata clade must be accepted with caution because of its strong nonstationarity of nucleotide composition. (2) Within Diplura, the monophyly of Campodeoidea and of Japygoidea were supported respectively, and all methods united Projapygoidea with Japygoidea. (3) Within Protura, Sinentomidae was the sister group to Acerentomata. (4) Within Collembola, the modern taxonomical hierarchy of Collembola (Poduromorpha, Entomobryomorpha, Symphypleona and Neelipleona) was confirmed.

Key words: Protura, Collembola, Diplura, Insecta, 18S rRNA gene, 28S rRNA gene, molecular phylogeny

INTRODUCTION

The "Pancrustacea" hypothesis uniting Crustacea and Hexapoda has gained growing credibility from molecular and morphological evidence (Giribet et al., 2001; Nardi et al., 2003; Luan et al., 2005; Mallatt and Giribet, 2006). Within Hexapoda, Insecta has been well characterized as a good monophyletic group, but the relationships of three basal hexapodan groups (Protura, Collembola, and Diplura) have been hotly argued for over a century. Based on mitochondrial-gene studies, Nardi et al. (2003) suggested Hexapoda is not monophyletic, and Collembola is basal to a clade of "crustaceans+insects". Recently, using more species and mitochondrial genes, Carapelli et al. (2007) "confirmed" non-monophyly of Hexapoda, and proposed crustaceans are more closely related to Insecta sensu stricto than are Collembola and Diplura. However, the previous studies based on ribosomal RNA and protein genes support Hexapoda as monophyletic (Luan et al., 2005; Mallatt and Giribet, 2006; Timmermans et al., 2008).

Ribosomal RNA genes are thought to be especially appropriate for resolving higher-level phylogenetic relationships within Arthropoda (Hillis and Dixon, 1991). By analyzing the 18S rRNA gene plus a small fragment (D3–D5 regions) of the 28S rRNA gene, Luan et al. (2005) studied the phylogeny of basal hexapods including 10 proturans, 12 diplurans, and 10 collembolans. Their results supported the monophyly of Hexapoda, a clade of "Protura+Diplura" as 'Nonoculata' ("no eyes"), and the traditional clade Entognatha as Collembola+Nonoculata. These results were upheld by Mallatt and Giribet (2006), who used nearly complete 18S and 28S rRNA genes, but only included one proturan, two diplurans, and three collembolans.

Protura is composed of Acerentomata, Eosentomata and Sinentomata (Yin, 1996), but the phylogenetic position of Sinentomata is controversial, due to the special morphological characteristics of its members. The finding of Luan et al. (2005) supported the monophyly of Acerentomata and of Eosentomata, but Sinentomata (including Sinentomidae and Fujientomidae) was paraphyletic. That is, the phylogeny of Protura was "Fujientomidae+[Sinentomidae+(Acerentomata +Eosentomata)]". However, the positions of Sinentomidae and Fujientomidae were not reliable due to low bootstrap values.

Collembolans live almost everywhere, with great variation in color and body shape. Deharveng (2004) summarized the modern four orders of Collembola (Poduromorpha, Entomobryomorpha, Symphypleona, and Neelipleona), but the validity and the phylogenetic relationships of these orders are still hotly debated, as are the monophyly of Entomobryomorpha and of Symphypleona, and the position of Neelipleona in Collembola. The results of Luan et al. (2005) supported the monophyly of Poduromorpha and of Entomobryomorpha, but found that Symphypleona may be paraphyletic. So far, no gene sequences from Neelipleona have been available.

Pagés (1959) established the higher taxonomic ranking of diplurans: Campodeoidea, Projapygoidea, and Japygoidea. The monophyly of Diplura was debated for a long time, but

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has been confirmed by some recent studies (Luan et al., 2005; Mallatt and Giribet, 2006), although we wanted to recheck it here with more gene sequences. In addition, few studies included Projapygoidea, and its phylogenetic position is still unclear. Luan et al. (2005) supported the projapygid *Octostigma* as close to Japygoidea, but the support values were not uniformly high.

Here, we use nearly complete 18S and 28S rRNA sequences to expand on the previous studies of Luan et al. (2005), which used much less of the 28S rRNA gene, and Mallatt and Giribet (2006), which used fewer taxa of basal hexapods, in order to obtain more evidence on the phylogenetic position of basal hexapods, as well as the systematic status of Projapygidae, Neelipleona, and Sinentomidae in Diplura, Collembola, and Protura, respectively. For this purpose, we sequenced the nearly complete 28S genes of 13 species, broadly sampling all three groups of basal hexapods.

MATERIALS AND METHODS

Proturans, diplurans, and collembolans were collected in 75% ethanol by using modified Tullgren funnels, and stored in 100% ethanol at –20°C after morphological identification. Genomic DNA was extracted from one individual of most species using the single-fly extraction method (Gloor et al., 1993), or with the DNeasy Tissue Kit (Qiagen inc., Valencia CA). The nearly complete 28S rRNA genes (D1–D11 regions) of four proturans, four diplurans, four collembolans, and one myriapod were each amplified in several pieces. PCR amplification and sequence assembly followed protocols described in past studies (Mallatt and Sullivan, 1998; Winchell et al., 2002).

The 18S rRNA gene sequences for these taxa were previously sequenced in our lab (Luan et al., 2005). To place the basal hexapod clades accurately within Arthropoda, we took advantage of the many complete arthropod rRNA sequences that are available in GenBank. Overall, we included four proturans, five diplurans, seven collembolans, five insects, six crustaceans, two myriapods, and two chelicerates. Table 1 lists details for all species used.

All sequences were aligned automatically with Clustal W in BioEdit v7.0.5 software (Hall, 1999), and then checked by eye strictly based on the 18S secondary-structure models of *Xenopus laevis* and *Strongylocentrotus purpuratus* (Gutell, 1994), and the 28S secondary-structure model of *Xenopus laevis* (Schnare et al., 1996), since rRNA secondary-structure is strongly conserved across eukaryotes (Mallatt and Giribet, 2006). Ambiguously aligned sites in the variable regions of 18S and the divergent domains of 28S, which comprised about 42% of the original alignment sites, were excluded from the analysis. The remaining alignment of 18S +28S rDNA contained 4149 sites across the taxa.

We combined 18S and 28S rDNA sequences together for all phylogenetic analyses, because these two genes are transcribed together and belong to the same gene family, so they evolve together (Mallatt and Giribet, 2006). Minimum-evolution analysis of LogDet-transformed distances was performed in PAUP* 4.0 beta 10 (Swofford, 2002) with 1000 bootstrap replicates. Additionally, the maximum likelihood (ML) algorithm was executed in PAUP, in which the GTR+G+I model was found to fit our sequence data best by the AIC approach in Modeltest 3.7 (Posada and Crandall, 1998). Support for clades was evaluated with ML bootstrapping (1000 replicates) in GARLI v0.95 (Zwickl, 2006). Likelihood-based Bayesian inference (Markov Chain Monte Carlo analysis) was also performed, using MrBayes 3.01 (Huelsenbeck and Ronquist, 2001) with the GTR+G+I model. No initial values were assigned to the model parameters, and empirical nucleotide frequencies were used. Four Markov chains were run for 10^6 generations, sampled every 100 generations, and posterior probabilities were calculated from the last 80% of these trees, with the rest discarded as burn-in. Majority-rule (50%) consensus trees were constructed, to produce posterior probabilities.

We also examined the relationships of taxa within Collembola by ME/LogDet, ML, and Bayesian analyses. The rDNA sequences of the immediate outgroups, proturan and dipluran, are too divergent, so introducing an outgroup would reduce the usable base pairs for phylogenetic analysis. Here, we did a within-group analysis without an outgroup, so that a larger number of alignable characters (5185 nt) could be recognized and included.

We accepted clades in the Bayesian tree having ≥98% posterior probability. While in the ML and ME/LogDet bootstrap trees, we accepted values ≥90% as strong support and 70% to 90% as moderate support.

RESULTS

Nucleotide Composition

The Chi-square test of stationarity of nucleotide frequencies in PAUP* was applied to our data set. The sequences of proturans and especially diplurans have a high proportion of C and G nucleotides (diplurans, 61.3%; proturans, 55.6%), which resulted in the highly nonstationary of frequencies across the 31 taxa (χ^2=738.07, df=90, P=0.00000000). The LogDet method is designed to minimize the "long-branch-attraction" artifact this can cause (Lockhart et al., 1994), so our findings from the LogDet method added to the credibility of the ML/Bayesian results (Fig. 1).

When nucleotide frequencies were tested within each of the three basal hexapod groups, they were always stationary (P=0.45 within Protura, 0.47 within Diplura, 0.99 within Collembola). Therefore, the phylogenetic relationships within Protura, Diplura, and Collembola respectively avoid any artifacts of nucleotide nonstationarity.

Trees

The same topological structures of 31 species were obtained by ME/ LogDet, ML, and Bayesian inference (Fig. 1). Hexapoda was always monophyletic within Pancrustacea, with good support values. Our results also reaffirmed that extant Hexapoda are arranged in four well-supported monophyletic lineages: Protura, Diplura, Collembola, and Insecta. Within the basal-hexapod groups, Protura grouped strongly with Diplura as Nonoculata, with universal 100% support, and Nonoculata joined with collembolans with good support (100%, 100% and 99% respectively in Bayesian, ML, and LogDet analyses).

Within Diplura, the monophylies of Campodeoidea and of Japygoidea were supported, and all methods united Projapygoidea with Japygoidea with strong support (100%, 100%, and 91%, respectively, in the Bayesian, ML, and LogDet analyses). Within Protura, *Sinentomon* was the sister group to Acerentomata, with good support (100%, 80% and 100%, respectively, in the Bayesian, ML, and LogDet analyses). Within Collembola, Poduromorpha and Entomobryomorpha were both monophyletic, with strong support, and these two clades grouped together with some support (66% for ML, 100% for Bayesian); Neelipleona was separate from Symphypleona; the two species of Symphypleona always grouped together in the analyses of 31 arthropod species (4149 characters), with very low support values

Table 1. Information on species used in this study.

Classification	Species	Locality	GenBank Numbers		Reference
			18S rDNA	28S rDNA	
Hexapoda					
Protura					
Acerentomata					
Berberentulidae	*Baculentulus tianmushanensis*		AY037169		Luan et al., 2003
		Shanghai, China		EF192433	present study
	Gracilentulus shipingensis		AY596354		Luan et al., 2005
	Gracilentulus maijiawensis	Shanghai, China		EF192435	present study
Eosentomata					
Eosentomidae	*Eosentomon sakura*		AY596355		Luan et al., 2005
		Guangdong, China		EF192434	present study
Sinentomata					
Sinentomidae	*Sinentomon erythranum*		AY596358		Luan et al., 2005
		Jiangsu, China		EF192442	present study
Diplura					
Projapygoidea					
Octostigmatidae	*Octostigma sinensis*		AY145134		Luan et al., 2005
		Guangdong, China		EF192439	present study
Japygoidea					
Japygidae	*Occasjapyx japonicus*		AY596365		Luan et al., 2005
		Shanghai, China		EF192438	present study
Parajapygidae	*Parajapyx emeryanus*		AY037168		Luan et al., 2003
		Shanghai, China		EF192440	present study
Campodeoidea					
Campodeidae	*Campodeidae sp.*		AY859561	AY859560	Mallatt and Giribet, 2006
	Lepidocampa weberi		AY037167		Luan et al., 2003
		Shanghai, China		EF192436	present study
Collembola					
Poduromorpha					
Poduridae	*Podura aquatica*		AY596363		Luan et al., 2005
				AY210838-39	Mallatt et al., 2004
Hypogastruridae	*Triacanthella sp.*		AY859610	AY859609	Mallatt and Giribet, 2006
Entomobryomorpha					
Isotomidae	*Folsomia candida*		AY555515		Giribet et al., 2004
		Shanghai, China		EF392699	present study
Entomobryidae	*Sinella curviseta*		DQ016565		Xiong et al., unpublished data
		Shanghai, China		EF192441	present study
Neelipleona					
Neelidae	*Neelides minutus*		DQ016567		Xiong et al., unpublished data
		Shanghai, China		EF422366	present study
Symphypleona					
Sminthuridae	*Sminthurus viridis*		AY859604	AY859603	Mallatt and Giribet, 2006
Sminthurididae	*Sphaeridia pumilis*		AY145140		Luan et al., 2004
		Shanghai, China		EF192443	present study
Insecta					
Archeognatha					
Machilidae	*Dilta littoralis*		AF005457		Giribet et al., 2000
				AY859570–71	Mallatt and Giribet, 2006
Zygentoma					
Lepismatidae	*Ctenolepisma longicaudata*		AY210811	AY210810	Mallatt et al., 2004
Palaeoptera					
Baetidae	*Callibaetis ferrugineus*		AF370791		Giribet et al., 2001
				AY859557	Mallatt and Giribet, 2006
Neoptera					
Mantodea					
Mantidae	*Mantis religiosa*		AY859586	AY859585	Mallatt and Giribet, 2006
Coleoptera					
Tenebrionidae	*Tenebrio molitor*		X07801		Hendriks et al., 1988
	Tenebrio sp.			AY210843	Mallatt et al., 2004
Crustacea					
Branchiopoda					
Anostraca					
Artemiidae	*Artemia salina*		X01723		Nelles et al., 1984
	Artemia sp.			AY210805	Mallatt et al., 2004
Cladocera					
Daphniidae	*Daphnia pulex*		AF014011		Crease and Colbourne, unpublished data
	Daphnia pulicaria			AF346514	Omilian and Taylor, 2001
Malacostraca					
Decapoda					
Nephropidae	*Homarus americanus*		AF235971		Crandall et al., 2000
				AY859581	Mallatt and Giribet, 2006
Mysidacea					
Mysidae	*Heteromysis sp.*		AY859580	AY859578–79	Mallatt and Giribet, 2006
Maxillopoda					
Branchiura					
Argulidae	*Argulus nobilis*		M27187		Abele et al., 1989
	Argulus sp.			AY210804	Mallatt et al., 2004
Pentastomida					
Cephalobaenidae	*Raillietiella sp.*		AY744887		Giribet et al., 2005
				AY744894, DQ013856–57	Giribet et al., 2005; Mallatt and Giribet, 2006
Myriapoda					
Diplopoda					
Polyxenidae	*Monographis sp.*		AY596371		Luan et al., 2005
		Shanghai, China		EF192437	present study
Xystodesmidae	*Cherokia Georgiana*		AY859563	AY859562	Mallatt and Giribet, 2006
Chelicerata					
Merostomata					
Limulidae	*Limulus polyphemus*		U91490		Giribet and Ribera, 1998
				AF212167	Winchell et al., 2002
Arachnida					
Scorpionidae	*Pandinus imperator*		AY210831	AY210830	Mallatt et al., 2004

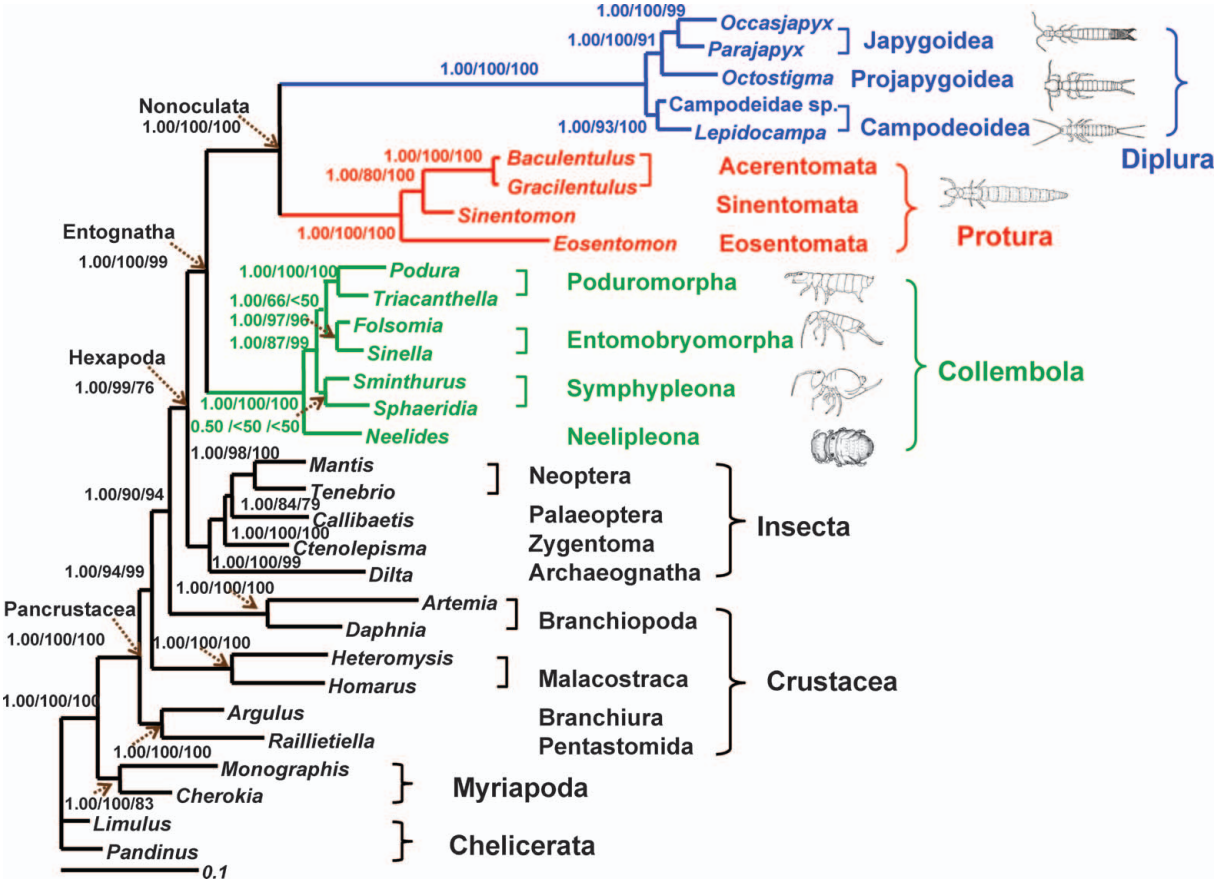

Fig. 1. Bayesian tree calculated from combined nearly complete 18S+28S rRNA gene sequences from 31 taxa, based on the alignment of 4149 characters. The numbers at each node are Bayesian posterior probability/ML bootstrap value (1000 replications)/ME-Logdet-bootstrap value (1000 replicates).

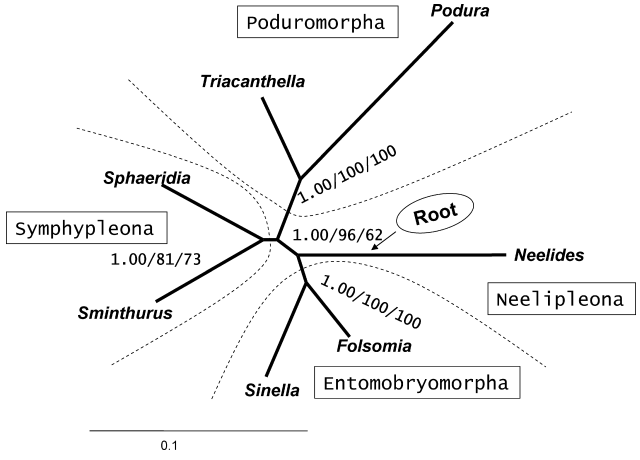

Fig. 2. Bayesian tree including seven collembolan species (genus name only; see Table 1 for species names), without outgroup taxa, based on an expanded alignment of 5185 characters, which should reveal the relationships within collembolans better than the tree in Fig. 1. The numbers at each node are Bayesian posterior probabilitiy/ML bootstrap value (1000 replications)/ME-Logdet-bootstrap value (1000 replicates). The arrow shows where the tree can be rooted, as determined from Fig. 1.

(50%, <50%, and <50%, respectively, in the Bayesian, ML, and LogDet analyses) (Fig. 1), but gained much higher support values (100%, 81%, and 73%, respectively, in the Bayesian, ML, and LogDet analyses) in the presumably better (5185 characters), unrooted tree of seven collembolan species (Fig. 2).

The other arthropod taxa showed the same relationships as determined previously from these same sequences (Mallatt et al., 2004; Mallatt and Giribet, 2006). Most notably, Crustacea was paraphyletic, with monophyletic Branchiopoda close to Hexapoda.

DISCUSSION

Hexapoda

Monophyly of hexapods has been obtained by many morphological and molecular data (Kristensen, 1981, Wheeler et al., 2001, Mallatt and Giribet, 2006), although some molecular data has contradicted this (Giribet et al., 2001; Nardi et al., 2003; Carapelli et al., 2007). Compared with previous studies (Luan et al., 2005; Mallatt and Giribet, 2006), our expanded study with nearly complete 18S and 28S further supported the monophyly of hexapods, with the same internal arrangement of Entognatha (Nonoculata+Collembola) and Ectognatha (Insecta), as well as Protura and Diplura as sister taxa, and with diplurans monophyletic. Therefore, in

adding more 28S sequences and more basal hexapods, we confirmed that Protura, Diplura, and Collembola are basal to a monophyletic Insecta.

'Nonoculata' (Protura+Diplura)

Luan et al. (2005) first recognized the problem of nonstationarity of nucleotide frequencies across the hexapod taxa. Both the dipluran and proturan sequences are CG rich, so these two sequences might have been united artifactually by homoplasy for which the LogDet method may not be able to compensate; see p.1587 in Luan et al. (2005) for a full discussion of this problem. Therefore, our support for 'Nonoculata' is not absolutely solid.

Protura

Protura includes three subgroups, Acerentomata, Eosentomata, and Sinentomata, with three different types of pseudoculus (false eyes; Yin, 1996). In addition, Eosentomata possess spiracles, while Acerentomata does not. Within Sinentomata, fujientomids lack a tracheal system, but sinentomids possess one (though this system differs obviously from that of Eosentomata). The position of Sinentomidae has been debated since Yin (1965) established this taxon. Some experts suggested that Sinentomidae is a special group between Acerentomata and Eosentomata (Imadaté, 1966; Yin 1996), but others placed *Sinentomon* in the Protentomidae of Acerentomata (Tuxen, 1977). Based on complete18S rRNA genes plus partial 28S rRNA genes (D3–D5 regions), Luan et al. (2005) found that the phylogeny of Protura was "Fujientomidae+[Sinentomidae +(Acerentomata+Eosentomata)]". By contrast, the present, expanded, analysis of the complete 18S rRNA gene plus the nearly complete 28S rRNA gene strongly supported Sinentomidae as the sister group of Acerentomata, although we did not include species of Fujientomidae. Further studies will be needed to discern the exact phylogenetic position of the Sinentomidae and Fujientomidae.

Diplura

Diplura is composed of Campodeoidea, Japygoidea, and Projapygoidea. Due to obvious differences in sperm morphology and ovarian structure between Campodeoidea and Japygoidea, the monophyly of Diplura has been questioned (Štys and Bilinski, 1990; Jamieson et al., 2000). Recent studies based on different molecular data and using different analytical methods have not come to an agreement. Campodeidae and Japygidae were apart in both the phylogenetic tree in Shultz and Regier (2000) based on the nuclear EF-1a and Pol II genes, and in the analyses by Giribet et al. (2001) based on a synthesis of eight molecular loci and 303 morphological characters. Conversely, high support for the monophyly of Diplura was obtained from the analyses of rRNA genes (Luan et al., 2005; Mallatt and Giribet, 2006), and the present study strengthened the case for this monophyly (Fig. 1).

Most previous studies were limited to a restricted number of species of Campodeoidea and Japygoidea, and did not include the third dipluran group, Projapygoidea. Specimens of Projapygoidea are quite difficult to find. Different morphological studies have concluded that they are basal diplurans (Rusek, 1982), or that they group with Japygoidea (Štys and Bilinski, 1990), or with Campodeoidea (Pagés, 1997). In the present study, the nearly complete 18S and 28S genes placed Projapygoidea as the sister group to Japygoidea, with high support values (100%, 100%, and 91%, respectively, in the Bayesian, ML, and LogDet analyses). In addition, Luan et al. (2004, 2005) found that the 18S genes in Projapygoidea and Japygoidea were longer by more than 300 bp than this gene in Campodeoidea. The present study obtained the lengths of 28S rDNA genes: 28S from the projapygid *Octostigma sinensis* was 300 bp longer than in two species of Campodeoidea, but 140 bp and 250 bp shorter than in the japygoids *Parajapyx emeryanus* and *Occasjapyx japonicus*, respectively. Thus, in the length of their 28S gene, projapygids are intermediate between Japygoidea and Campodeoidea.

Collembola

Collembola is the most diverse of the basal hexapods, and its internal relationships are complicated. Traditional concepts of the classification of collembolan subgroups have been challenged in recent years. Arthropleona was replaced by two orders, Poduromorpha and Entomobryomorpha (Cassagnau, 1971); Neelidae was separated from Symphypleona as Order Neelipleona (Massoud, 1971). The four modern orders of Collembola (Poduromorpha, Entomobryomorpha, Symphypleona, and Neelipleona) were summarized by Deharveng (2004).

In our phylogenetic trees, Poduromorpha is monophyletic, as is Entomobryomorpha; Neelipleona is separate from Symphypleona (Figs. 1, 2). The two species of Symphypleona grouped together with high support in the unrooted tree of seven collembolans with the most characters included (Fig. 2). Therefore, the paraphyly of Symphypleona suggested by D'Haese (2002) and Luan et al. (2005) was probably an artifact due to including too few nucleotide characters. Still, further studies based on more species and more data will be needed to confirm the monophylies of Poduromorpha, Entomobryomorpha and Symphypleona.

This is the first rDNA study to include Neelipleona, so the position obtained for *Neelides* (Figs. 1, 2) should be discussed further. Neelids and sminthurids have globular bodies, so they were traditionally combined into Symphypleona sensu lato (which includes today's Symphypleona and Neelipleona). However, some authors (Massoud, 1971; Christiansen and Bellinger, 1998) pointed out that the globular body in Neelidae is totally different from that in Symphypleona. Based on the character of an absent protergite, Janssens (2005) proposed a "Neocollembola" clade (Symphypleona, Entomobryomorpha, and Neelipleona), and tentatively suggested Neelipleona is a derived form of Entomobryomorpha.

Our tree based on nearly complete 18S+28S rDNA data showed *Neelides* as a separate clade sister to all other collembolans, with good support (100%/87%/99% for Bayesian/ML/LogDet) (Fig. 1). This confirmed that Neelipleona can be used as a valid order in Collembola. Nobody ever suggested Neelidae is a basal group based on morphological evidence, so ours is an interesting finding that will bear further study.

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