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Monophyly of the ring-forming group in Diplopoda (Myriapoda, Arthropoda) based on SSU and LSU ribosomal RNA sequences

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Abstract

Two controversies exist in the phylogeny of the derived millipedes (Diplopoda). The first is whether millipedes with a fusion ring, including Polydesmida, Spirobolida, Spirostreptida and Julida, form a monophyletic group (the ring-forming group). The second concerns the phylogenetic relationship within the three orders of Juliformia, i.e. Julida, Spirostreptida and Spirobolida. To resolve these phylogenetic controversies, we sequenced 18S and 28S rDNA from six millipede orders and retrieved several homologous sequences from GenBank. Our results give robust support to the monophyly of the ring-forming group based on maximum parsimony methods, maximum likelihood methods and Bayesian inference. The monophyly of the ring-forming group suggests that the fusion of segment sclerites might have occurred only once during millipede evolutionary history. We also established a sister-group relationship between Spirobolida and Spirostreptida within Juliformia after eliminating a short-branch attraction phenomenon, which is consistent with that from the mitochondrial genome analysis.

Keywords: Diplopoda; Ring-forming group; Ribosomal RNA; Monophyly; Short-branch attraction

1. Introduction

Diplopoda is the third most diversified arthropod class [1], next to Insecta and Arachnida, with an estimation of about 80,000 species [2,3]. It is also one of the earliest known terrestrial arthropod groups, with body fossils and trace fossils found in late Silurian (ca. 426–421 Ma) and late Ordovician (ca. 450 Ma), respectively [4,5]. The diversification of millipedes is believed to be related to a series of specific morphological innovations, such as the fusion of segment sclerites (tergite, pleurite and sternite), making a more rigid, ring-form trunk that is better adapted to burrowing and feeding in soil [6]. This character only occurred in the most derived groups, including Polydesmida and Juliformia (Julida, Spirobolida and Spirostreptida).

Enghoff et al. [7] argued that those groups with fused sclerites might constitute a monophyletic taxon referred to as "the ring-forming group". However, such grouping has been controversial. A recent morphological phylogenetic study resolved Polydesmida and Nematophora (Stemmiulida, Callipodida and Chordeumatida) as sister groups [8]. While molecular phylogeny based on the most comprehensive compilation of three protein-coding genes (*EF-1* α, *Pol II* and *EF-2*) placed Polydesmida as a sister taxon to Colobognatha (Polyzoniida, Siphonophorida and

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Platydesmida) [9,10], a reanalysis of the combining data largely confirmed this grouping [2]. Another controversy is the phylogenetic relationship within Juliformia, with all the three possible topologies proposed [2,8,10,11].

There are many problems in previous phylogenetic studies on Diplopoda phylogeny, especially those based on molecular data. First, although the most extensive data compilation [10] claimed to include three genes (*EF-1* α, *EF-2* and *Pol II*) and more than 4000 nucleotide sites, the actual alignable sites are much fewer. Only a short fragment of *EF-2* and *Pol II* is shared among all species. So their contribution to phylogenetic resolution of the diplopods is minimal. The *EF-1* α sequences contain more than 1000 aligned sites, with 503 polymorphic sites. Unfortunately, more than 78% of the polymorphic sites are at the third codon position. The third codon position is of little value for resolving deep phylogenies because they would have experienced full substitution saturation [12,13]. However, no effort was spent on previous studies to determine the degree of substitution saturation. Furthermore, the maximum parsimony (MP) method is employed in previous molecular phylogenetic studies although the high divergence among the sequences would have rendered the MP method inappropriate because MP is prone to long-branch attraction [14–16].

Ribosomal RNA genes (18S and 28S) are popular markers in molecular phylogeny [17,18]. In recent years, combining analysis of 18S and 28S rDNA is widely used in exploring deep systematic problems, such as the phylogeny of arthropod [19–21] and metazoan [22–24]. In this paper, we sequenced 18S rDNA of 14 millipede species and 28S rDNA of 15 millipede species, together with other sequences retrieved from GenBank, to investigate the phylogeny of the ring-forming and related diplopod groups.

2. Materials and methods

Fifteen millipede species, belonging to 10 families of six orders (Table 1), were collected from the Jiangsu and Yunnan Provinces of China. Specimens were stored in 90% ethanol at ambient temperature prior to storage at −20 °C. Sequences of *Cherokia georgiana* (Xystodesmidae, Polydesmida), *Orthoporus* sp. (Spirostreptidae, Spirostreptida) and the outgroup Polyxenid sp. (Polyxenidae, Polyxenida) were retrieved from GenBank.

Genomic DNA was isolated with the DNeasy Blood & Tissue Kit Qiagen Inc., Valencia, CA. Primers for 18S rDNA follow Refs. [25,26] and those for 28S rDNA follow Refs. [17,24,27].

A 50 μl system was used in PCR amplification, which comprised 1× PCR buffer, 1.5–2.5 mM/l Mg^{2+} (optimized for each reaction), 0.2 mM/l for every four dNTP, 0.2 μM/l for each of the two primers, 1 U *TaqE* and 1 μl template. PCR kits were provided by Biocolor Bioscience & Technology Company (Shanghai, China) or TranGen Biotech (Beijing, China). Reactions run on the Perkin-Elmer GeneAmp PCR System 9600 follow the program below: first denature for 3 min at 94 °C, then run 40 amplification cycles (denature for 30 s at 94 °C, anneal for 30 s at 48–54 °C, extend for 30–90 s at 72 °C), finally incubate for 10 min at 72 °C for full extension. Annealing temperature and extension time in the cycle reaction depend on primer set and target sequence length, respectively.

PCR products were purified with the 3S Spin PCR Product Purification Kit provided by the Biocolor Bioscience & Technology Company and the PCR Cleanup Kit and DNA Gel Extraction Kit provided by Axygen Inc. (California, U.S.) and sequenced with the ABI 3730 and ABI 377 automated DNA sequencer. Finally, blocks were assembled using SeqMan Pro 7.1 [28] (see GenBank Accession Nos. in Table 1).

The secondary structure of rRNA sequences is highly conserved among highly diverged taxa and, for this reason, has often been used to guide sequence alignment [29,30] in molecular phylogenetics [31–35]. As no rRNA secondary structure model of millipedes has been experimentally determined, the secondary structure of *Loricera foveata* (Carabidae: Coleoptera: Hexapoda) from The European ribosomal RNA database [36] and 28S rRNA of *Apis mellifera* (Apidae: Hymenoptera: Hexapoda) [37] were used to guide sequence alignment in our study.

18S and 28S rDNA were first aligned with ClustalX [38] under default settings. Then, conserved regions and vari-

Table 1
List of taxa and GenBank Accession numbers used in this study.

Classification	Species	GenBank Accession No.	
		18S rDNA genes	28S rDNA genes
Polyxenida			
Polyxenidae	Polyxenid sp.	AY859596	AY859595
Polyzoniida			
Siphonotidae	?*Dawydoffia* sp.	–	FJ605294*
Callipodida			
Paracotinidae	Paracotinid sp. A	FJ605278*	FJ605292*
	Paracotinid sp. B	FJ605283*	FJ605298*
Polydesmida			
Xystodesmidae	*Cherokia georgiana*	AY859563	AY859562
Polydesmidae	Polydesmid sp.	FJ605279*	FJ605293*
Pyrgodesmidae	Pyrgodesmid sp.	FJ605277*	FJ605291*
Paradoxosomatidae	*Sigipinius* sp.	FJ605274*	FJ605288*
	Nedyopus sp.	FJ605275*	FJ605289*
	Oxidus sp.	FJ605276*	FJ605290*
Spirostreptida			
Spirostreptidae	*Orthoporus* sp.	AY210829	AY210827-8
Harpagophoridae	*Junceustreptus* sp.	FJ605272*	FJ605286*
	Uriunceustreptus sp.	FJ605273*	FJ605287*
Cambalopsidae	*Glyphiulus* sp.	FJ605280*	FJ605295*
	Podoglyphiulus sp.	FJ605282*	FJ605297*
Spirobolida			
Spirobolidae	Spirobolid sp. A	FJ605284*	FJ605299*
	Spirobolid sp. B	FJ605271*	FJ605285*
Julida			
Julidae	Julid sp.	FJ605281*	FJ605296*

*, new sequences; ?, probably taxonomic assignment with uncertainties.

able regions were identified with BioEdit 7.0 [39], with reference to the secondary structure model of *L. foveata* and *A. mellifera*. The procedure mainly followed that of the jRNA website [40] with some modifications. Secondary structures of some ambiguity regions are re-estimated with RNA Structure 4.5 [41]. Overall, 1463 sites were used in 18S rDNA and 1887 sites in 28S rDNA.

The best-fitting substitution model was determined by using ModelTest 3.7 [42]. The congruence between 18S and 28S rDNA is strong ($p = 0.987$, tested with the 'hompart' command of PAUP 4.0b10 [43]), suggesting that the two sets of rRNA sequences can be combined in a single analysis.

PAUP 4.0b10 was employed to build the MP tree (under the graphic interface of PaupUP 1.0.3 [44]). Step matrices were used to weight the *Tv*/*Ti* ratio or the A/T/G/C substitution rates of 18S and 28S rDNA. The heuristic strategy was used in searching trees with simple sequence addition and TBR branch-swapping. Branches were collapsed if maximum branch length was zero. Topological constraints were not enforced. Non-parameter bootstrap was performed 1000 times to test the robustness of the final tree. RAxML 7.0.3 [45] was employed for Maximum Likelihood (ML) tree building. GTR+I+Γ was selected as the best model of 18S and 28S rDNA by ModelTest. The Rapid Bootstrap Algorithm [46] was taken for calculating node support (1000 times). Bayesian Inference (BI) was constructed with MrBayes 3.1.2 [47], in which parameter priors were estimated with Modeltest 3.7. Two analyses were run, each one with four Markov chains (one cold chain and three heated chains). The analyses run two million generations (with the average standard deviation of split frequencies = 0.000431). Trees and parameters were sampled every 100 generations. Stationary sampling was determined using Tracer 1.4 [48], discarding the first 5000 samples with burnin command.

3. Results and discussion

3.1. Monophyly of the ring-forming group

Partition analyses in this study (MP, ML and BI) based on 18S and 28S rDNA support that Polydesmida and Juliformia (see Section 3.2) group together, with moderate to strong supporting values by different methods (Figs. 1–3). This grouping was first advanced by Dohle [49] based on the fusion of segment sclerites into a ring and a similar appendage distributing pattern of the four orders. Enghoff et al. [7] accepted this scheme and named it the 'ring-forming millipedes' informally.

However, recent morphological analyses grouped Polydesmida with Nematomorpha instead of Juliformia [8]. Characters that support this hypothesis include: (1) abrupt development of the gonopod; (2) male gonopore location at the coxa of the second leg. As there are still questions about homology of the gonopod of Polydesmida (the eighth pair of legs) and Nematomorpha (the ninth pair of legs) [4], and the gonopod development of some Nematomorpha (some group of Chordeumatida) is gradual instead of abrupt [50], it is thus improper to use such a character in determining the sister relationship between Polydesmida and Nematomorpha. On the other hand, because the male gonopore of some other Chilognatha groups (such as Sphaerotheriida and Siphonocryptida) is also located at the coxa of the second leg [51], there exists the possibility of homoplasy between the male gonopores of Polydesmida and Nematomorpha. In addition, the monophyly of Nematomorpha is questionable [1].

Molecular phylogenic analyses based on coding genes *EF-1 α*, *EF*-2 and *Pol II* indicate that Polydesmida and Colobognatha are closer to each other [9,10]. This result is repeated by the 'total evidence' analysis based on these three genes and morphological data [2]. However, as pointed out above (under Introduction), phylogenetic signals from those markers are minimal and their suitability for analyzing such deep phylogeny as the ordinal relationships among the diplopods is questionable. The topologies from amino acid sequences and nucleotide sequences are not congruent even when the dataset was decreased to the millipedes range and analyzed methods were optimized [52]. Furthermore, although the 'total evidence' analysis supported this topology scheme, Sierwald and Bond [2] admitted that there was no morphological character that could be evaluated as synapomorphy of Polydesmida and Colobognatha.

In summary, critical morphology (fusion of sclerites into a ring and the appendage distribution pattern) and molecular phylogeny as recovered in this study support the monophyly of the ring-forming group and Polydesmida

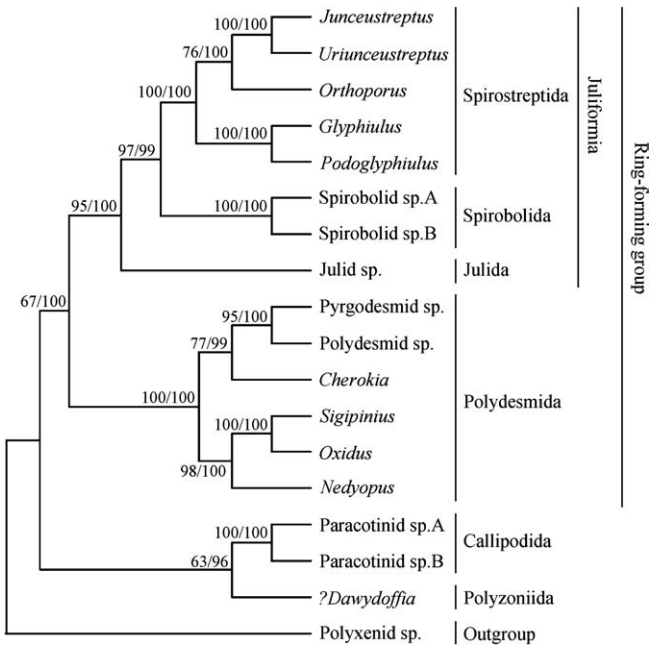

Fig. 1. ML/BI tree based on partition analysis of 18S and 28S rDNA. Numbers on each node are rapid bootstrap values and the Bayesian posterior probabilities (multiplied by 100).

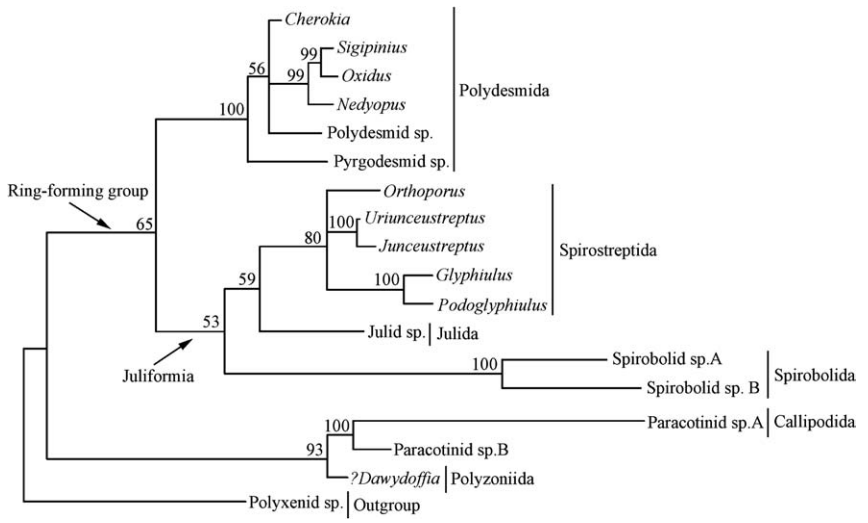

Fig. 2. MP tree based on partition analysis of 18S and 28S rDNA (*Tv*/*Ti* weighting). Note the artifact relationship of Spirostreptida and Julida due to the effect of short-/long-branch attraction often confronted in maximum parsimony analyses of highly diverged sequences. Tree length = 7823, $CI = 0.7599$, $RI = 0.7522$, $RC = 0.5716$. Numbers on each node are non-parametric bootstrap values (multiplied by 100). Only nodes with a support value >50% are shown.

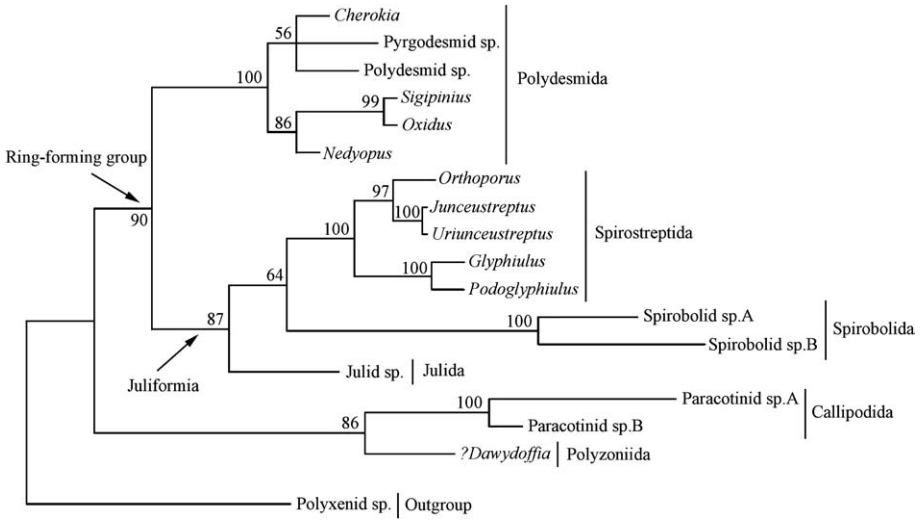

Fig. 3. MP tree based on partition analysis of 18S and 28S rDNA (A/T/G/C substitution rate weighting). Tree length = 73,407, $CI = 0.7043$, $RI = 0.7355$, $RC = 0.5180$. Numbers on each node are non-parametric bootstrap values (100×). Only nodes with a support value >50% are shown.

and Juliformia as the two major components within the ring-forms.

3.2. Phylogenetic relationship within Juliformia

Our results strongly support monophyly of the superorder Juliformia (Figs. 1–3), a scheme that has been advocated by most morphological classifications [7,8,53]. The main controversy is the relationship among the three component orders. Hoffman [51] suggested that Julida and Spirostreptida might compose a monophyletic group, Diplocheta, based on the facts that the tergite of these two orders is divided into the meta- and pro-parts, there is no pleurite, and their sternites do not fuse. Whereas some of these synapomorphies for Diplocheta are still disputable (e.g., their sternites do fuse), some of these characters, such as the tergite and pleurite, are apomorphies for all Juliformia. Although Sierwald and Bond [2] on the basis of the so-called 'total evidence' resolved Julida as a sister to Spirostreptida, they pointed out that it lacks explicit synapomorphy for the grouping. In addition, as discussed above, the insufficiency of the dataset and inconsistent results of amino acid and nucleotide sequences hampered further evaluation of the result based on *EF-1 α*, *EF-2* and *Pol II*.

On the other hand, pure morphologic analyses suggest the close relationship of Spirobolida and Julida [8]. This grouping is mainly based on the gonopod. The eighth pair legs of these two orders are modified to facilitate gonopods (the ninth pair legs) in transferring sperms; while the eighth pair legs of Spirostreptida are modified to be the gonopods

and the ninth pair modified for facilitation. However, among the higher diplopod group Helminthomorpha (an infraclass including Juliformia, Polydesmida and other taxa), the gonopods, which are thought of as modified from appendages, located at variable positions for different helminthomorph groups (see a detailed discussion in Ref. [4]; Fig. 12). Therefore, it is questionable to group Spirobolida and Julida on the basis of the gonopod positions.

Another morphological study suggests the sister-group relationship of Spirobolida and Spirostreptida when some characters are oriented, i.e., given evolutionary directions [11]. This proposed relationship has been supported by a molecular phylogenetic analysis based on the mitochondria genome and the same gene arrangement pattern under the DNL model [54]. There is also an indication that similar geographic distribution of Spirobolida and Spirostreptida might reflect the close relationship between the two orders, i.e. Spirostreptida and Spirobolida consist primarily of tropical species, while the order Julida is distributed widely in the Holarctic region [54].

Our comprehensive analyses of 18S and 28S rDNA, including the MP method after eliminating the short-branch attraction (see discussion below), support the sister-group relationship of Spirostreptida and Spirobolida with Julida as the basal group among Juliformia (Figs. 1 and 3).

3.3. The phenomenon of short-branch attraction

An interesting issue in our analyses is the incongruence between ML/BI trees (Fig. 1) and the MP tree (Fig. 2), for which 18S and 28S rDNA were partitioned with *Tv*/*Ti* weighting matrices during tree building. In the MP tree, Julida and Spirostreptida, both with significantly shorter branches, were grouped together, which is similar to the MP tree based on the three protein-coding genes (*EF-1* α, *EF-2* and *Pol II*) in a previous study although the monophyly of Juliformia was not recovered [10]. This branch length pattern prompted the possibility of the short-branch attraction interfering with the topology in the MP tree, as the MP method usually does not correct for multiple substitutions and is prone to long-branch attraction. As shown above, the problem of long-branch attraction is really due to the sharing of plesiomorphs (unchanged nucleotide sites) among slowly evolving sequences and should be more appropriately termed short-branch attraction [15,16]. While model-based methods such as ML and BI use the substitution model to correct for multiple hits, they are expected to be relatively resistant to the long-branch attraction problem. This expectation is substantiated by analysis of simulated data [55,56]. Incongruence in topology between Figs. 1 and 2 is attributable to the short-branch attraction problem inherent in MP analysis of highly diverged sequences. Therefore, we suggest that the grouping of Julida and Spirostreptida in Fig. 2 is an artifact due to short-branch attraction.

It is noted that although the estimated *Tv*/*Ti* ratio of 18S and 28S rDNA is nearly equal (1.9 and 2.0 respectively), the substitution rates of A/T/G/C of these two genes are quite variable (Table 2). To better accommodate this difference, we used the step matrices, which is the reciprocal of estimated substitution rates (multiplied by 100), instead of the *Tv*/*Ti* ratio. It should be noted that the step matrix of PAUP requires triangle inequality, which may not be satisfied by the substitution rate matrix estimated with Modeltest. Under this circumstance, PAUP will automatically adjust the matrix to satisfy the requirement. Despite all these, our parsimonious reanalysis with refined step matrices broke the sister relationship of the short-branch taxa, resolved Spirostreptida as a sister to Spirobolida (Fig. 3), which is congruent with the ML/BI trees (Fig. 1). In addition, the refined MP analysis also increase node supporting values for the ring-forming group and for Juliformia (Figs. 2 and 3).

4. Concluding remarks

As pointed out above, millipedes probably represent the earliest terrestrial arthropods so far known, which appeared in the terrestrial environment almost as early as the terrestrial higher plant according to the fossil record. The recent discovery of fossil Juliformia in Early Devonian [57] suggests a very early diversification of millipede's higher groups, including Polydesmida and Juliformia, apparently before Early Devonian. The monophyly of the ring-forming group and their fossil record [57] indicate that the morphological innovation of sclerite fusion should have occurred during Silurian or earlier. A preliminary estimation on the origin of the ring-forming group centers in middle Ordovician [58], possibly related with trace markers of the late Ordovician burrowing trace fossil (*Scoyenia beerboweri*) [59]. With the establishment of the internal phylogeny in all major

Table 2
Substitution rates and step matrices weighting of 18S and 28S rDNA.

		A ↔ C	A ↔ G	A ↔ T	C ↔ G	C ↔ T	G ↔ T
18S rDNA	Sub. rates*	1.0158	2.5647	0.8781	0.2856	3.4887	1
	SM wt.#	98	39	114	350	28	100
28S rDNA	Sub. rates	0.9246	2.8230	1.6949	0.4796	5.3110	1
	SM wt.	108	35	59	208	19	100

* Substitution rates: estimated with Modeltest.
\# Step matrix weighting: reciprocal of the corresponding substitution rate multiplied by 100.

groups of Myriapoda and through interdisciplinary approaches of molecular systematics, relaxed molecular clock approaches and increasingly active paleontological discoveries, it will be possible to establish the chronology of major diplopod and myriapod phylogenic events, i.e., the myriapod phylochronology [60,61], in the context of the paleoenvironmental evolution of the Earth, especially in relation to the establishment of the complex terrestrial ecosystem most likely in the early Paleozoic Era.

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