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Analysis of 18S rRNA gene of *Octostigma sinensis* (Projapygoidea: Octostigmatidae) supports the monophyly of Diplura

Yun-Xia Luan[a], Yong-Gang Yao[b], Rong-Dong Xie[a], Yi-Ming Yang[a], Ya-Ping Zhang[b], Wen-Ying Yin[a,*]

[a]*Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, P.R. China*
[b]*Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, P.R. China*

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Summary

The phylogenetic position of Diplura within Hexapoda has been controversial. There are three major lineages in Diplura: Campodeoidea, Projapygoidea, and Japygoidea. However, most of the previous studies were restricted to Campodeoidea and Japygoidea. Until now, only preliminary morphological study on Projapygoidea was reported, and no sequence data from Projapygoidea was available. The main aim of the present study was to investigate the phylogenetic position of Octostigmatidae, one of the three families of Projapygoidea, in Diplura and to test if Diplura are monophyletic. The complete 18S rRNA gene sequences of *Octostigma sinensis* (Projapygoidea: Octostigmatidae) from subtropical China, together with representative species of Campodeoidea and Japygoidea, and several species of Protura and Collembola were analyzed. The phylogenetic trees were obtained by different methods (neighbor-joining, maximum parsimony, and maximum likelihood) with a chelicerate species as outgroup. Our results suggested that *Octostigma* was closer to the genus *Parajapyx* (Japygoidea: Parajapygidae) than to the representative genus of Campodeidae (Campodeoidea). All phylogenetic trees supported the monophyly of Diplura.

*Corresponding author. Tel.: +86-21-54924181; fax: +86-21-54924180.
E-mail address: wyyin@sibs.ac.cn (W.-Y. Yin).

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Introduction

Hexapoda include four groups: Protura, Collembola, Diplura, and Insecta *s. str.* Among these four groups, the Diplura constitutes a key taxon for understanding the evolution of Hexapoda (Kristensen, 1991; Koch, 1997). Pagés (1959) established the higher taxonomic rank of dipluran families: Campodeoidea (Campodeidae+Procampodeidae), Projapygoidea (Anajapygidae+Projapygidae), and Japygoidea (Japygidae+Parajapygidae), which was generally accepted. In 1982, Rusek established Octostigmatidae as a new family of Projapygoidea based on the new species *Octostigma herbivora* from the Tonga Islands. Later, Xie and Yang (1991) described the second species, *Octostigma sinensis*, from South China. Recently, Pagés (2001) described the third species, *Octostigma spiniferum*, from Java.

Whether the order Diplura being monophyletic or not is an unresolved question, because Campodeidae show several obvious differences from Japygidae in the structures of sperm and ovary. The sperm axoneme pattern of Campodeidae, 9+9+2, resembles that of Insecta *s. str.*, whereas the pattern in Japygidae (9+2), is similar to that of Collembola (Jamieson et al., 2000). On the other hand, the meroistic polytrophic type of ovary of Campodeidae shows essential similarities to that of Collembola, but Japygidae has an entirely different type of ovary that is more closely related to that of Insecta *s. str.* (Bilinski, 1994). These anatomical characteristics thus blurred the phylogenetic position of Diplura within Hexapoda. Stys and Bilinski (1990) proposed that Diplura was a paraphyletic group, in which Campodeidae and Japygidae were considered as two independent monophyletic taxa, and especially that Campodeidae was the sister-taxon to Ellipura (Protura+Collembola). This suggestion was supported by a series of following studies (Stys et al., 1993; Bilinski, 1994; Stys and Zrzavỳ, 1994).

Doubts concerning some synapomorphies of dipluran taxa were also raised by the study of *Testajapyx thomasi* (Japygoidea: Japygidae) found in Lower Carboniferous deposit (Kukalová-Peck, 1991). This fossil has well-developed compound eyes and exposed mouthparts, which are different from the existent dipluran species. However, Bitsch (1994) thought that it was probably erroneous to classify *T. thomasi* into Diplura, as its pincer cerci may be a homoplastic character. Based on the fact that the entognathous condition of campodeids and japygids showed remarkable similarities in details and differed from those of Protura and Collembola, Koch (1997) argued for the monophyly of Diplura.

Recent studies based on different molecular data by using different analytical methods have not yet come to an agreement concerning the above points. Campodeidae and Japygidae were apart in the phylogenetic tree of Shultz and Regier (2000) based on nuclear EF-1α and Pol II genes. Conversely, fairly highly supported trees for the monophyly of Diplura were obtained from the analyses of DNA segments of mitochondrial 12S rRNA and nuclear EF-1α genes (Carapelli et al., 2000). Our recent analyses of 18S and 28S rDNA data of representative species in Hexapoda from China (Luan et al., 2003) also supported the suggestion for monophyly of Diplura and agreed with the results of Giribet and Ribera (2000). However, most of the foregoing molecular studies were limited to a restricted number of species of Campodeoidea and Japygoidea. None of them included the third dipluran group, the Projapygoidea.

In the present study, through comparison of the conserved segments of the 18S rDNA gene of representative species of Octostigmatidae (*O. sinensis*), Campodeoidea, and Japygoidea, we aimed at assessing the phylogenetic position of Octostigmatidae in Diplura and testing the monophyly of Diplura.

Materials and methods

Samples collection

All the species sequenced in this study were collected from China (Table 1). In total, seven dipluran species (including *O. sinensis*) were analyzed. In addition, two proturan species, two collembolan species, and one locust species were sequenced for comparison. Specimens were collected in 75% ethanol by Tullgren funnels, and stored in absolute ethanol at $-20\,^{\circ}C$ after identification. An air-dry locust, *Oxya chinensis*, was stored at room temperature. A thysanuran *Lepisma* sp. GG-1997 (GenBank accession no. AF005458) was used as another representative species of Insecta *s. str.* A chelicerate *Nesticus cellulanus* (accession no. AF005447) was selected as the outgroup in the phylogenetic analyses. A crustacean *Artemia salina* (accession no. X01723) and a myriapodan *Epicyliosoma* sp. GG-2001 (accession no. AF370785) were also included for comparison.

DNA amplification and sequencing

Genomic DNA was extracted according to the method described by Gloor et al. (1993). After a single individual was adequately mashed in an Eppendorf tube containing 30 μl Buffer SB (10 mM

Table 1. Classification and accession number of the species considered

Classification	Species	18S rDNA accession no.	Reference
Hexapoda			
Diplura			
Projapygoidea			
Octostigmatidae	*Octostigma sinensis*	AY145134	This study
Japygoidea			
Parajapygidae	*Parajapyx emeryanus*	AY037168	This study
	Parajapyx isabellae	AY145135	This study
Campodeoidea			
Campodeidae	*Lepidocampa weberi*	AY037167	This study
	Lepidocampa takahashii	AY145136	This study
	Pseudlibanocampa sinensis	AY145137	This study
	Campodea mondainii	AY145138	This study
Protura			
Berberentulidae	*Kenyentulus ciliciocalyci*	AY145139	This study
Protentomidae	*Neocondeellum dolichotarsum*	AY037170	This study
Collembola			
Onychiuridae	*Onychiurus yodai*	AY037171	This study
Sminthuridae	*Sphaeridia pumilis*	AY145140	This study
Thysanura			
Lepismatidae	*Lepisma* sp. GG-1997	AF005458	Giribet and Ribera (2000)
Orthoptera			
Catantopidae	*Oxya chinensis*	AY037173	This study
Crustacea			
Anostraca			
Artemiidae	*Artemia salina*	X01723	Nelles et al. (1984)
Myriapoda			
Sphaerotheriida			
Sphaerotheriidae	*Epicyliosoma* sp. GG-2001	AF370785	Giribet et al. (2001)
Chelicerata			
Arachnida			
Araneae			
Nesticidae	*Nesticus cellulanus*	AF005447	Giribet and Ribera (2000)

Tris–Cl pH 8.2, 1 mM EDTA pH 8.0, 25 mM NaCl, and 200 μg/ml Proteinase K), the tube was incubated at 55 °C for 6 h; then, digestion was inactivated by heating at 95 °C for 2 min.

The whole sequence of 18S rDNA was amplified in three overlapping fragments (each of about 800 bp) using primer pairs 1L (5′-TACCTGGTTGATCCTGC CAGT-3′)/1R (5′-TAATATACGCTATTGGAGCTGG-3′), 4f (5′-ACATCCAAGGAAGGCAGCAG-3′)/2R (5′-GAGG TTCCCCGTGTTGAGTC-3′), and 5f (5′-CTCGAAGGC GATCAGATACC-3′)/3R (5′-CCTACGGAAACCTTGTTACG -3′), respectively. A hotstart (98 °C for 5 min before adding Taq polymerase) was used before the PCR cycles [94 °C for 60 s, 50–56 °C (varied according to the different primer pairs) for 60 s, and 72 °C for 60 s, totally 35 cycles], then, ended the cycles by incubation at 72 °C for 10 min for full extension. PCR products were purified with DNA Purification Kit (Beyotime Biotechnology Co.) and were sequenced directly using the BigDye™ Terminator Kits (ABI Applied Biosystems). The sequencing products were analyzed on an ABI 3700 Automated Sequencer (ABI Applied Biosystems, Foster City, CA). Both strands of all fragments were sequenced for at least two independent times by using the primers for amplification and the following internal primers: for fragments amplified by 1L/1R, the internal primer 4R (5′-TAATTTGCGCGCCTGCTGCC-3′) was used; for fragments amplified by 5f/3R, the internal primer 6f (5′-AGAACAGGTCCGTGATGCCC-3′) was used.

Phylogenetic analysis

DNA sequences were edited and aligned by using DNAStar software package (DNASTAR Inc., Madison, WI). For proper alignment, 16 sequences obtained in this study and retrieved from GenBank were checked against available 18S rRNA secondary-structure model of *Drosophila melanogaster* (Hancock et al., 1988).

Three phylogenetic methods, including neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML), were employed to construct the phylogenetic trees using PAUP4.0 (Swofford, 2001). All characters were treated as unordered and gaps were treated as missing data. Bootstrap test (1000 replicates) was used to test the robust of the branches in the trees. We performed phylogenetic analyses based on the aligned data set and a modified data set that discarded the highly divergent regions. The NJ tree was constructed by using uncorrected P-distance (proportional distance, pairwise distance) to show the general cluster pattern of the species, with special attention to see whether dipluran species form a single clade compared with other species from Hexapoda. The branch-swapping algorithm for the MP and ML tree construction was tree-bisection-reconnection (TBR). We used the F84 model for ML tree construction. The ML tree under this model was then tested for several other settings and models of sequence evolution.

Results

Sequence variation

Among the species sequenced, the 18S rDNA fragments showed length differences: in *O. sinensis* it was 2138 bp, which was close to that of the two species of Parajapygidae (2120 and 2144 bp, respectively), but longer than in each of the species of Campodeidae (1837 and 1847 bp, respectively). The length of the aligned sequences was 2358 bp, but with a total of 898 bp in alignment gaps (including missing data). Further alignment checking against the 18S rRNA secondary-structure model of *D. melanogaster* indicated that most of the length differences were located in the loop of region V4 (Hancock et al., 1988).

Table 2 presents the pairwise distances between the species in Hexapoda (excluding gaps and missing data). As expected, small distances (<0.10) were generally found between the species of the same genus compared with those from different genera, which suggested high conservation of 18S rRNA gene at lower taxonomic level (Field et al., 1988; Hillis and Dixon, 1991). The average distance between species within Diplura, Protura, and Collembola was 0.033, 0.042, and 0.051, respectively. These values were far smaller than the distances between the species from each of the three comparisons (Diplura vs. Protura, Diplura vs. Collembola, and Protura vs. Collembola).

Phylogenetic trees

The trees obtained by different phylogenetic methods with respect to the modified alignment data set that discarded highly divergent regions demonstrated similar topologies. The species from Diplura clustered together into a monophyletic group with high bootstrap support (100%). Octostigmatidae showed closer relationship to Parajapygidae than to Campodeidae, although the bootstrap

Table 2. Genetic distances between the species from Hexapoda[a]

Species	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *O. sinensis*		0.005	0.006	0.005	0.006	0.006	0.005	0.010	0.010	0.011	0.010	0.011	0.011
2. *P. isabellae*	0.049		0.002	0.004	0.005	0.005	0.005	0.010	0.010	0.011	0.010	0.011	0.010
3. *P. emeryanus*	0.049	*0.009*		0.005	0.005	0.005	0.005	0.010	0.010	0.011	0.010	0.011	0.010
4. *C. mondainii*	0.046	0.032	0.034		0.003	0.003	0.002	0.010	0.010	0.010	0.010	0.010	0.010
5. *L. takahashii*	0.054	0.041	0.042	0.019		0.002	0.004	0.010	0.010	0.010	0.010	0.011	0.010
6. *L. weberi*	0.051	0.038	0.039	0.016	*0.004*		0.003	0.010	0.010	0.010	0.010	0.011	0.010
7. *P. sinensis*	0.047	0.035	0.037	0.005	0.021	0.017		0.010	0.010	0.010	0.010	0.010	0.010
8. *K. ciliciocalyci*	0.214	0.206	0.209	0.198	0.206	0.204	0.201		0.005	0.010	0.009	0.009	0.009
9. *N. dolichotarsum*	0.210	0.201	0.203	0.196	0.203	0.199	0.199	0.042		0.009	0.009	0.009	0.009
10. *O. yodai*	0.229	0.222	0.219	0.210	0.214	0.212	0.211	0.171	0.165		0.006	0.008	0.008
11. *S. pumilis*	0.212	0.201	0.201	0.189	0.197	0.194	0.191	0.154	0.148	0.051		0.007	0.007
12. *Lepisma* sp.	0.233	0.225	0.227	0.216	0.222	0.218	0.217	0.166	0.159	0.112	0.092		0.006
13. *O. chinensis*	0.220	0.212	0.213	0.203	0.210	0.207	0.205	0.150	0.143	0.104	0.085	0.059	

[a]Below diagonal, P-distances based on the aligned 18S rDNA sequences excluding gaps and missing data; above diagonal, standard errors of the distances.

values supporting the clade (*Octostigma*+*Parajapyx*) varied between the trees (95%, ML; 82%, MP; 71%, NJ) (Fig. 1).

Diplura and Protura formed a sister-group with high bootstrap support in all the trees. This result gave no support to the monophyly of Ellipura (Protura+Collembola) and suggested that this traditional taxon should be considered carefully in phylogenetic analyses. The crustacean *A. salina* was intermixed with the species from Hexapoda in the rooted trees, which was in general agreement with the result of Giribet et al. (2001). We observed similar clustering pattern for the dipluran species when the whole aligned data set were used for tree construction (data not shown). The topological structures of the ML trees remained consistent when F84+G, F81, F81+G, HKY, HKY+G, GTR, and GTR+G models were employed (data not shown).

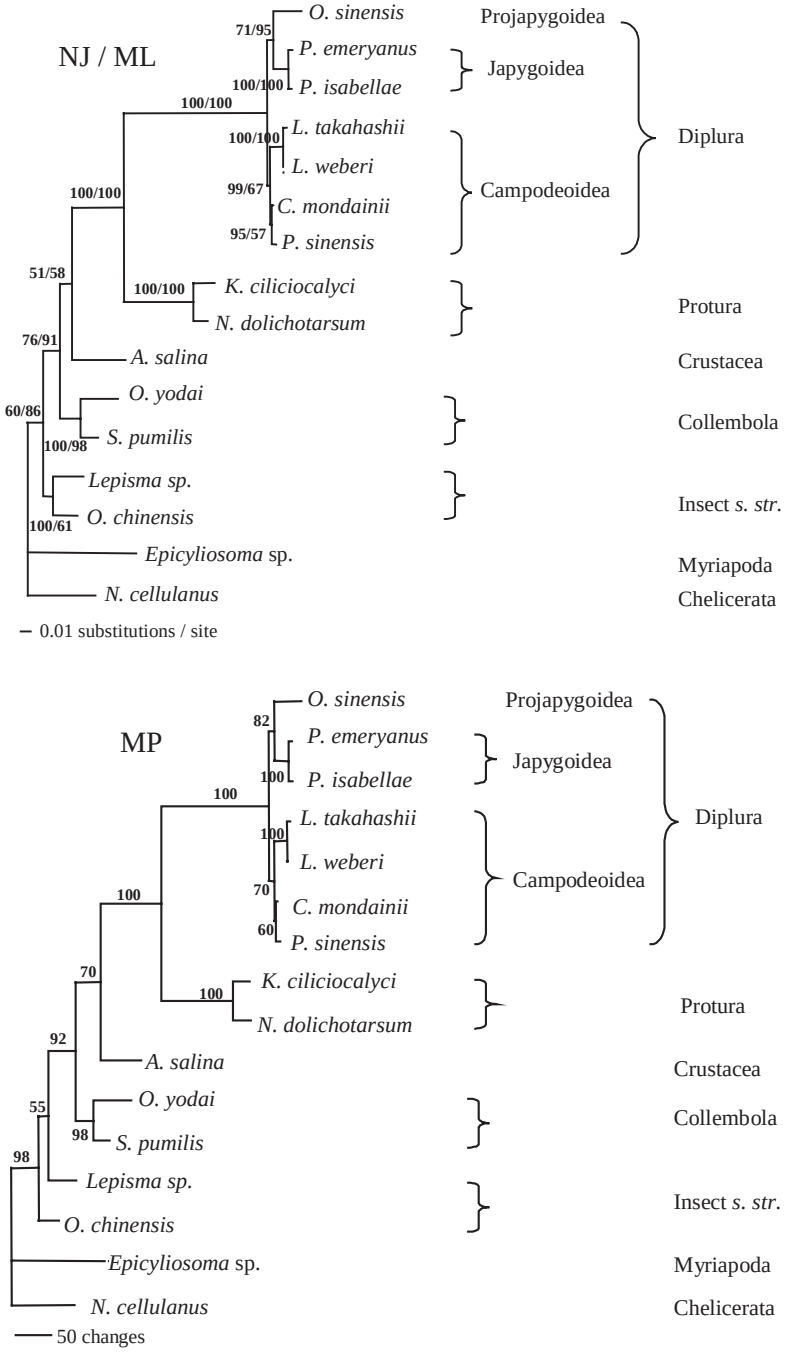

Figure 1. Phylogenetic trees of species in Diplura. The NJ and ML trees had the same topology. The MP tree (tree length=1111, CI=0.744, RI=0.846) had a similar topology to the NJ and ML trees. The numbers on the branches refer to bootstrap values based on 1000 replications.

Discussion

Prior to the study of Wheeler (1997), no molecular analysis considered both groups of Diplura (Campodeidae and Japygidae). In this study, we analyzed seven species of Diplura, including a species of Projapygoidea, *O. sinensis*, into the analysis. Octostigmatidae harbors many morphologically intermediate characters between Anajapygidae and Projapygidae, but it resembles the Japygidae in the number of thoracic spiracles (four pairs) (Rusek, 1982). Moreover, Rusek (1982) thought that the Projapygoidea had more variation in the morphological characters than the other two taxa, Campodeoidea and Japygoidea, such as in cerci and lacinia. Therefore, he suggested that Campodeoidea and Japygoidea were younger groups compared with the Projapygoidea, and Projapygoidea was a relict group of ''living fossils'' of the Diplura.

Pagés (1997) proposed that the Projapygoidea (Projapygidae+Anajapygidae+Octostigmatidae) was more close to Campodeoidea. This hypothesis was supported by the study of Bitsch and Bitsch (2000) that was also based on external morphological evidence. However, comparison of the structures of ovarioles in *Anajapyx* gave a different suggestion: Projapygoidea had closer relationship to Japygoidea than to Campodeoidea (Stys and Bilinski, 1990; Stys et al., 1993). Our results showed that *Octostigma* was closer to *Parajapyx* of Japygidae than to Campodeidae (Fig. 1). Moreover, the length of 18S rDNA sequences of *Octostigma* and *Parajapyx* was approximately equal, but longer than any of the species in Campodeoidea by more than 300 bp.

Previous studies on morphological characters, including the special structure of the labium and of the oral folds, the coaptation between the superlingua and the maxillary galea, unique muscles and pivot in legs (Manton, 1972, 1977), and eyeless and the absence of tentorium (Bitsch and Bitsch, 2000), all suggested that Diplura was monophyletic. However, evidence from the ultra-structure of germ cells indicated that the sperm of Campodeidae was close to that of Insecta *s. str.*, yet its ovarian structure was similar to that of Collembola and differed from that of Insecta *s. str.* In contrast, Japygidae was similar to Collembola in sperm ultrastructure but resembled Insecta *s. str.* in ovarian structure. Based on these discrepancies, Stys and Bilinski (1990) suggested that ''Diplura'' represented a paraphyletic group. In our study, the suggestion for a monophyly of Diplura was supported by two results. Firstly, the genetic distances between the species within Diplura were smaller than those of any comparison of species between Diplura and Collembola or between Diplura and Protura. Secondly, the dipluran species clustered together, and the clade received 100% bootstrap support in all the trees constructed. Further studies on the phylogenetic position of Projapygoidea and the inter-relationships among Octostigmatidae, Anajapygidae, and Projapygidae are still needed.

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