Mitochondrial cytochrome *b* sequences variation of Protura and molecular systematics of Apterygota

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Abstract Mitochondrial cytochrome *b* genes of about 450 bp fragments from 3 proturan species, 5 collembolan species and 2 dipluran species have been sequenced. The number of nucleotide substitutions and Kimura 2-parameter distances have also been calculated, and a series of molecular phylogenetic trees reconstructed by using parsimony and distance methods. The proturan, collembolan and dipluran species have evolved monophyletic groups. The results suggest that Protura and Collembola are sister groups, while Diplura is more or less demonstrating a closer phylogenetic relationship to the pterygotan insects. The phylogeny and their systematic position of protura and other groups are also discussed.

Keywords: **Protura, Apterygota, mitochondrial cytochrome *b* sequence, evolution.**

COMPARED with winged insects, there are lots of arguments on the taxonomic placement or evolutionary relationship of the lower wingless insects, especially on the taxonomic placement of Protura, Collembola, Diplura! in Arthropoda and their relationships with the living pterygotan insects[1]. Based on the results of

post-embryonic development and camparative spermatology, Yin[2,3] posed the question of whether Protura is really an insect. Furthmore, recent study of comparative morphology shows that Protura, Collembola and Diplura seem not to fall into insects, and should be elevated to the classes in parallel with the monophyletic Class Insecta, under the Superclass Hexapoda[1,4]. Fossile data support the phylogeny of Hexapoda, but different inferences were made from the comparative morphology study on the systematic position of those groups[5]. So far, there have been no fossile samples for direct evidence of origin and evolution of Protura. For lack of direct evidence the evolutionary process of Apterygota is unclear. It is thus important to offer novel evidence on DNA level in settling the problems of phylogenetic systematics on higher taxa of Apterygota.

The utility of mitochondrial DNA sequences as useful molecula markers for inferring phylogenetic relationship is now well established[5]. Only 9—10 mtDNA encoded polypeptides are needed to make up complex Ⅲ of the mitochondrial oxidative phosphorylation system. Cytochrome *b* is assumed to harbour two redox centers, which are involved in electron transfer from dihydroubiquinone to cytochrome *c*[6]. Due to such important functions these genes are more conservative, which makes mitochondrial cytochrome *b* an effective tool for the distant phylogenetic relations study.

Up to now, study on the molecular systematics of Protura has not been reported yet. It is a tiny soil-living animal less than 2 mm in length. In the present study, mitochondrial *cytb* fragments of Protura, Collembola and Diplura were sequenced, and compared with those of winged insects. New evidence was given for illustrating some arguments for phylogeny of lower wingless insects.

1 Materials and methods

(ⅰ) Samples. All specimens were collected with the Tullgren funnel method. Sequence data were collected from 9 morphological species of Apterygota (table 1).

Table 1 List of proturan species and other apterygotan species used in this study

Taxa[a)]	Serial	Collecting sites
1 *Gracilentulus maijiawensis*	971225	Kunshan, Jiangsu
2 *Baculentulus tienmushanensis*	970926	Tianmushan, Zhejiang
3 *Kenyentulus japonicus*	970926	Tianmushan, Zhejiang
4 *Onychiurus orientalis*	960911	Suburbs of Shanghai
5 *Onychiurus foliatus*	970324	Western suburbs of Shanghai
6 *Homidia sauteri*	970926	Tianmushan, Zhejiang
7 *Neanura latior*	971129	Fengshan, Chaozhou, Guangdong
8 *Metriocampa sahi*	971226	Kunshan, Jiangsu
9 *Lepidocampa weberi*	980315	Beigaofeng, Hangzhou, Zhejiang

a) 1—3, Protura; 4—7, Collembola; 8—9, Diplura.

(ⅱ) DNA preparation, PCR amplification and DNA sequencing. Genomic DNA was extracted from single individuals of each species, which were ground in Chelex 100 buffer or in homogenizing buffer and incubated overnight in sodium dodecylsulfate (SDS) and proteinase K. DNA was than isolated by phenol-chloroform extraction. The mitochondrial cytochrome *b* gene sequence was amplified with two modified primers CB1/CB2[6]. PCR amplifications were performed in a 50 μl reaction volume for 35 cycles. PCR reaction product was purified in 2.0% low melting SeaPlaque agarose, double stranded DNA was directly sequenced by the dideoxy method using heat denaturation.

(ⅲ) Data analysis. Sequence data were aligned by software package PC/GENE6.6. Basic sequence statistics and Kimura 2-parameters distances were computered using MEGA1.01[8]. Phylogenetic analysis was based on the maximum-parsimony and the distance methods. The molecular tree reconstruction was performed, using program MP from MEGA and Neighbor program from PHYLIP3.5c software package[9].

2 Results and discussion

Each sequenced fragment of mitochondrial *cytb* is about 445—464 bp in length. All these DNA sequences are submitted to Genbank, and compared with the sequences of *Lacusta migratoria*, which belong to winged insects (Heterometabola), and were selected as outgroup (figure 1).

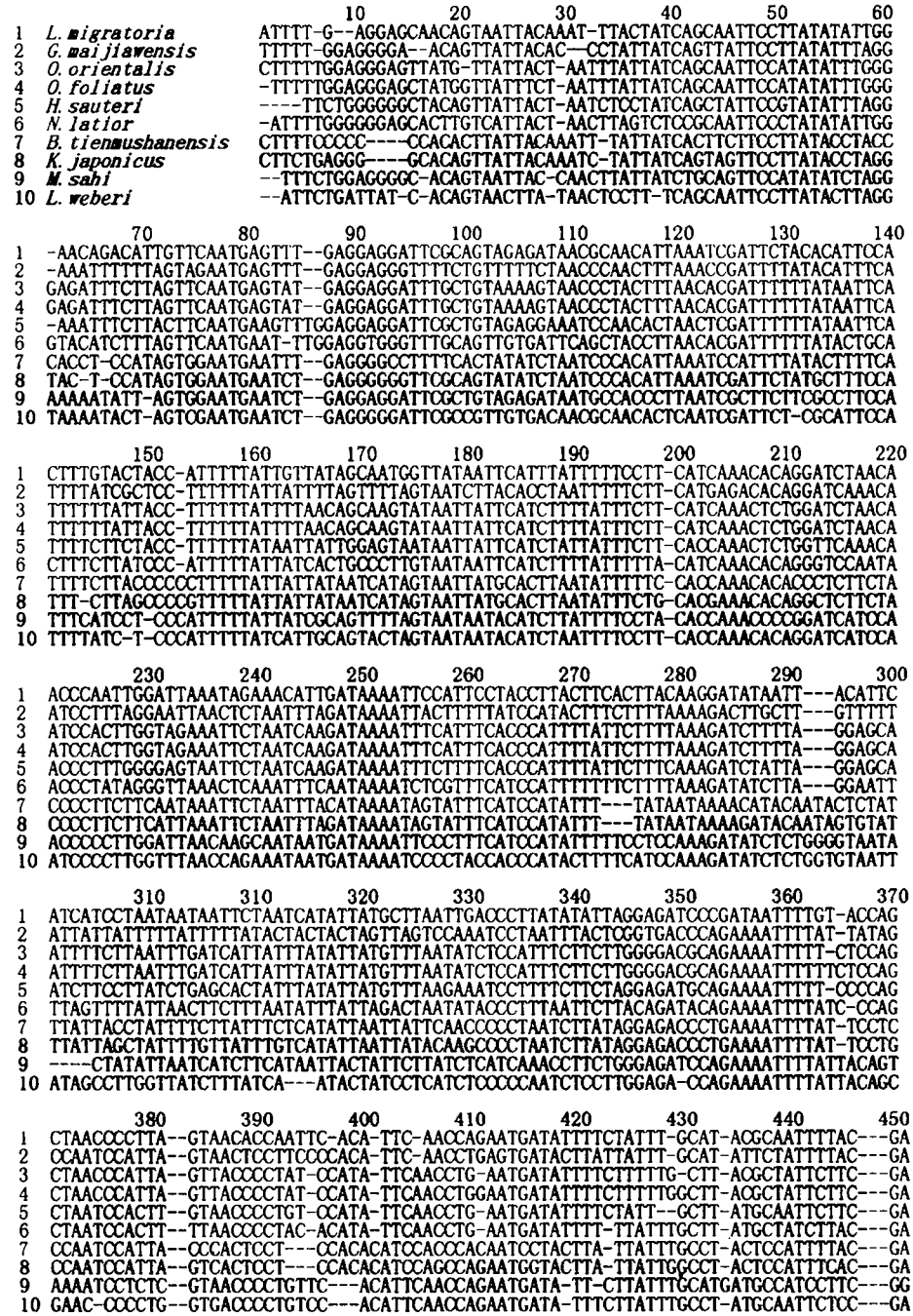

Fig. 1. Aligned mitochondrial cytochrome *b* gene sequences of the Protura and other Apterygotan species. The sequence of *Locusta migratoria* was obtained from Rippe R. M. (1994).

It is worth notice that the transversion number is higher than the transition number among these sequences of the wingless insects. Taking *Neanura latior* and *Baculentulus tienmushanensis* for example, the number of transversion is 114 from 159 nucleotide substitutions——2.5 times higher than the transition number (table 2). In Protura, the number of substitution between *B. tienmushanensis* and *Kenyentulus japonicus* is 43 while the transversion number is 35—4.3 times greater than the transition number. These results differ from those of vertebrate, whose transition number of mtDNA *cytb* sequence is usually higher than the transversion number.

Table 2 Mitochondrial cytochrome *b* gene sequence variation of proturan species and other apterigotan species

Species	1	2	3	4	5	6	7	8	9	10
1 *L. migratoria*		122	112	113	113	122	149	130	120	116
2 *G. maijiawensis*	78		118	119	115	131	122	112	130	133
3 *O. orinentalis*	76	78		1	60	102	143	132	137	143
4 *O. foliatus*	77	79	1		62	103	144	133	138	142
5 *H. sauteri*	77	71	37	38		112	140	131	138	140
6 *N. latior*	74	86	66	67	79		159	147	126	138
7 *B. tienmushanensis*	110	78	94	95	97	114		43	153	159
8 *K. japonicus*	83	65	79	80	84	93	35		134	141
9 *M. sahi*	74	76	82	83	89	76	102	81		70
10 *L. weberi*	70	76	86	85	89	82	102	87	36	

Number of nucleotide substitutions and number of transversions are above and below the diagonal, respectively.

As a result of long-term evolution, the accumulated rich genetic variation in DNA molecules, provided us plentiful genetic information both for phylogenetic reconstruction and probe into the mechanism of molecular evolution. As far as the evolution of protein-coding gene sequence is concerned, the rate of synonymous substitution should be higher than that of nonsynonymous substitution with the limitation of function before the polymorphic site is saturated. Commonly, the transition of the third codon site, the partial transition of the first codon site in mitochondrial *cytb* fragment and the transvertion of the third codon are classified as synonymous substitution while the rest are classified as the nonsynonymous substitution. In vertebrates the transition rate is obviously higher than the transvertion rate, which is caused by more synonymous substitutions in the coding sequence of those closer related species. Up to now there has been no such data in insects and other invertebrates. From the comparison with wingless insects, we could conclude that transvertion rate is higher than the transition rate in mitochondrial *cytb* sequences. In comparison with winged insects, these wingless insects seem to belong to lower groups, such as *Rhyniella praecursor*, which was found from the base of the L. Devonian of Scotland. Genetic divergence of those groups caused by DNA sequence variation is in accordance with the long-term history of their lineage divergence. In order to determine the mean value of genetic divergence degree, according to the variation among sequences (table 3), the Kimura 2-papameters distances are calculated. In Protura, the value of distance between *G. maijiawensis* and *B. tienmushanensis* is 0.5145, which equals the value between *G. maijiawensis* and *Lacusta migratoria*. The genetic distance between *B. tienmushanensis* and *K. japonicus* is 0.1261, which implies little genetic divergence.

Finally, the molecular phylogenetic trees were constructed based on the sequence data, and *L. migratoria* was selected as an outgroup to determine the root of the tree (figures 2, 3).

The phylogenetic tree shows that a monophyletic lineage composed of *G. maijianwensis*, *B. tianmushanensis* and *K. japonicus* of Protura was more closely related with that composed of *O. orientalis*, *O. foliatus*, *H. sauteri* and *N. latior* of Collembola. Both lineages formed a sister group and were related with the lineage composed of *M. sahi* and *L. weberi* of Diplura. Having *L. migratoria* as root, the phylogenic tree presents the evolutionary forms of these three lineages in wingless insects and their relationship with living winged insects.

The neighbor-joining (NJ) tree is in accordance with the maximum-parsimony (MP) tree with *L.*

migratoria as outgroup. And these three groups have strong support (84%, 99% and 100% bootstrap values), which proves that the fragment sequence of mitochondrial cytochrome *b* is a good molecular marker for inferring phylogenic relationships among higher taxa, and brings us new evidence for phylogeny of wingless insects.

Table 3 Kimura 2-parameter distances in the upper-right matrix, standard errors in lower-left matrix

Species	1	2	3	4	5	6	7	8	9	10
1 *L. migratoria*		0.5145	0.4469	0.4533	0.4533	0.5164	0.7490	0.5742	0.5015	0.4750
2 *G. maijiawensis*	0.0656		0.4863	0.4931	0.4675	0.5815	0.5145	0.4502	0.5792	0.6056
3 *O. orientalis*	0.0572	0.0620		0.0025	0.1917	0.3865	0.6832	0.5935	0.6353	0.6886
4 *O. foliatus*	0.0580	0.0628	0.0025		0.1956	0.3922	0.6924	0.6012	0.6435	0.6798
5 *H. sauteri*	0.0580	0.0599	0.0285	0.0288		0.4475	0.6571	0.5819	0.6394	0.6574
6 *N. latior*	0.0662	0.0740	0.0501	0.0507	0.0574		0.8518	0.7223	0.5461	0.6446
7 *B. tienmushanensis*	0.0988	0.0656	0.0875	0.0888	0.0841	0.1132		0.1261	0.7806	0.8472
8 *K. japonicus*	0.0732	0.0583	0.0764	0.0774	0.0741	0.0933	0.0213		0.6091	0.6681
9 *M. sahi*	0.0642	0.0749	0.0821	0.0832	0.0817	0.0701	0.1012	0.0784		0.2306
10 *L. weberi*	0.0610	0.0789	0.0895	0.0883	0.0843	0.0835	0.1113	0.0861	0.0331	

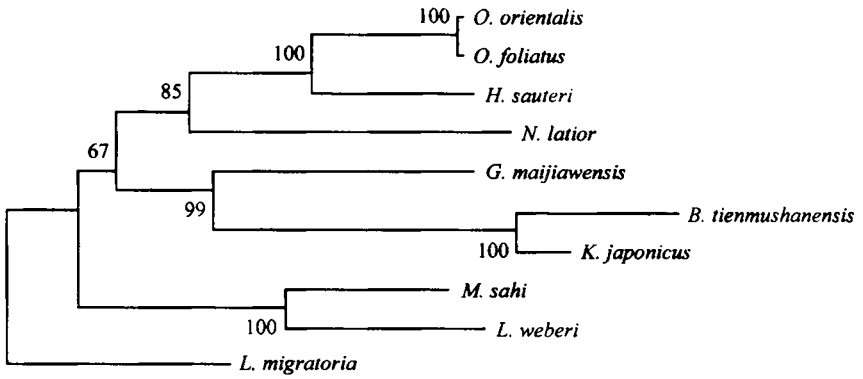

Fig. 2. Neighbor-joining phylogram constructed by the Kimmura-2-parameter distance based on the sequence data. The figures above of the branches are the supporting percentage by 1000 replicates.

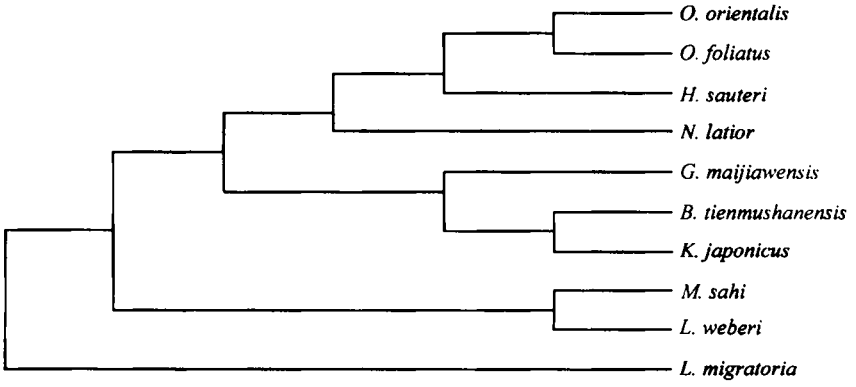

Fig. 3. Maximum parsimony phylogram obtained based on the sequence data.

The Hexapoda is conventionally composed of Protura, Collembola, Diplura, Archaeognatha, Zygentoma and Pterygota. Protura, Collembola and Diplura have been most debated in systematic entomology

and their relationship with winged insects. After summarizing morphological data Hennig[11] and Kristensen[12] considered that these three orders should be classified as Ellipura instead of Insecta s.str. Later Kristensen[4] modified his view and claimed that Ellipura consists of Protura and Collembola only, while Diplura should be classified as an independent group and placed between Ellipura and Insecta s.str.. The morphology and structure of the fossil Diplura, *Testajapyx thomasi*, which was found in the Lower Carboniferous, are different from those of Protura and Collembola. Fossil data demonstrate that Protura and Collembola are sister groups under Parainsecta. Diplura, a much disputed order, is regarded as the most primitive one under Insecta. From the study of post-embryonic development and comparative spermatology, Protura differs greatly in metamerism, structure of respiratory organs, and the axonemal pattern of spermatozoon from other orders of Insecta, so Protura should be raised in order to the rank of class[2,3]. Our study on higher taxa phylogeny by DNA sequencing agrees with the past view that these three groups are independent lineages in the molecular tree and Protura is most closely related with Collembola as a sister group. Diplura is closer to the winged insects than Protura and Collembola. Compared with *Lacusta migratoria*, the NJ tree shows that Protura branch is the longest, and probably this group has the deepest divergence among the three groups.

Since the mean value of the degree of genetic divergence provides valuable evidence for the classification of higher taxa, Frati[13] found that the mean values of genetic divergence of mitochondrial COII gene sequences among the families of Collembola are higher than that of winged insects, rendering strong support for views on evolution of Collembola to the rank of class. Therefore the genetic distances between different species of Protura are even equal to those of other groups. That means compared with winged insects (*L. migratoria*), Protura, Collembola and Diplura should be on a level with class Insecta. This view needs further proving and studying.

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