A preliminary phylogeny of *Tipula* and taxonomic placement of the subgenus *Tipula* (*Sivatipula*) (Diptera: Tipuloidea) based on mitochondrial COI gene

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Abstract: The large genus *Tipula* Linnaeus, 1758 contributes heavily to the biodiversity of the family Tipulidae. However, the monophyly of *Tipula* has not yet been verified. The subgenus *Sivatipula* Alexander, 1964 is possibly the most confusing subgeneric taxon in the genus *Tipula* because of its members' particularly long antenna and one-armed posterior immovable apodeme on semen pump, which makes its subgeneric position uncertain. In this research, the sequences of cytochrome oxidase I (COI) for 19 *Tipula* species and five taxa from other genera are analyzed. Considering the molecular evidence on genetic distance as well as phylogenetic analysis and morphological information, our results indicate that (1) the genus *Tipula* is not resolved as monophyletic in phylogeny based on neighbor joining (NJ) and maximum likelihood (ML) trees because the subgenus *Sivatipula* doesn't form a monophyletic clade with the remaining subgenera of *Tipula*; and (2) *Sivatipula* may deserve a generic status since it forms an independent phylogenetic line.

Key words: crane flies; mitochondrial gene; genetic distance; molecular phylogeny

基于线粒体 COI 基因的大蚊属 *Tipula* 系统发育及长角大蚊亚属 *Tipula* (*Sivatipula*) 分类地位初步研究（双翅目：大蚊总科）

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摘要：大蚊属 *Tipula* Linnaeus, 1758 是大蚊科中种类最多的属，目前其单系性尚未得到全面验证。此外，长角大蚊亚属 *Tipula* (*Sivatipula*) Alexander, 1964 因其极长的触角以及独有的精子泵结构，明显不同于大蚊属其他亚属，使其亚属的分类地位存在争议。本研究基于 COI 序列对 19 个大蚊属物种及 5 个其他属物种进行了系统发育分析，并计算了物种间的遗传距离。研究结果表明：（1）邻接树（NJ）和最大似然树（ML）均显示长角大蚊亚属与大蚊属其他亚属未形成单系，大蚊属的单系性没有得到支持；（2）基于遗传距离和系统发育分析并结合形态信息，结果显示长角大蚊亚属独立于大蚊属内其他亚属，应将其提升为属级分类单元。

关键词：大蚊；线粒体基因；遗传距离；分子系统学

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Introduction

Tipula Linnaeus, 1758, is the largest genus in the family Tipulidae, with more than 2400 species assigned to 40 subgenera, and accounting for over 65 percent of all Tipulidae species (Oosterbroek 2017). It is extremely abundant in the Palaearctic Region with about 1010 species.

Although the species diversity of this genus is rich, very little research has been conducted on its phylogenetic relationships. Advances in the phylogenetics of *Tipula* over the past few decades have been made by a relatively small number of researchers attempting to verify relationships at subgeneric taxonomic levels using morphological characters, such as *Tipula* (*Tipula*) Linnaeus, 1758, *Tipula* (*Vestiplex*) Bezzi, 1924, *Tipula* (*Lunatipula*) Edwards, 1931, and *Tipula* (*Acutipula*) Alexander, 1924 (Theowald 1984; de Jong 1994a, 1994b, 1995a, 1995b; Starkevic 2012). Savchenko (1979) investigated the phylogeny of the family Tipulidae based on morphological features of 14 subgenera from the Western Palaearctic Region, and results indicated that *Tipula* was a monophyletic group. However, monophyly of *Tipula* has never been verified based on molecular data. Lack of molecular phylogenetic studies on *Tipula* has resulted in a limited understanding of relationships at higher levels as well as classification within the family Tipulidae.

Tipula (*Sivatipula*) Alexander, 1964, known as long-horned crane fly, is a small subgenus with *Tipula mitocera* Alexander, 1927 from the east Himalayas, India as its type species. All 11 species in this subgenus are restricted to the Oriental Region, including south China, the east Himalayas of India, and northern regions of Myanmar and Thailand (Oosterbroek 2017). The species of *Tipula* (*Sivatipula*) had been previously placed in the subgenus *Tipula* (*Acutipula*) Alexander, 1924, but were subsequently treated as a distinct group on the basis of the combined structural characters of the antenna, hypopygium and wing (Alexander 1964). However, this subgenus seems to bear little relation with any other subgenus of *Tipula* because of its particular long antenna and one-armed posterior immovable apodeme of the semen pump. These characters make the subgeneric position of *Tipula* (*Sivatipula*) uncertain.

The COI gene has been widely used to evaluate interspecific and intraspecific relationships in Insecta (Barcenas *et al*. 2005; Pan *et al*. 2006; Men & Qin 2011; Dai *et al*. 2012). The aims of this study are therefore to infer phylogenetic relationships within the genus *Tipula* using the COI gene, and to examine the subgeneric position of *Tipula* (*Sivatipula*) based on genetic distance and phylogenetic analysis.

Material and methods

Sample collection and species identify

In this study, partial mitochondrial COI gene sequences of 25 species were used, 10 of which were newly obtained in present study and were submitted to GenBank with the accession numbers listed in Table 1. The remaining sequences were downloaded from GenBank with the accession numbers shown in Table 2. Species in this study were collected from a variety of locations in China (Table 1). An unidentified species in the family Psychodidae (Diptera: Psychodomorpha) was assigned as the outgroup taxon. For identification, the hypopygium of each male was removed and macerated in 10% NaOH for 5

min at 100 °C or overnight at room temperature, and observed in glycerin under a SOIF XTZ-E stereomicroscope (SOIF, Shanghai, China). Specimens and DNA templates were deposited in the Laboratory of Systematics and Evolution, the Provincial Key Laboratory of the Biodiversity Study and Ecology Conservation in Southwest Anhui, Anqing Normal University.

Table 1. Species used in this study and specimen information

Species	Locality	Collector and date	Accession NO.
Dictenidia leigongshanensis	Leigongshan, Guizhou	Qiulei Men 2016.05.17	KY861853
Holorusia basiflava	Dayaoshan, Guangxi	Guoxi Xue 2015.05.12	KY861847
Holorusia oosterbroeki	Diaoluoshan, Hainan	Guoxi Xue 2015.04.20	KY861848
Tangptera hubeiensis	Huangshan, Anhui	Qiulei Men 2014.06.05	KY861852
Tipula (*Formotipula*) *holoserica*	Dayaoshan, Guangxi	Qiulei Men 2016.05.14	KY861856
Tipula (*Formotipula*) *maolana*	Fanjingshan, Guizhou	Guoxi Xue 2015.06.12	KY861854
Tipula (*Formotipula*) *vindex*	Huangshan, Anhui	Qiulei Men 2015.06.01	KY861855
Tipula (*Sivatipula*) *biprocessa*	Cenwanglaoshan, Guangxi	Guoxi Xue 2015.05.07	KY861850
Tipula (*Sivatipula*) *parvauricula*	Cenwanglaoshan, Guangxi	Guoxi Xue 2015.05.11	KY861849
Tipula (*Sivatipula*) *tongbiguanensis*	Tongbiguan, Yunnan	Guoxi Xue 2016.05.10	KY861851

Table 2. The COI sequence downloaded from GenBank with its accession number

Species	GenBank accession NO.	Preference
Ctenophora apicata	KR436394	Hebert *et al*. 2016
T. (*Labiotipula*) *macrolabis*	KM905901	Barcoding Canada Data Release
T. (*Lindnerina*) *senega*	KR427137	Hebert *et al*. 2016
T. (*Lindnerina*) *serta*	KJ087741	Barcode of Life Data Systems Release
T. (*Lunatipula*) *parshleyi*	KR462313	Hebert *et al*. 2016
T. (*Lunatipula*) *saxemontana*	KR742319	Hebert *et al*. 2016
T. (*Platytipula*) *pendulifera*	KR740139	Hebert *et al*. 2016
T. (*Platytipula*) *ultima*	KM569746	Barcoding Canada Data Release
T. (*Pterelachisus*) *wahlgreni*	JQ912055	Pilipenko *et al*. 2012
T. (*Pterelachisus*) *winthemi*	JQ912056	Pilipenko *et al*. 2012
T. (*Savtshenkia*) sp.	KM569864	Barcoding Canada Data Release
T. (*Vestiplex*) *bicalcarata*	KU844262	Men *et al*. 2017
T. (*Vestiplex*) *leigongshanensis*	KU844261	Men *et al*. 2017
T. (*Vestiplex*) *maoershanensis*	KU844263	Men *et al*. 2017
Psychodidae sp.	KT119222	Hebert *et al*. 2016

DNA extraction and PCR amplification

Genomic DNA was extracted from any leg of dry preserved specimens using a Biomiga

Insect gDNA Kit (Biomiga, USA). The partial sequence of the mitochondrial COI gene was amplified using the universal primers for metazoan invertebrates, LCO1490 (5'-GGTCAACAAATCATAAAGATATTG-3') and HCO2198 (5'-TAAACTTCAGGGTGA CCAAAAAAT-3') (Folmer *et al*. 1994). PCR amplifications were carried out using a final volume of 20 μl containing 10 μl 2×PCR Super Master Mix (biotool, Shanghai, China), 0.25 μl of each primer, and 1μl genomic DNA (10–30 ng/μl). Initial denaturation was implemented for 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C for denaturation, 1 min at 52 °C for annealing and 1 min at 72 °C for extension, with a final extension at 72 °C for 10 min. All PCR sets included a negative control reaction tube in which all reagents were contained except the template DNA. After electrophoresis, the target DNA samples were sequenced by Tianyi Huiyuan Biotechnology Co., Ltd. (Wuhan).

Data Analysis

The partial COI gene sequences were aligned with CLUSTAL X (Thompson *et al*. 1997), and then saved as the forms of PHYLIP and FASTA. The pairwise genetic distances were calculated based on the Kimura-2-parameter (Kimura 1980) model using MEGA 6.0 (Tamura *et al*. 2013). For exploring the degree of nucleotide saturation present in the datasets, we plotted raw sequence divergence, p distance based on Transition+Transversion vs. p distance only based on transition or transversion, for all pairwise comparisons among taxa (Huang 2012). Phylogenetic analysis was conducted by the neighbor joining (NJ) method using MEGA 6.0 based on the Kimura-2-parameter model for the bootstrap test repeated 1,000 times, and also the maximum likelihood (ML) method using PHYLIP 3.2 (Felsenstein 1989) for the bootstrap test repeated 1000 times. The CONSENSE subroutine within PHYLIP 3.2 was then applied to generate a consensus tree that provided estimates of robustness at each node based on the bootstrapping of the gene frequencies. The tree was visualized using Treeview 1.6.6 software.

Results

The COI gene sequences and variations

The nucleotide composition was analyzed using MEGA 6.0. There was no gap in the sequences of 603 sites, which included 205 variable sites and 398 conserved sites. In the variable sites, 176 were informative in parsimony analysis, including 139 in the 3rd codon position, 4 in the 2nd codon position and 33 in the 1st position of codons. The average nucleotide compositions of guanine, adenine, thymine, and cytosine were 16.5%, 29.0%, 37.9%, and 16.6%, respectively. Nucleotide frequencies over 603 sites of 24 sequences showed a distinct A+T bias ranging from 64.9% to 68.8%. This bias was stronger for the 3rd codon position (86.1%–94.5%) than for the 1st codon position (50.5%–54.9%) and 2nd codon position (56.2%–57.7%). Substitutions included 31 transitions and 39 transversions among the entire nucleotide sequences.

Analysis of nucleotide saturation

The relationships between p distance calculated from transition+transversion (horizontal axis) and p distance based only on transition or transversion (longitudinal axis) of the COI

sequences were plotted for all pairwise species comparisons (excluding outgroup taxa) (Fig. 1). The results showed linear relations between the two sets of p distance data. All plots indicated that no saturation was found in the COI gene in this study. Results also revealed that the number of transitions was less than that of transversions.

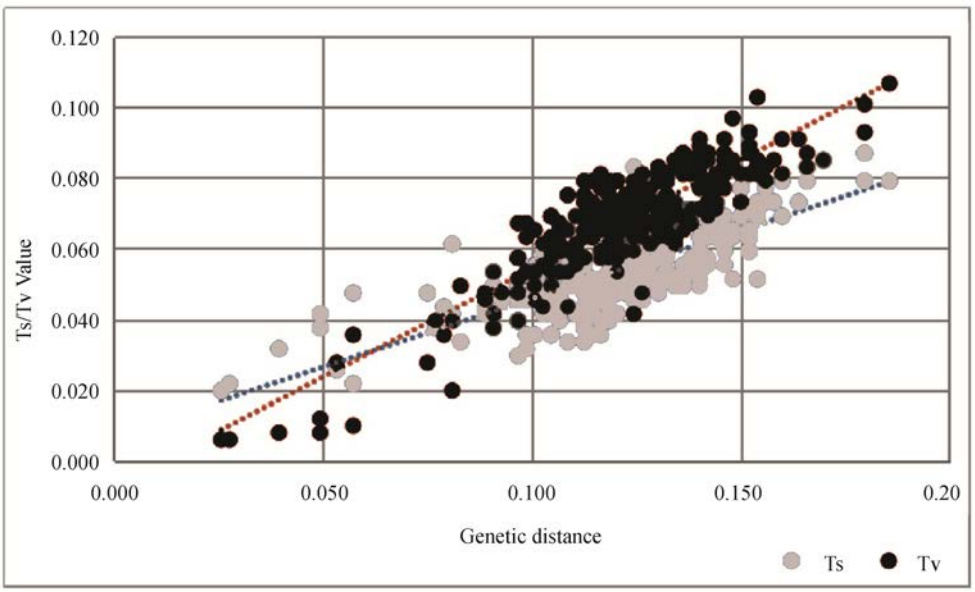

Figure 1. Relationships of p distance based on transition+transversion (horizontal axis) and p distance based only on transition or transversion (longitudinal axis).

Phylogenetic analysis

NJ and ML trees were built using MEGA and PHYLIP software. The phylogenetic analyses by these two trees show the same conclusion. Species in subgenera *Tipula* (*Labiotipula*), *Tipula* (*Lindnerina*), *Tipula* (*Lunatipula*), *Tipula* (*Pterelachisus*), *Tipula* (*Platytipula*), *Tipula* (*Vestiplex*), *Tipula* (*Formotipula*) and *Tipula* (*Savtshenkia*) cluster together to form a monophyletic group (bootstrap values: A, 63; B, 63.5), which is separated from the clade of *Tipula* (*Sivatipula*). Given the above, the genus *Tipula* is not resolved as monophyletic in the phylogeny inferred from the COI sequence. *Tipula* (*Sivatipula*) does not cluster with other subgenera of *Tipula*, therefore forming an independent phylogenetic line (Fig. 2).

Genetic distances

The pairwise genetic distances of five genera including nine subgenera were calculated using COI sequence based on the Kimura-2-parameter model (Table 3). The genetic distance between *Tipula* (*Sivatipula*) and *Tipula* (*Labiotipula*) is 0.188, bigger than the comparison values between *Tipula* (*Sivatipula*) and the other genera including *Ctenophora* (0.140), *Dictenidia* (0.143), *Holorusia* (0.135) and *Tanyptera* (0.121). The same situation is found in comparisons of *Tipula* (*Sivatipula*) with other subgeneric members of *Tipula*, such as *Tipula* (*Lindnerina*) (0.156), *Tipula* (*Lunatipula*) (0.163), *Tipula* (*Platytipula*) (0.151), *Tipula*

(*Formotipula*) (0.171) and *Tipula* (*Savtshenkia*) (0.148).

Table 3. Genetic distances between examined taxa based on COI sequences

	Cte	*Dic*	*For*	*Hol*	*Lab*	*Lin*	*Lun*	*Pla*	*Pte*	*Sav*	*Siv*	*Tan*
Cte												
Dic	0.127											
For	0.180	0.166										
Hol	0.128	0.118	0.151									
Lab	0.139	0.160	0.157	0.151								
Lin	0.146	0.134	0.137	0.111	0.129							
Lun	0.156	0.146	0.151	0.139	0.118	0.107						
Pla	0.143	0.139	0.143	0.121	0.160	0.121	0.141					
Pte	0.129	0.130	0.134	0.126	0.144	0.094	0.118	0.098				
Sav	0.146	0.146	0.148	0.119	0.111	0.097	0.111	0.129	0.115			
Siv	0.140	0.143	0.171	0.135	0.188	0.156	0.163	0.151	0.138	0.148		
Tan	0.115	0.115	0.162	0.108	0.173	0.119	0.140	0.142	0.119	0.120	0.121	
Ves	0.157	0.135	0.137	0.131	0.158	0.129	0.135	0.130	0.125	0.135	0.149	0.126

Abbreviation: *Cte*—*Ctenophora*; *Dic*—*Dictenidia*; *For*—*Formotipula*; *Hol*—*Holorusia*; *Lab*—*Labiotipula*; *Lin*—*Lindnerina*; *Lun*—*Lunatipula*; *Pla*—*Platytipula*; *Pte*—*Pterelachisus*; *Sav*—*Savtshenkia*; *Siv*—*Sivatipula*; *Tan*—*Tanyptera*; *Ves*—*Vestiplex*.

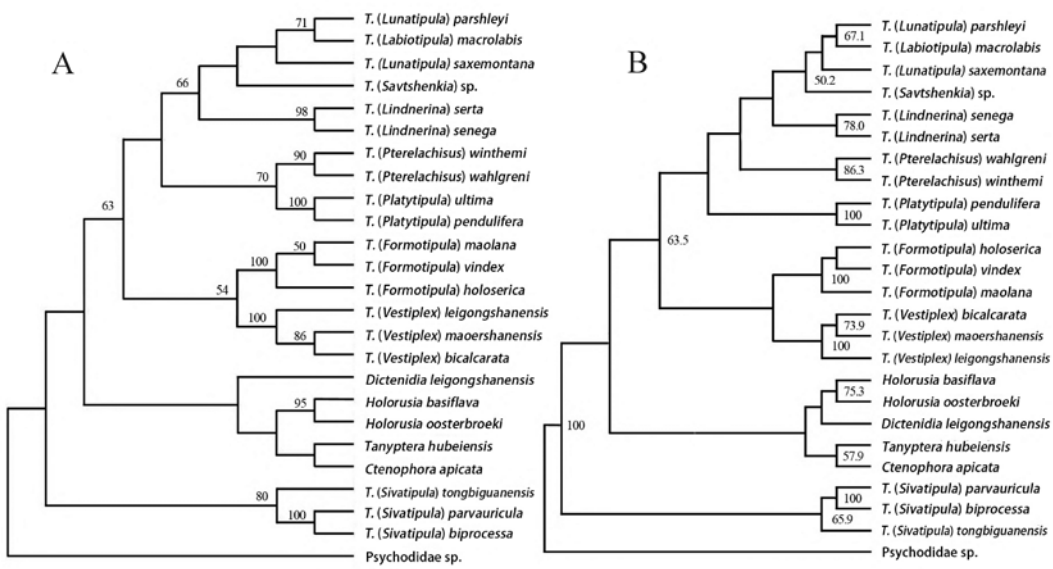

Figure 2. Phylogenetic trees based on the COI gene sequence data. A. NJ tree; B. ML tree. The numbers on each node represent bootstraps. Bootstrap percentage >50 are given.

Discussion

In this study, results show that the number of transitions is lower than those of transversion at codon positions as a whole. In general, comparisons of the DNA sequences of metazoa show an excess of transitional over transversional substitutions, which was due to the relatively high rate of mutation of methylated cytosines to thymine and selection for codon-usage bias in coding regions (Keller *et al*. 2007). However, transition/transversion bias was not observed in some species; for example *Podisma pedestris* vs. *Drosophila melanogaster* based on nuclear and ribosomal DNA sequences (Keller *et al*. 2007), and lycaenid species in three subfamilies based on mitochondrial DNA sequences (Xia *et al*. 2016). Our result provides further evidence that transition/transversion bias is not universal in Insecta species. With the increase in the mutation rate, the numbers of transitions and transversions significantly increases in the COI gene in our study. Our investigation demonstrates that saturation has not yet occurred, and the COI gene is an effective data source for resolving the phylogenetic relationships of *Tipula*.

In our study, the monophyly of *Tipula* is not well supported by NJ and ML trees. *Tipula* (*Sivatipula*) was not grouped with the remaining subgenera of *Tipula* to form a monophyletic clade. Previous phylogenetic analysis based on 28S rRNA and CAD sequenced data also indicated that three subgenera of *Tipula* were variously nested in a clade including *Nephrotoma eucera*, *Holorusia hespera*, *Tanyptera dorsalis* and *Ctenophora* sp. (Petersen *et al*. 2010). Traditionally, the taxonomic rank of subgeneric groups within *Tipula* has mainly relied on morphological similarity among taxa rather than phylogenetic hypotheses, which has likely resulted in polyphyletic relationships among subgeneric groups. Savchenko (1979) indicated that *Tipula* was a monophyletic group based on morphological features of 14 subgenera from the Western Palaearctic Region. However, some Oriental subgenera, such as *Tipula* (*Sivatipula*) and *Tipula* (*Formotipula*), were not included in his study, which made the result less comprehensive.

The genetic distances of five genera including nine subgenera were calculated using COI sequence data based on the Kimura-2-parameter model. The results revealed that the genetic distance between *Tipula* (*Sivatipula*) and other subgenera of *Tipula* is bigger than the distances of *Tipula* (*Sivatipula*) compared with other genera. In addition, the phylogenetic evidence also shows that the *Tipula* (*Sivatipula*) clade forms an independent phylogenetic line. All the molecular evidence above supports promoting the subgeneric positon of *Tipula* (*Sivatipula*) to a generic position.

The structure of the semen pump shows substantial variation in shape and color at both specific and generic levels, and is a traditional source of phylogenetic characters (de Jong 1995b). Three types of semen pumps were defined by Frommer (1963) based on morphological studies of the reproductive system of North American crane flies. Type III is the most common type characterized by the strongly bowed intromittent organ and by a posterior immovable apodeme which is generally equipped with a pair of separate arms (Frommer 1963). According to the overall morphology of the anterior immovable apodeme and compressed apodeme, the semen pump of *Tipula* (*Sivatipula*) should be assigned to Type III. However, its posterior immovable apodeme has only one arm, which differs from those of other *Tipula* subgenera (Men *et al*. 2016; Xue & Men 2016). Moreover, the male of *Tipula*

(*Sivatipula*) generally has very elongated antennae (equal to, slightly shorter or longer than body length), which is not observed in other subgenera of *Tipula*. The unique characters of the posterior immovable apodeme and antenna also provide significant morphological evidence for promoting the subgeneric positon of *Tipula* (*Sivatipula*) to a generic position. As a newly discovered lineage, *Sivatipula* would certainly provide novel perspectives in studying the evolution and diversity of species in the family Tipulidae.

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