Molecular Systematics of the Issidae (Hemiptera: Fulgoroidea) from China Based on *Wingless* and *18S rDNA* Sequence Data

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Abstract: In this study, phylogenetic relationships of 33 species belonging to 19 genera of Issidae and 1 outgroup (*Paravarcia decapterix*) were analyzed using maximum parsimony (MP) and Bayesian inference (BI) methods based on partial sequences of the nuclear *18S rDNA* and *wingless* genes (*Wg*). The topologies of the phylogenetic trees generated from different methods were quite similar. The phylogenetic analysis divided Issidae into five subfamilies: Caliscelinae, Tonginae, Parahiraciinae, Hemisphaeriinae and Issinae. The results also supported that the genus *Eusudasina* should be transferred from Issinae to Hemisphaeriinae.

Key words: Fulgoromorpha; Phylogeny; toplogy

基于 *18S rDNA* 和无翅基因的中国瓢蜡蝉科分子系统发育关系研究（半翅目：蜡蝉总科）

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摘要：研究测定了 19 属 33 种中国瓢蜡蝉和 1 种外群 (*Paravarcia decapterix*) 的 *18S rDNA* 及无翅基因 (*Wg*) 部分 DNA 序列，并利用最大简约法 (maximum parsimony，MP) 和贝叶斯法 (bayesian inference，BI) 进行系统发育关系分析。研究结果表明：采用最大简约法和贝叶斯法 2 种分析方法获得的瓢蜡蝉科 (Issidae) 系统发育树的拓扑结构基本一致，将该科分为 5 个亚科：杯瓢蜡蝉亚科 (Caliscelinae)、汤瓢蜡蝉亚科 (Tonginae)、帕瓢蜡蝉亚科 (Parahiraciinae)、球瓢蜡蝉亚科 (Hemisphaeriinae) 和瓢蜡蝉亚科 (Issinae)。并支持将角唇瓢蜡蝉属 (*Eusudasina*) 归入球瓢蜡蝉亚科 (Hemisphaeriinae)。

关键词：蜡蝉次目；系统发育；拓扑结构

Introduction

The family Issidae Spinola *s. l.* is one of the largest families of fulgoroid planthoppers with approximately 270 genera and 1200 species. All species in this family are phytophagous, of which some are important pests of fruits and damage crops by sucking and spawning (Chou *et al*. 1985; Yan *et al*. 2005; Che 2006; Meng *et al*. 2011).

The Issidae was first established as a subfamily of the family Fulgoridae in 1839 by Spinola. Schaum (1850) promoted it to the family level. The history of notions about superageneric system of the family Issidae has more than one hundred years (Spinola 1839;

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Distant 1906, 1916; Kirkalday 1906, 1907; Melichar 1906; Fennah 1954; Muir 1923, 1930; Schaum 1850; Chan &Yang 1994). Melichar (1906) considered three subfamilies in the family Issidae (Issinae Spinola, Hysteropterinae Melichae, and Thioniinae Melichar) and treated Calicelidae Amyot & Serville and Hemisphaeiidae Melichar as separate families by morphological characteristics of the body configuration, the relative length of fore wing, fore wing with claval suture or without, and protibiae of the male expanding foliaceous or not. According to the classification system proposed by Muir (1930), the family Issidae consists of three subfamilies: Issinae, Caliscelinae and Hemisphaeriinae. Muir (1930) recognized in the subfamily Issinae three tribes: Issini, Hysteropterini and Thioniini. However, Fennah (1954) included in Issidae five subfamilies: Issinae, Tonginae, Trinopinae, Caliscelinae, and Acanaloniiinae on base of synthetical characteristics. Metcalf (1958) revised the classification system of family Issidae proposed by Melichar(1906), Muir (1923, 1930) and Fennah (1954), divided the family Issidae into five subfamilies and six tribes: Caliscelinae (Ahomocnemiellini, Caliscelini, Omamatidiotini), Hemishaerinae, Issinae (Hysteropterini, Issini, Thionini) and Tonginae.

Since then, classification and systematics of the family Issidae has undergone considerable changes which included some genera and species previously listed within Issidae (Metcalf 1958) were transferred to the other family or that previously listed within the other family transferred to Issidae. Relevant researches mainly included the following aspects in the group.

Dlabola(1980) erected a tribe Adenissini in the subfamily Issinae for the genera *Adenisssus* Linnavuori, 1973 and *Anissussis* Dlabola, 1980. Cheng & Yang (1991) on the basis of larval features erected the subfamily Paraheiaciinae for the genus *Parahiracia* Ôuchi, 1940, which was transferred to Issidae from Tropiduchidae by Fennah (1982) and was downgraded to tribe by Gnezdilov (2003a). According to the phylogenetic research of Issidae, Emeljanov (1990) considered the family Issidae *s. l.* included Issinae, Tonginae, Trienoppinae, Caliscelinae, Acanaloniinae, and Bladininae, and upgraded Acanaloniinae Amyot et Serville and Caliscelinae Amyot et Serville to family level. However, a study of the phylogenetic relationship of 38 genera of Issidae from China based on morphological characteristics supported that Caliscelinae was a subfamily of the family Issidae (Che 2006).

Genzdilov (2002) treated Hysteropterini as a subtribe of the Issini and erected a new subtribe Agalmatiina after the study of the ovipositor structure. Soon after, he erected again a tribe Colpopterini based on the features of the ovipositor structure and hind wings and arranged in the subfamily Issinae, and definitely proposed that the family Issidae *s. str.* included the nominotypical subfamily Issinae with five tribes Hemisphaeriini Melichar, Parahiraciini Cheng & Yang, Issini Spinola, Thioniini Melichar and Colpopterini Gnezdilov (Gnezdilov 2003a). The Thioniini was erected by Melichar (1906), as a subfamily Thioniinae of the family Issidae, based on a well developed trilobed hind wings but the taxon was treated as a tribe in subfamily Issinae by (Muir 1930; Gnezdilov 2003a), according to the relevant research, the condition of the hind wing cannot be treated as apomorphies of Issini and Thioniini, thus, the tribe Thioniini Melichar, 1906 was treated as a junior synonym of Issini Spinola, 1839 (Gnezdilov 2009; Gnezdilov & Fletcher 2010). Gnezdilov and Wilson (2006) transferred the tribe Adenissini Dlabola and the genera *Pterilia* Stål and *Coinquenda* Distant from Issidae to Caliscelidae and proposed a tribal system of the family. The subfamily

Trienopinae Fennah, 1954 was transferred to the family Tropiduchidae Stål, 1866, while the subfamily Tonginae Kirkaldy, 1907 and the tribe Colpopterini Gnezdilov, 2003 were again transferred to the family Nogodinidae Melichar, 1898 with their ranks changed to those of a tribe and a subfamily by male and female genitalia charactecrics (Gnezdilov 2007, 2012, 2013). Recently Gnezdilov (2013) suggested the scope of the family Issidae *s. str.* was reduced to three tribes: Issini Spinola, 1839; Hemisphaeriini Melichar, 1906; Parahiraciini Cheng et Yang, 1991, included into the nominotypical subfamily with 161 genera and 997 species and subspecies.

From the above it is not difficult to see that there are many different opinions about the classification of the family Issidae based on morphological data, even same author has different views in the different periods on a group. Meanwhile, recent studies using molecular evidence about phylogenetic analysis of Fulgoroidea also questioned the monophyly of Issidae (Bourgoin *et al.* 1997; Yeh *et al.* 1998, 2005; Urban & Cryan 2007; Song & Liang 2013).

The *18S rDNA* gene is a commonly used marker in molecular phylogenetic investigations due to its universality and evolutionary rate heterogeneity. This gene is easily detected for most insect taxa and therefore often used to address relationships among a wide variety of taxonomic levels (Hillis & Dixon 1991; Hamby & Zimmer 1992; Cryan *et al*. 2000). Similarly, the nuclear protein coding gene *wingless* is useful for phylogenetic analysis of insect relationships at levels of divergence from species to subfamilies (Brower *et al*. 1998).

At the present time, molecular study has not yet been conducted within the family Issidae. To go further in our understanding of the phylogenetic relationships of the family and to better assess the monophyly of the family, the subfamilies, we carried molecular phylogenetic analyses using data from the partial sequences of the *18S rDNA* and the *wingless* gene in 33 species belonging to 19 genera of Issidae and 1 outgroup (*Paravarcia decapterix*). The phylogenetic relationships among taxa were reconstructed using both BI and MP inference methods. The main questions we intend to answer in this paper are: (1) how many subfamilies of Issidae are there? and (2) are the subfamilies monophyletic?

Material and methods

Taxon sampling

Insect specimens (Table 1) were collected and immersed in 95–100% ethanol and were stored at –20°C in the Entomological Museum, Northwest A&F University. The 33 ingroup specimens included in this analysis represent 19 genera of Issidae, and 1 species of Nogodinidae, which is a sister group of Issidae, was chosen as outgroup taxon in consideration of Urban and Cryan (2007) and Gnezdilov (2013b).

DNA extraction and PCR amplification

Genomic DNA was extracted from the legs of adults. Legs were homogenized in a 1.5 μL centrifuge tube in 600 μL extracting solution that contained 0.5M NaCl, 0.1M EDTA, 0.05M Tris (PH = 7.0–8.5), 5% SDS and 2 mg/ml proteinase K. This mixture was incubated at 50°C for 4 hours, then extracted with phenol-chloroform-isoamylol. Extracted crude DNA was precipitated with absolute ethanol and eluted in 800 μL 70% ethanol. Finally, the crude DNA was dissolved in 30 μL TE buffer and stored in –20°C to be used as the DNA template in the following amplification reaction.

The polymerase chain reaction (PCR) was employed to amplify the *18S rDNA* and *wingless* gene fragments. The primers for amplification the *18S rDNA* gene were designed based on the conserved nucleotide sequences of tettigometrid *T. sulphurea* (Bourgoin *et al.* 1997); the forward primer was 18S-mid, 5'-GAT ACC GCC CTA GTT CTA ACC-3' and the reverse primer was 18S-2200, 5'-CGG CAG GTT CAC CTA CGG-3'. The primers for amplification of the *wingless* gene were developed by Urban and Cryan (2007), and the forward primer was Wg 1A, 5'-GAR TGY AAR TGY CAY GGY ATG TCT GG-3' and the reverse primer was Wg DelR1, 5'-GTC CTG TAR CCR CGK CCA CAA CAC AT-3'.

Amplification was carried out for 35 cycles in a 25 μL volume reaction that contained 10×PCR buffer, 25 mmol/L $MgCl_2$, 2.5 mmol/L of each dNTP, 10 μmol/L primers, 5 U/μL Taq polymerase and 1 μL DNA template. All PCR sets included a negative control reaction tube in which all reagents were included except the template DNA. And the temperature profile included: initial denaturation for 3 min at 94°C, denaturation for 1 min at 94°C, annealing for 1 min at 51–55°C and extension for 1 min at 72°C. Amplified DNA was visualized using 1% agarose gel electrophoresis with ethidium-bromide staining, and the DNA products were sequenced directly.

Sequence alignment and phylogenetic analysis

All sequence chromatogram were visually inspected, edited and assembled in contiguous sequences using BioEdit 7.0.9 (Hall 1999). The *wingless* sequences were further checked after the derived amino acid sequences were obtained. Multiple sequence alignment was performed using Clustal X 1.83 (Thompson *et al.* 1997). Highly variable regions of *18S rDNA* that differed in base composition and sequence length across the sampled issid taxa were excluded from phylogenetic analysis due to the extreme ambiguity involved in any possible alignment, following that of previous studies using this marker (Sanderson & Shaffer 2002; Cryan *et al.* 2004; Urban *et al.* 2010). Within the aligned regions, gaps in *18S rDNA* sequences were coded as missing data. Multiple sequence alignment of the nuclear protein coding gene *wingless* was unambiguous, and no gaps detected. Codon position was determined by comparison of amplified *wingless* sequences with sequences available on GenBank for which the amino acid identity and reading frame had been determined (Urban & Cryan 2007).

Aligned DNA sequences were analyzed using MEGA5.05 (Tamura *et al.* 2011) for calculating the average content of A, T, C, G variable sites, conserved sites and parsimony-informative sites.

Analyses of the combined data were conducted under the criterion of maximum parsimony (MP) using Paup*4.0b10 (Swofford 2002). Heuristic tree searches were performed using 1000 replications of random additions with the tree bisection and reconnection (TBR) option. Bootstrap analyses were conducted using 1000 standard replicates to provide an estimate of support for individual nodes in parsimony-based strict consensus tree.

Bayesian analysis (BI) was conducted for the combined data using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best-fitting model SYM+I+G was determined by Modeltest3.7 based on results of the Akaike information criterion (AIC; Akaike 1974). Bayesian analysis was run for 2 million generations, with four chains (one cold and three heated) and flat priors. Trees were sampled at intervals of 100 generations, and a total of

20,000 sampled trees were finally obtained. The first 5000 sampled trees were discarded as burn-in, and the remaining trees were used to construct a 50% majority rule consensus tree.

Table 1. Taxa sampled and the collected data

Subfamily	Genus	Species	Collection locality	Accession no.	
				18S rDNA	*wingless*
Hemisphaeriinae	*Gergithus* Stål	*Gergithus carbonarius*	Hainan: Bawangling	JX196138	JX196179
		Gergithus spinosus	Hainan: Jianfengling	JX196134	JX196180
		Gergithus tesselatus	Zhejiang: Fengyangshan	JX196140	JX196185
	Mongoliana Distant	*Mongoliana sinuata*	Yunnan: Lvchun	JX196139	JX196181
		Mongoliana triangularis	Yunnan: Menglun	JX196141	JX196182
		Mongoliana recurrens	Hunan: Mangshan	JX196137	JX196183
		Mongoliana serrata	Zhejiang: Qingliangfeng	JX196160	JX196184
	Gergithoides Schumacher	*Gergithoides undulatus*	Zhejiang: Qingliangfeng	JX196162	JX196168
		Gergithoides carinatifrons	Hainan: Bawangling	JX196167	JX196170
		Gergithoides rugulosus	Hunan: Mangshan	JX196163	JX196169
	Neogengithoides Sun *et al.*	*Neogengithoides tubercularis*	Hainan: Diaoluoshan	JX196166	JX196171
	Hemisphaerius Schaum	*Hemisphaerius trilobulus*	Yunnan: Longmen	JX196161	JX196187
		Hemisphaerius testaceus	Hainan: Jianfengling	JX196135	JX196186
	Eusudasina Yang	*Eusudasina nantouensis*	Yunnan: Lvchun	JX196136	JX196173
	Choutagus Zhang *et al.*	*Choutagus longicephalus*	Hainan: Jianfengling	JX196154	JX196172
Issinae	*Eusarima* Yang	*Eusarima* sp.1	Hunan: Mangshan	JX196146	JX196192
		Eusarima sp.2	Hainan: Yinggeling	JX196147	JX196191
		Eusarima sp.3	Hainan: Yinggeling	JX196148	JX196198
		Eusarima sp.4	Hainan: Jianfengling	JX196164	JX196193
		Eusarima sp.5	Hainan: Diaoluoshan	JX196145	JX196195
		Eusarima sp.6	Zhejiang: Qingliangfeng	JX196143	JX196194
	Kodaiana Distant	*Kodaiana* sp.	Fujian: Wuyishan	JX196151	JX196178
	Thabena Stål	*Thabena hainanensis*	Hainan: Bawangling	JX196152	JX196177
	Kodaianella Distant	*Kodaianella damnosus*	Yunnan: Yexianggu	JX196156	JX196189
		Kodaianella bicinctifrons	Zhejiang: Qingliangfeng	JX196155	JX196190
				JX196144	JX196197
	Jagannata Distant	*Jagannata* sp. 1	Hainan: Limuling	JX196142	JX196196
		Jagannata sp. 2	Hainan: Diaoluoshan	JX196157	JX196175
Parahiraciinae	*Fortunia* Distant	*Fortunia byrrhoides*	Yunnan: Menglun	JX196158	JX196176
	Flavina Stål	*Flavina hainana*	Hainan: Jianfengling	JX196159	JX196174
	Mincopius Distant	*Mincopius* sp.	Hainan: Yinggeling	JX196150	JX196200
Caliscelinae	*Caliscelis* de Laporte	*Caliscelis rhabdocladis*	Hebei: Langfang	JX196149	JX196201
	Symplanella Fennah	*Symplanella unipuncta*	Hainan: Yinggeling	JX196165	JX196188
Tonginae	*Tonga* Kirkaldy	*Tonga westwoodi*	Hainan: Jianfengling	JX196153	JX196199
Outgroup	*Paravarcia* Schmidt	*Paravarcia decapterix*	Hainan: Yinggeling		

Results and Discussion

Sequence variation

Approximately 1078 bp in length of the combined *18S rDNA* and *wingless* gene sequence were analyzed for 34 taxa (33 ingroup and 1 outgroup). Among these sites, 221 were variable, 853 were conserved and 120 were parsimony-informative. The average nucleotide composition proportions for the combined data partition were: T, 23.9%; C, 23.5%; A, 25.1%; G, 27.5%. Regression analysis of total substitutions versus transitions (Ts) and transversions (Tv) revealed that the substitution saturation due to multiple hits has not yet occurred in issids examined in this study.

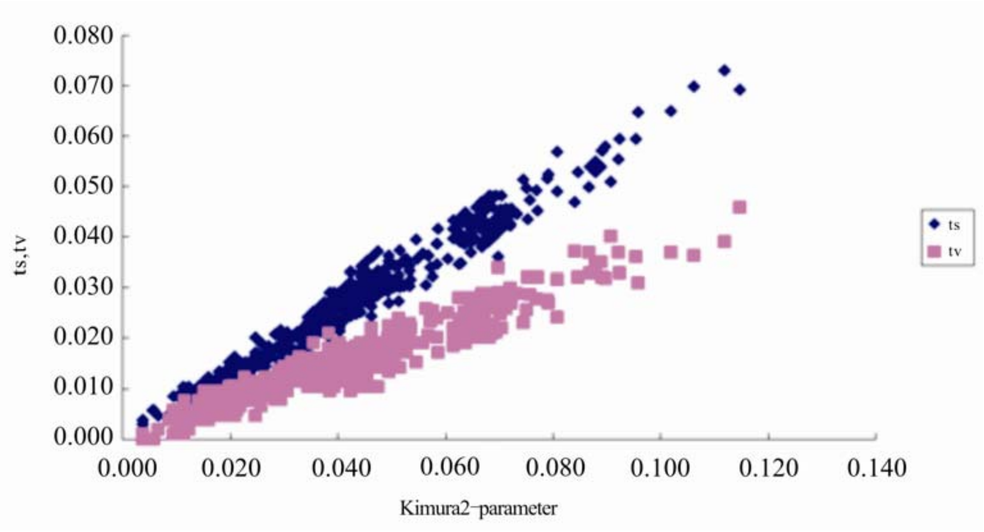

Figure 1. Regression analysis of total substitutions versus transitions (Ts) and transversions (Tv) in the combined genes of *18S rDNA* and *wingless* of Issidae.

Phylogenetic analysis

Figures 2 and 3 show the phylogram of the issids examined in this study recovered from the MP and Bayesian analyses respectively. Both trees show generally concordant topology in the pattern of main groups representing different subfamilies. The phylogenetic relationships of them show both similarities and differences compared with previous studies based on morphology. Both trees show Issidae is a monophyletic group. The most basal lineage is Caliscelinae Amyot et Serville, as is sister to the remaining subfamilies with strong nodal support. Therefore, the placement of the Caliscelinae as a subfamily within the family Issidae was well supported in this study, herein, consistent with the hypothesis of Metcalf (1958). However, it is clear that these results above do not support Emeljanov's (1999) placement of the Caliscelidae as a family outside of Issidae based on morphology and specifically the female and male genitalia-based hypotheses (Gnezdilov 2003b; Gnezdilov & Wilson 2006).

The subfamily Parahiraciinae, represented in these analyses by three genera, is treated as sister to the Hemisphaeriinae + Tonginae (Figs. 2, 3). This result concurs with that of Cheng and Yang (1991), rather than the placement of the Parahiraciini as a tribe of Issinae as proposed by the ovipositor characteristic-based hypotheses (Gnezdilov 2003).

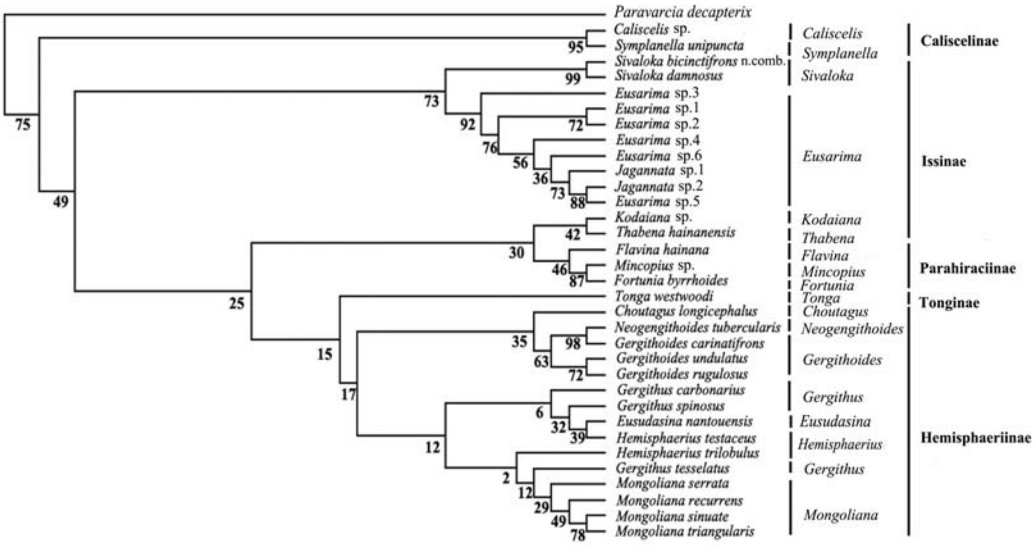

Figure 2. Maximum parsimony tree (MP) based on analysis of combined data *18S rDNA* + *wingless* genes among 33 species of Issidae and 1outgroup. The number on the node indicates bootstrap value.

Until now, morphological data had been unable to resolve the placement of Tonginae. Tonginae was erected and placed in the family Flatidae due to the unique morphology and its uncertain classification (Kirkaldy 1907). Baker (1927), Fennah (1954) and Metcalf (1958) proposed that Tonginae should be placed in the family Issidae. However, Tonginae was transferred to Nogodinidae based on features of the male and female genitalia (Gnezdilov 2007). In this investigation, the placement of Tonginae as a subfamily of Issidae was supported by both parsimony and Bayesian trees except that the related nodes are lowly supported (Figs. 2, 3).

The monophyly of Issinae was not supported by either parsimony or Bayesian analyses, with the two genera *Kodaiana* and *Thabena*, both formerly included within Issinae, being placed in a clade sister to the parahiraciid clade in the current study. And Gnezdilov (2003) degraded the subfamily Parahiraciinae to Parahiraciini as a tribe of the Issinae based on the characters of the ovipositor. So, more detailed investigation is needed to test the monophyly of Issinae and its affinity to Parahiraciinae.

In both parsimony and Bayesian analyses, a *Choutagus* + *Neogengithoides* + *Gergithoides* clade was supported within the Hemisphaeriinae group. Based on morphological characters, they share certain common characters: body hemispherical, median carina in frons and pronotum, wing well-developed, translucent and netlike. Both molecular and morphological data support *Choutagus* Zhang, Wang et Che, *Neogengithoides* Sun, Meng et Wang and *Gergithoides* Schumacher having close relationships.

Three species representing the genus *Gergithus* were mixed with two species which represents the genus *Hemisphaerius* in these analyses. The morphologies used to distinguish these two genera are mainly based on the ratio of the size of tegmen and wing (e.g. wing longer than half length of tegmen in *Gergithu*s, but shorter than half length of tegmen in *Hemisphaerius*). However, this feature has limitations in distinguishing between species, and therefore additional data are clearly needed to more thoroughly resolve placement of the genera *Gergithus* and *Hemisphaerius*.

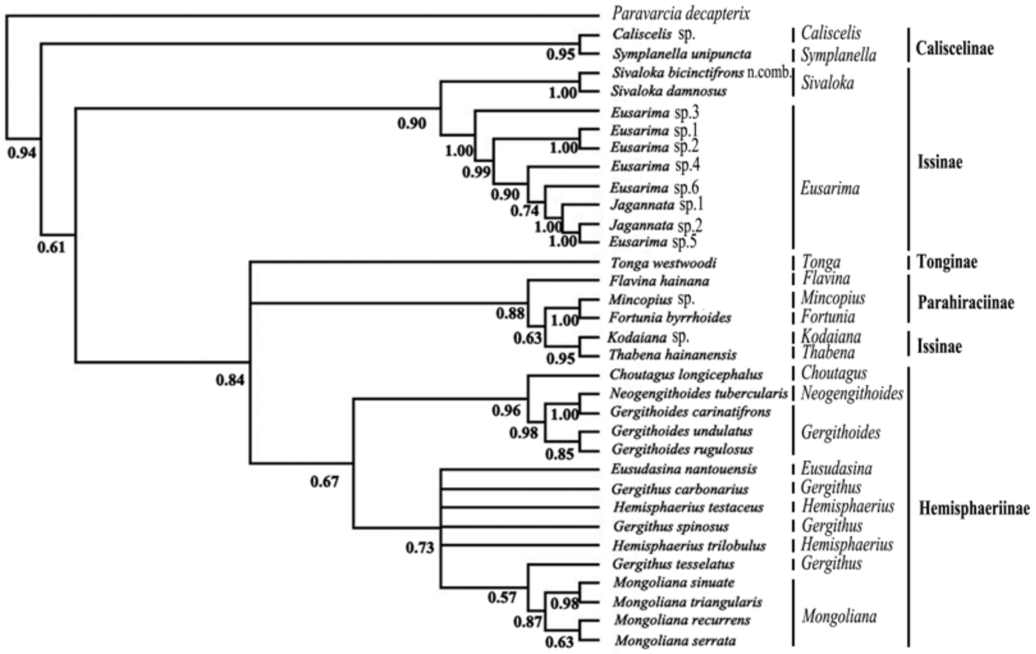

Figure 3. Bayesian tree (BI) resulting from analysis of combined data *18S rDNA* + *wingless* genes among 33 species of Issidae and 1 outgroup. The number on the node indicate posterior probability value.

Both parsimony and Bayesian trees show that *Eusudasina* are sister to the genus *Hemisphaerius* in the Hemisphaeriinae clade which accord with the result of phylogenetic relationship of Issidae from China based on morphological characteristics (Che 2006). This result rejects the hypothesis of putting the *Eusudasina* into Issinae (Yang & Fang 1993).

As previously described, the two species *Jagannata* sp. 1 and *Jagannata* sp. 2 were in the genus *Jagannata*. But, in both parsimony and Bayesian topologies, *Jagannata* sp. 1 and *Jagannata* sp. 2 are placed within the genus *Eusarima*. In reexamining their morphology (tegmen with Sc + R diverging near base, "Y" shorter than clavus, aedeagus somewhat U-shaped with sword processes), their features are more coincidence with the taxonomic characteristics of *Eusarima*. Both molecular and morphological data support *Jagannata* sp. 1 and *Jagannata* sp. 2 belonging within the genus *Eusarima*. Based on molecular data, the genus *Jagannata* Distant is closely related to the genus *Eusarima* Yang, as is different to the results based morphological study. The relationship between these two genera needs to be further adressed based on combined molecular and morphological data.

Conclusions

The following conclusions were supported after analyzing both parsimony and Bayesian trees constructed based on *18S rDNA* and wingless combined data.

(1) The placement of Caliscelinae as a subfamily of Issidae is strongly supported by the

molecular data.

(2) Subfamily Tonginae is placed within Issidae by both parsimony and Bayesian trees.

(3) The tribe Parahiraciini should be promoted to the subfamily Parahiraciinae outside of Issinae.

(4) *Eusudasina* should be transferred to Hemisphaeriinae and is supported by both parsimony and Bayesian trees.

Our limited taxon sampling only can provide a preliminary phylogenetic picture of Issidae, and the reseach results confirms that the *18S rDNA* and the *wingless* genes are available in resolving phylogenetic relationships of Issidae. Obviously, further research that integrates more taxon sampling including more outgroups, and data from other molecular markers would provide greater insight into the evolution of Issidae.

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