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Systematic position of *Gomphogyne* (Cucurbitaceae) inferred from ITS, *rpl16* and *trnS-trnR* DNA sequences

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Abstract This paper assessed the systematic position of the genus *Gomphogyne*. The nuclear ITS, the chloroplast *rpl16*, and *trnS-trnR* sequences were used to reconstruct the phylogeny of *Gomphogyne* and its related genera. Analyses of three separate and combined datasets provided a good amount of informative characters and resolved the systematical relationships of *Gomphogyne* well. The maximum parsimony analyses revealed that: (1) *Gomphogyne* was a natural genus and was different from the genera *Hemsleya* and *Gynostemma*; (2) *Hemsleya delavayi* and *H. macrocarpa* did not belong to the genus *Gomphogyne*, but to the genus *Hemsleya*; and (3) *Gomphogyne* was sister to *Hemsleya*. It was assured that *Gomphogyne* was a monotypic genus. These results were largely in agreement with the systems of classification of the Cucurbitaceae of Jeffrey in 1990 and in 2005 and with that of Li for *Hemsleya* in 1993, but were different from the previous studies in which *Gomphogyne* and *Gynostemma* together were suggested to be sister to *Hemsleya*.

Key words *Gomphogyne*, ITS, molecular phylogeny, *rpl16*, *trnS-trnR*.

Gomphogyne Griff., a member of subtribe Gomphogyninae of tribe Zanonieae (Cucurbitaceae) (Jeffrey, 1990, 2005), was established in 1841 by Griffith with one species *G. cissiformis* Griff. Henceforth, a few new species were reported, namely, *G. delavayi* Gagnep., *G. macrocarpa* Cogn. and *G. heterosperma* (Wall.) Kurz. However, later studies revealed that all the new species belonged to the genus *Hemsleya* Cogn., in which they were named as *H. delavayi* (Gagnep.) C. Jeffrey ex C. Y. Wu & C. L. Chen, *H. macrocarpa* (Cogn.) C. Y. Wu ex C. Jeffrey and *H. heterosperma* (Wall.) C. Jeffrey. As a result, the genus *Gomphogyne* became a monotypic genus comprising only one species, i.e., the type species, *G. cissiformis* (Li, 1993; Lu et al., 2007). It is principally an Indo-Malayan genus with its northernmost distribution in China. Besides *Gomphogyne*, subtribe Gomphogyninae also harbored *Hemsleya* and *Gynostemma* and they formed a monophyletic group (Jeffrey, 1990, 2005; Li, 1993). *Neoalsomitra* Hutch. or *Neoalsomitra* and *Zanonia* L. together were suggested to be the sister to the subtribe (Li, 1993). *Hemsleya* (Li, 1993) comprised about 24 species and *Gynostemma* (Chen, 1995; He, 1996) consisted of 17 species. The genus *Gomphogyne* is characterized by monoecia and differentiates the dioecism of *Hemsleya* and *Gynostemma*. The male flowers possess five stamens, while female flowers bear three stigmas bifurcating at the apex, in a raceme or panicle. Five densely imbricate petals build the rotatable pale green corollas. Fruits are capsular, turbinate, venose and ribbed. The plants are herbaceous vines, with palmately compound and alternate leaves comprising seven leaflets (Lu, 1986).

As *Gomphogyne* is monotypic, there are few studies involving the genus. We can only acquire data of *Gomphogyne* from the studies on Cucurbitaceae. Moreover, all the data focused mainly on either morphology (Lu, 1986) or chromosome numbers (Singh, 1990). Two questions remained unanswered. First, whether the taxon should be recognized as an independent genus; second, whether it is sister to *Hemsleya* as suggested earlier (Li, 1993) or not. Although studies of molecular phylogeny of many genera of the Cucurbitaceae have been comprehensively carried out, *Gomphogyne* has not yet been included in previous studies (Sanjur et al., 2002; Clark et al., 2006).

This study addressed to the systematic position of *Gomphogyne*. We sequenced the nuclear ITS regions, the chloroplast *rpl16* intron and flanking regions, and the chloroplast *trnS-trnR* regions of the genus and its relatives. These sequences have been useful in clarifying phylogenetic relationships in the Cucurbitaceae (Jobst et al., 1998; Jarret & Newman, 2000; Garcia-Mas et al., 2004). The major objective of this paper is to confirm the systematic position of *Gomphogyne,* particularly its relationships with *Hemsleya* and *Gynostemma.*

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1 Material and methods

1.1 Plant samples

For this study, the only *Gomphogyne* species and representatives of the remaining genera in subtribe Gomphogyninae (Jeffrey, 1990, 2005; Li, 1993; Chen, 1995; Kocyan et al., 2007), *Hemsleya* and *Gynostemma,* were sampled including the type species of *Gynostemma*. As *Hemsleya* is a monophyletic group based on morphological evidence (Li, 1993) and molecular data (Li, 2007), five *Hemsleya* species were sampled, including the type species, and *H. delavayi* and *H. macrocarpa* that were previously regarded as *Gomphogyne* species. *Hemsleya heterosperma* was not sampled because of inaccessibility. We used a species of *Neoalsomitra,* which was suggested to be sister to subtribe Gomphogyninae (Li, 1993), as outgroup to root the tree. Samples were all obtained from wild plants (Table 1). Healthy, clean leaves were collected and quickly dried in silica gel, with voucher herbarium specimens deposited at the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

1.2 DNA extraction, amplification and sequencing

For each sample, total genomic DNA was isolated from 0.2 g silica-gel-dried or 0.4 g fresh leaves using the modified CTAB method (Doyle & Doyle, 1987), with 4% CTAB instead of 2% CTAB. Leaf tissue was ground in liquid nitrogen before using CTAB.

Following extraction, DNA was amplified using the polymerase chain reaction (Saiki et al., 1988). The ITS region includes the ITS1, 5.8S, and ITS2 nuclear rDNA regions. It was amplified with primers ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS-5 (5′-GGA AGG AGA AGT CGT AAC AAG G-3′) (White et al., 1990). The *rpl16* intron region was amplified as described in Jordan et al. (1996) with primers *rpl16*-F71 (5′-GCT ATG CTT AGT GTG TGA CTC GTT G-3′) and *rpl16*-R1516 (5′-CCC TTC ATT CTT CCT CTA TGT TG-3′). The *trnS-trnR* region was amplified with primers $trnS^{GCU}$-F (5′-CGC CGC TTT AGT CCA CTC A-3′) (Doyle et al., 1992) and *trnR*-R (5′-ATT GCG TCC AAT AGG ATT TGA A-3′) (Dumolin-Lapegue et al., 1997). The thermal cycler (PE9600 or PE9700) for *trnS-trnR* was programmed for an initial step of 4 min at 94 ℃, followed by 36 cycles of 1 min at 94 ℃, 90 s at 50 ℃, 90 s at 72 ℃, and a final extension of 7 min at 72 ℃. The PCR products were visualized by agarose gel electrophoresis, cleaned with Wizard PCR preps DNA Purification system (Promega, Madison, WI, USA), and both strands were sequenced using the same primer combination as for PCR amplifications.

1.3 Phylogenetic analyses

Clustal X (Thompson et al., 1997) was used to produce an aligned matrix, which was corrected manually using the BioEdit program (Hall, 1999). The indels were coded using GapCoder method (Simmons & Ochoterena, 2000) in the data matrix.

Maximum parsimony (MP) analyses were conducted using PAUP 4.0b10 (Swofford, 2001). Characters were treated as unordered and unweighted. Heuristic searches were conducted with Tree-Bisection Reconnection (TBR) branch swapping, MulTrees ON, and 1000 random taxon addition replicates holding 20 trees at each step. Branch support (BS) values for individual clades were calculated by running 1000 bootstrap replicates of the data, with starting trees acquired by a single replicate of random stepwise addition of taxa, under TBR branch swapping, and MulTrees ON. The consistency index (*CI*), retention index (*RI*) and rescaled consistency index (*RC*) were obtained with PAUP 4.0b10.

Incongruence among multiple data partitions (ITS, *rpl16* and *trnS-trnR*) was evaluated with the partition homogeneity test (Farris et al., 1994, 1995) implemented in PAUP 4.0b10 (Swofford, 2001). The partition homogeneity test used 1000 resampling replicates under the maximum parsimony criterion, and all characters were equally weighted.

2 Results

2.1 Sequence comparisons

Sequences information for the four datasets (ITS, *rpl16*, *trnS-trnR* and the combined ITS+*rpl16*+*trnS-trnR*) of this study is shown in Table 2. The aligned ITS region has a length of 703 characters, with 139 (19.8%) variable sites, 65 (9.2%) of which were parsimony-informative. The aligned *rpl16* sequence has 1062 characters, with 97 (9.1%) variable characters, 34 (3.2%) were parsimony-informative characters. The aligned *trnS-trnR* sequence has 1701 characters, 132 (7.8%) of which were variable characters, and 39 (2.3%) were parsimony-informative characters.

2.2 Phylogenetic analyses

Separate analyses of the ITS, *rpl16* and *trnS-trnR* datasets produced well resolved trees. The partition homogeneity test suggested that the ITS, *rpl16* and *trnS-trnR* datasets were not significantly incongruent (P=1). At the same time, each of three dataset produced identical topologies, thus these three datasets were combined for phylogenetic analyses. The aligned combined sequences including coded gaps had 3466

Table 1 The list of taxa and voucher specimens used in this study.

Species	GenBank #			Locality information	Voucher
	ITS	*rpl16*	*trnS-trnR*		
Gomphogyne cissiformis Griff. (锥形果)	EF621663	EF621641	EF621684	Yongde, Yunnan, China (云南永德)	H. T. Li (李洪涛) 835 (KUN)
Gynostemma pentaphyllum (Thunb.) Makino. (绞股蓝)	EF621662	EF621622	EF621683	Huangshan, Anhui, China (安徽黄山)	H. T. Li (李洪涛) 005 (KUN)
Hemsleya graciliflora (Harms) Cogn. (马铜铃)	EF621654	EF621640	EF621675	Pengzhou, Sichuan, China (四川彭州)	H. T. Li (李洪涛) 069 (KUN)
Hemsleya delavayi (Gagnep.) C. Jeffrey ex C. Y. Wu & C. L. Chen. (短柄雪胆)	EF424063	EF424072	EF424080	Songming, Yunnan, China (云南嵩明)	H. T. Li (李洪涛) 048 (KUN)
Hemsleya macrocarpa (Cogn.) C. Y. Wu ex C. Jeffrey (圆锥果雪胆)	EF621652	EF621632	EF621672	Yongde, Yunnan, China (云南永德)	H. T. Li (李洪涛) 003 (KUN)
Hemsleya lijiangensis Lu ex C. Y. Wu & C. L. Chen. (丽江雪胆)	EF424065	EF424075	EF424078	Lijiang, Yunnan, China (云南丽江)	H. T. Li (李洪涛) 047 (KUN)
Hemsleya chinensis Cogn. (雪胆)	EF424064	EF424073	EF424081	Mt. Emei, Sichuan, China (四川峨眉山)	H. T. Li (李洪涛) 023 (KUN)
Neoalsomitra integrifoliola (Cogn.) Hutch. (棒锤瓜)	EF621642	EF621620	EF621664	Xishuangbanna, Yunnan, China (云南西双版纳)	H. T. Li (李洪涛) 803 (KUN)

Table 2 DNA site variation and tree statistic for the four data sets used in the phylogenetic analyses of taxa presented in this study

	Number of characters	Number of variable sites	Number of informative sites	No. trees	Tree length	*CI*	*RI*	*RC*
ITS	703	139	65	1	275	0.916	0.744	0.682
rpl16	1062	97	34	1	144	0.972	0.915	0.889
trnS-trnR	1701	132	39	1	178	0.962	0.865	0.833
ITS + *rpl16*+ *trnS-trnR*	3466	368	138	1	606	0.941	0.810	0.761

CI, consistency index; *RC*, rescaled consistency index; *RI*, retention index.

characters, with 368 (10.6%) variable and 138 (4%) parsimony-informative sites.

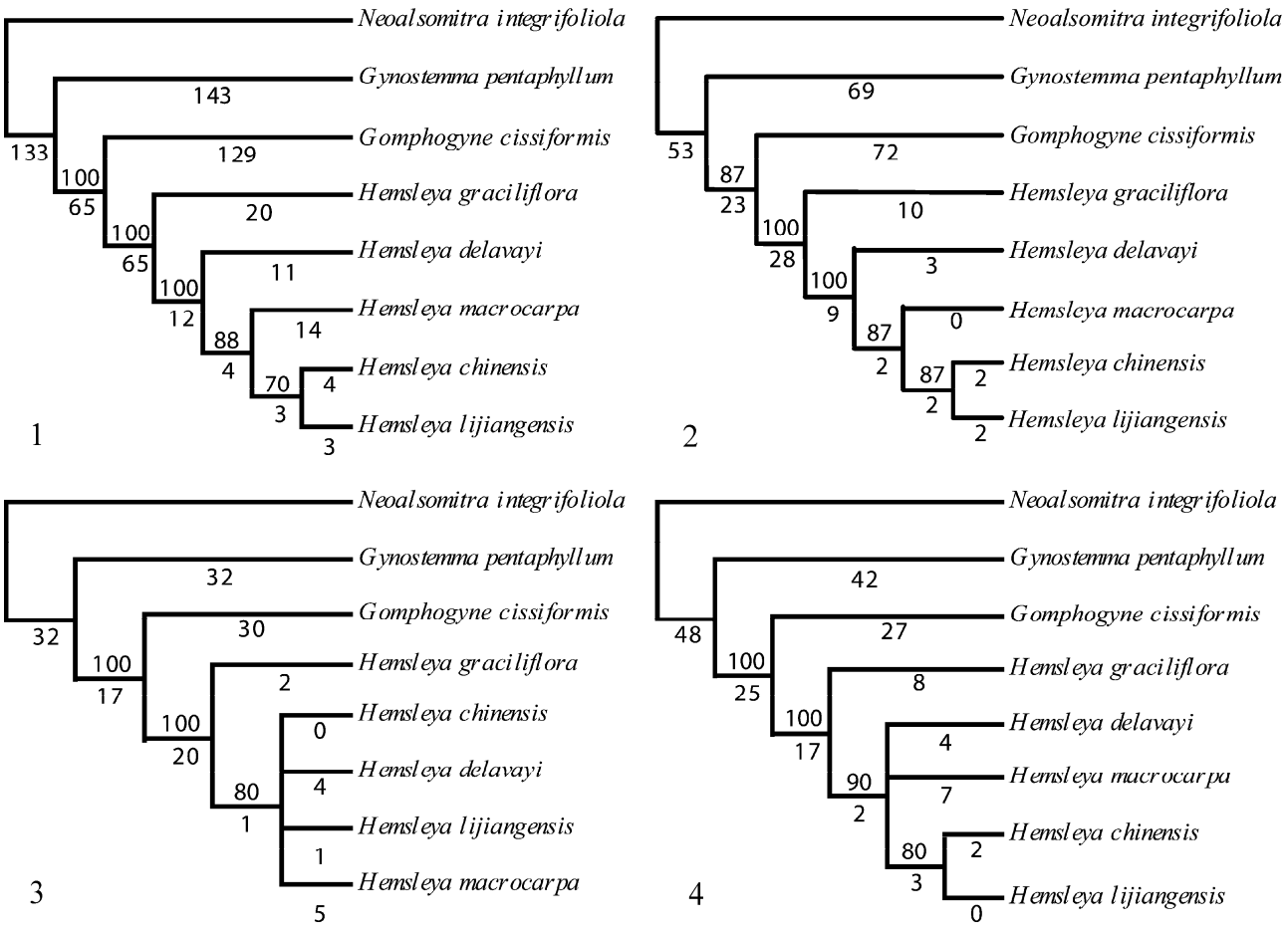
In our study, the MP analyses of four datasets each produced only a tree and the resulting topologies were identical (Figs. 1–4). All MP analyses recovered well-resolved and strongly supported topologies. The MP analysis of combined data yielded one most parsimonious tree of 606 steps (*CI*=0.941, *RI*=0.810, *RC*=0.761). The tree was well resolved with strong branch support (Fig. 1), as shown by the bootstrap values. In the analyses three clades were identified (Fig. 1), all with strong bootstrap support (BS= 100%). Each clade represents a different genus of subtribe Gomphogyninae. The basal group was *G. pentaphyllum* representing the genus *Gynostemma*. The monotypic genus *Gomphogyne* followed as sister to the genus *Hemsleya*.

3 Discussion

ITS, *rpl16* and *trnS-trnR* sequences were useful for resolving the intergeneric even interspecific relationships in this study. The separate and combined analyses of the three DNA regions all recovered well-resolved and strongly supported trees, in which the combined tree was most strongly supported.

Our study revealed that *H. delavayi* and *H. macrocarpa* were members of *Hemsleya*, though they were previously treated as *Gomphogyne* species. It corresponded to Li's (1993) classification of *Hemsleya*, and consequently, it was assured that *Gomphogyne* was a monotypic genus. In comparison with rest of the taxa sampled in the study, *Gomphogyne* had many indel singletons in the three DNA regions. There were five indels ranging from 1 to 5 bps in the ITS sequences, and five indels of 1 bp in the *rpl16* intron, and four indels in *trnS-trnR*, one of which was a 99-bp long deletion. These indel singletons are the unique molecular characteristics to *Gomphogyne*.

In the separate and combined DNA analyses, the MP systematic trees presented three main strongly supported clades (Fig. 1). Moreover, each clade represented a different genus of the subtribe Gomphogyninae, respectively. These relationships also corresponded to the previous classification (Jeffrey, 1990, 2005). Certainly, the results of our analyses suggested a different scheme of relationships. Only the *Gomphogyne* was sister to *Hemsleya*. It did not corroborate the results of previous studies, such as Li (1993), in which *Gomphogyne* and *Gynostemma* together were suggested to be sister to *Hemsleya*.



Figs. 1–4. Single most parsimonious trees of *Gomphogyne* and related genera based on combined data of three DNA regions (Fig. 1), ITS (Fig. 2), *rpl16* (Fig. 3) and *trnS-trnR* separate data sets (Fig. 4). Numbers above the lines are bootstrap values. Numbers below the lines are branch lengths.

The phylogeny of subtribe Gomphogyninae presented here for the first time provided a valuable picture of the relationships among the three genera, i.e., *Gomphogyne, Hemsleya* and *Gynostemma,* of the subtribe Gomphogyninae as defined by Jeffrey. Further studies with increased species sampling, especially of more *Gynostemma* species, are needed to ultimately resolve the relationships of *Gomphogyne* among the three genera of the subtribe Gomphogyninae.

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基于 ITS、*rpl16* 和 *trnS-trnR* DNA 序列讨论锥形果属的系统位置

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摘要 基于核基因ITS和叶绿体基因*rpl16*、*trnS-trnR*的DNA序列讨论了锥形果属的系统位置, 3个基因片段独立以及联合的分析为锥形果属*Gomphogyne*的系统进化研究提供了足够的信息。结果表明: (1)锥形果属是一个自然属; (2)雪胆属*Hemsleya*的短柄雪胆*H. delavayi*和圆锥果雪胆*H. macrocarpa*曾经被作为锥形果属的种, 分子证据表明它们确实隶属于雪胆属; (3)锥形果属单独构成雪胆属的姊妹群, 而并非是与绞股蓝属*Gynostemma*共同构成。

关键词 锥形果属; ITS; 分子系统学; *rpl16*; *trnS-trnR*