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Molecular Phylogeny of *Ophiopogon* (Asparagaceae) Inferred from Nuclear and Plastid DNA Sequences

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Abstract—The East and Southeast Asian genera *Ophiopogon*, *Liriope*, and *Peliosanthes* are classified in the tribe Ophiopogoneae (Asparagaceae). Phylogenetic relationships of this group were explored using maximum parsimony, Bayesian and maximum likelihood analyses of nuclear ITS and plastid psbA-trnH, matK, rbcL, and trnL-trnF sequences. These analyses supported the monophyly of *Ophiopogon*, *Liriope*, and *Peliosanthes* within the Ophiopogoneae, although tree topologies based on nuclear and chloroplast DNA differed in the placement of many taxa. Incongruence between these two datasets may be a result of hybridization and introgression. Our results reveal that *Ophiopogon* consists of two major lineages, which we recognize at the sectional level: *L. lancangensis*, *O. multiflorus*, *O. yunnanensis*, *O. reversus*, *O. longibracteatus* (sect. *Ophiopogon*), and *O. inui* (sect. *Peliosanthes*).

Keywords—*Liriope*, *Ophiopogon*, Ophiopogoneae, *Peliosanthes*, phylogenetic reconstruction.

*Ophiopogon* Ker Gawler (Asparagaceae) is distributed mainly in East Asia, with some species occurring in Southeast Asia (Fig. 1). Species of *Ophiopogon* are perennial, rhizomatous, or sometimes stoloniferous. *Ophiopogon* comprises about 65 species, with 38 endemic to China (Chen and Tamura 2000) and a center of diversity in southern and southeastern Yunnan and southwestern Guangxi (Yang et al. 1990). Zhang (1991) indicated that the modern focus of differentiation in the genus extends from the Himalayas to the Hengduan Mountains through southern and western Sichuan.

The correct tribal placement of *Ophiopogon* has been disputed at length, and still remains unsettled. Krause (1930) transferred *Ophiopogon*, *Liriope* Lour., and *Peliosanthes* Andrews to the tribe Ophiopogoneae within Liliaceae because the fruit divides into two sections and five series based on inflorescence position and leaf shape. Genera related closely to *Ophiopogon* include *Liriope* and *Peliosanthes*. De Loureiro (1790) proposed the genus *Liriope* for plants from Cochinchina (presently Cambodia, Laos, and Vietnam), which is the origin of the plants known presently as *L. spicata* (Thunb.) Lour. Andrew (1810) established the genus *Peliosanthes* for a plant introduced from India into England, designating *P. tetra Andr.* as the type species.

Chromosome counts are reported for about 45 *Ophiopogon* species. The genus has two basic chromosome numbers: *x* = 18, which is present in most species, and *x* = 17, which represents *O. clarkii* Hook. f., *O. intermedium* D. Don, *O. japonicus* (L. f.) Ker Gawl., *O. olivii* (L. f.) Ker Gawl., and *O. umbretica* Hance (Wang et al. 2013). Most species are diploid, with the exception of 12 polyploid species (Zhang 1991; Wang et al. 2013). Moreover, Zhang’s (1991) karyotype analyses supported Yang and Li’s (1990) morphologically based classification.

Rudall et al. (2000), Tamura and Yamashita (2004), and Kim et al. (2010) included only two species of *Ophiopogon* in their broad phylogenetic analyses of Rusaceae and other monocotyledons based on chloroplast DNA (cpDNA). Thus, the phylogenetic relationships within *Ophiopogon* have been tested only minimally using molecular data and remain unclear. In this study, we undertook a comprehensive phylogenetic analysis of *Ophiopogon*, *Liriope*, and *Peliosanthes* using DNA sequence data derived from the nuclear internal transcribed spacer region (ITS) region and several chloroplast regions (psbA-trnH, matK, rbcL, and trnL-trnF). Our goals were to reconstruct the phylogenetic relationships among the three genera, to re-evaluate the monophyly of *Ophiopogon*, and to elucidate the interspecific relationships within *Ophiopogon*.
Single accessions of Convallaria L., Reineckea Kunth, Aspidistra Ker Gawl., Disporopsis Hance, Heteropolygonatum M. N. Tamura et Ogisu in M. N. Tamura et al. Maianthemum Web., and Polygonatum Mill. were chosen as outgroups, in accordance with Kim et al. (2010). Living plants were cultivated in a greenhouse at the Kunming Institute of Botany, Yunnan, China. Voucher specimens were deposited in KUN.

**DNA Extraction, PCR Amplification, and Sequencing**—Genomic DNA was extracted from 15 mg of desiccated leaf tissue using the CTAB method of Doyle and Doyle (1987) and a plant genomic DNA extraction kit (Bioteke, Beijing, China). Each PCR amplification was performed in a volume of 20 μl using 10 ng genomic DNA (quantified with a NanoDropTM2000c, Thermo Scientific, Waltham, Massachusetts) as template, 4 pmol each primer, 0.5 U Taq polymerase (Promega, Madison, Wisconsin), and 2.5 mM MgCl2 under the following conditions: 3 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; with a final extension of 10 min at 72°C.

To amplify the ITS region as a single fragment, the primers ITS4 and ITS5 (White et al. 1990) were used. However, if amplification of the entire ITS region was unsuccessful, the internal primers ITS2 and ITS3 (White et al. 1990) were used in the combinations ITS2/ITSmF and ITS3/ITS4 to obtain two shorter, overlapping fragments. The primers used to amplify the chloroplast DNA regions were as follows: psbA-F and trnH-R for psbA–trnH (Sang et al. 1997; Hamilton 1999); Z1 and 1024R for rbcL (Zarowski et al. 1981; Olmstead et al. 1993); 3F and 1R for matK (Kilian et al. 2009); and Tab-c and Tab-f for trnL–trnF (Taberlet et al. 1991).

The PCR products were purified using the polyethylene glycol (PEG) precipitation procedure following the manufacturer’s protocols. Cycle sequencing was carried out as follows: 35 cycles of 97°C for 15 s, 50°C for 5 s, and 60°C for 4 min. The products of cycle-sequencing reactions were cleaned using Sephadex columns (Amersham Pharmacia Biotech, Piscataway, New Jersey) and dried at 60°C in a rotary vacuum evaporator. Sequence data were generated with an ABI Prism 3100 capillary sequencer (Applied Biosystems, Foster City, California). Sequences were aligned with ClustalX 1.83 (PCversion, Thompson et al. 1997), then manually edited with Bioedit (Hall 1999).

**Phylogenetic Analyses**—Phylogenetic trees were reconstructed using maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML). Parsimony analyses were performed with heuristic searches of 1,000 replicates with random stepwise addition using tree bisection–reconnection (TBR) branch swapping, MulTrees, and the Collapse option selected in PAUP* v.4.0b10 (Swofford 2003). Gaps were treated either as missing data or as new characters. All characters and character state transformations were weighted equally. The bootstrap percentages (BP) were calculated from 1,000 replicates using a heuristic search with simple addition with the TBR and MULPARS options implemented.

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**Fig. 1.** Distribution of Ophiopogoneae in East and Southeast Asia. The number of species sampled and percentage of the total number of species in each geographic region are indicated.
The optimal model of molecular evolution under the Akaike information criterion (AIC) was determined with Modeltest 3.7 (Posada and Crandall 1998; Posada and Buckley 2004). For each analytic method the optimal model was the general time reversible model, with rate heterogeneity modeled by assuming that a proportion of sites were invariable and that the rate of evolution at other sites could be modeled using a discrete approximation to a gamma distribution (GTR + I + C). Bayesian inference using a Markov chain Monte Carlo (MCMC) algorithm was implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Bayesian analyses were run for 2,000,000 generations with four incrementally heated Markov chains, starting from random trees and sampling every 100 generations. The first 2,000–5,000 trees, depending on when chains appeared to have become stationary, were discarded as ‘burn-in’. The remaining trees were assumed to represent the posterior probability (PP) distribution. The 50% majority rule consensus tree and PP for each node were calculated in PAUP* 4.0 b10 (Swofford 2003). The ML bootstrap support (BS) values were estimated from 100 bootstrap replicates in GARLI. The ML bootstrap support (BS) values were estimated from 100 bootstrap replicates in GARLI.

To evaluate the congruence of the nuclear and plastid datasets, we employed the incongruence length difference (ILD) test (Farris et al. 1994). The ILD test was conducted using a heuristic search with 1,000 replicates and tree–bisection–reconnection branch–swapping with 10 random sequence additions in PAUP*.

**RESULTS**

The aligned sequences of the ITS region comprised a data matrix of 725 base pairs (bp) with 228 parsimony-informative sites (228/725, 31.45%). The psbA–trnH alignment consisted of 682 bp with 11 parsimony-informative sites (11/682, 1.61%). The aligned rbcL sequences were 1,146 bp in length with 30 parsimony-informative sites (30/1146, 2.62%). The aligned matK data comprised 869 bp, of which 44 were parsimony informative (44/869, 5.06%). The trnL–trnF alignment consisted of 1,029 bp, of which 38 sites were parsimony informative (38/1029, 3.69%) (Table 1). Given the absence of recombination in the chloroplast genome, we combined the plastidic sequences into a single dataset. The combined cpDNA data matrix comprised 3,726 characters including 123 parsimony-informative sites (123/3726, 3.30%). The ILD test indicated that the ITS and cpDNA (psbA–trnH, rbcL, matK, and trnL–trnF) datasets were significantly incongruent (p = 0.02); therefore, a combined analysis of the ITS and cpDNA sequence data was not performed.

The nrDNA tree with PP and BS support is shown in Fig. 2. The cpDNA tree is shown in Fig. 3. Our analyses resolve Ophiopogonoeae as monophyletic, but with low to moderate support. The genus Ophiopogon is monophyletic and consists of two major lineages, Clades A and B. Clade A consists of subclade A1 and A2, while Clade B is divided into subclades B1, B2, and B3. Based on the results of the phylogenetic analyses, Ophiopogon lancaignensis Wang et Tang, O. multilorus Y. Wan, O. yunnanensis S. C. Chen, O. reversus C. C. Huang, and O. longibracteatus D. Don were classified in sect. Ophiopogon, and O. isaii F. T. Wang et Tang was classified in sect. Peliosanthis. The monophyly of both Liriope and Peliosanthis was supported in all analyses.

**TABLE 1. Summary of the DNA sequence data used in this study of Ophiopogoneae.**

<table>
<thead>
<tr>
<th></th>
<th>ITS</th>
<th>psbA–trnH</th>
<th>rbcL</th>
<th>matK</th>
<th>trnL–trnF</th>
</tr>
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<tr>
<td>Length of aligned matrix (bp)</td>
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<td>682</td>
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<td>869</td>
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<td>Number of parsimony-informative characters</td>
<td>228</td>
<td>11</td>
<td>30</td>
<td>44</td>
<td>38</td>
</tr>
<tr>
<td>Percent of parsimony-informative sites</td>
<td>31.5%</td>
<td>1.6%</td>
<td>2.6%</td>
<td>5.1%</td>
<td>3.7%</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**Incongruence of Nuclear and Chloroplast Matrices**—The ILD test (Farris et al. 1994) indicated that the nuclear and combined plastid data sets were significantly incongruent (p = 0.02). For example, in the nrDNA tree, Ophiopogon changii Wang et Tang was sister to O. grandis W. W. Sm. and O. bodinieri Lévl., in which the cpDNA tree it was sister to O. latifolius Rodríg., O. platyphyllus Merr. et Chun and O. dracaemoides (Baker) Hook. f. Similarly, the clade O. multiflors and O. umbratiloca was internal to the nrDNA tree, but in the cpDNA tree, it was sister to all other clades. Theoretical and experimental studies have demonstrated that incongruence among gene trees or between gene trees and organismal phylogenies can result from a variety of factors, including sampling error, convergence, evolutionary rate heterogeneity, lineage sorting, hybridization, and introgression (Avise 1989; Rieseberg and Soltis 1991; Doyle 1992; Kadereit 1994; Rieseberg et al. 1996). One probable explanation for the differences in tree topologies is hybridization. Hybridization has been documented in Ophiopogon (Zhang 1991), as well as polyploidy (Zhang 1991; Wang et al. 2013). Most of the polyploid taxa within the genus occur within sect. Ophiopogon. Whether these are allopolyploids or auto-polyploids has not yet been evaluated using a molecular approach. Another probable explanation is introgression. Inflorences of O. szchuanensis Wang et Tang, O. latifolius, and O. mairei H. Lév. occur on the upper stem (unspecialized), whereas leaves are scattered (specialized); the leaf widths of O. clarkei, O. intermedius, O. megalanthis F. T. Wang et L. K. Dai, O. corfolius Wang et Dai, O. zingiberacens F. T. Wang et L. K. Dai, O. revolutus F. T. Wang et L. K. Dai, and O. platyphyllus are between sect. Ophiopogon and sect. Peliosanthis; inflorences of O. yunnanensis occur on the upper stem (unspecialized), while leaves are sessile (specialized). By their combination of specialized and unspecialized inflorescence features, we infer that these species are transitional taxa.

**Relationships among Ophiopogon, Liriope, and Peliosanthes of Ophiopogoneae**—Nuclear and chloroplast data indicated the monophyly of the tribe Ophiopogoeneae with strong to moderate support (PP 1.00, BS 99%, Fig. 2; PP 0.69, BS 87%, Fig. 3), a result consistent with findings from previous studies. The monophyly of Ophiopogoeneae also is supported by irregular dehiscence early during development to expose the immature seeds. The relationships of the three genera of Ophiopogoeneae have been studied using morphological, cytological, and molecular data. Dai and Liang (1991), Liang and Dai (1992), and Zhang (1991) hypothesized that Ophiopogon and Liriope were closely related based on leaf epidermal and pollen characters and chromosome numbers. Those authors placed Ophiopogon and Liriope in tribe Ophiopogoeneae and Peliosanthes in a separate tribe, Peliosantheae. Cutler (1992) also presented evidence for the close affinity of Ophiopogon and Liriope in a vegetative anatomical study of the tribe
Fig. 2. Strict consensus tree of Ophiopogoneae based on nuclear ITS Sequence data (tree length = 760 steps, CI = 0.57, RI = 0.83, RC = 0.47). Values above each branch represent Bayesian posterior probabilities and those below branch (> 50%) are maximum likelihood bootstrap values from 100 replicates.
Fig. 3. Strict consensus tree of Ophiopogoneae based on chloroplast DNA sequence data (tree length = 326, CI = 0.82, RI = 0.90, RC = 0.73). Values above each branch represent Bayesian posterior probabilities and those below branch (> 50%) are maximum parsimony values.
Ophiopogoneae. Rudall et al. (2000) and Kim et al. (2010) showed that Ophiopogon and Liriope were closely related in their phylogenetic analyses of matK and rbcL sequence data. However, some authors argued that Ophiopogon and Liriope had no close relationship (Bailey 1929; Hume 1961; Skinner 1971; Mcharo et al. 2003). Furthermore, Tamura and Yamashita (2004) suggested that Liriope was more closely related to Peliosanthes based on analyses of matK and rbcL sequences. Cytological data supports the monophyly of Ophiopogon, Liriope, and Peliosanthes (Wang et al. 2013). In the present study, Ophiopogon was a distinct clade and had moderate support, while the isolated clades of both Liriope and Peliosanthes were highly supported (Figs. 2 and 3). All three genera resolve within one clade, with relatively strong support. Therefore, we suggest that Ophiopogon, Liriope, and Peliosanthes be maintained as distinct but closely related genera.

**Monophyly of Ophiopogon**—Our results show low to moderate support for the monophyly of Ophiopogon (ITS PP = 0.96, BS = 53%, Fig. 2; cpDNA PP = 0.69, BS = 54%, Fig. 3). The monophyly of the genus also is supported by morphological and chromosomal evidence. For example, drooping flowers, free overlapping tepals, short filaments, and a semi-inferior ovary are synapomorphic for Ophiopogon (Bailey 1929; Chen and Tamura 2000), whereas erect flowers, free tepals, long filaments, and a superior ovary are diagnostic of Liriope, and basally overlapping tepals, filaments dilated and connate in a fleshy ring, and an inferior ovary are synapomorphic in Peliosanthes (Chen and Tamura 2000). Most species of Ophiopogon share a basic chromosome number of \( x = 17 \) is known in a few species (e.g. *O. umbraticola*, *O. japonicus*, *O. clarkei*, *O. intermedius*, and *O. shiuii*) (Malik 1962; Sharma and Chaudhuri 1964; Nagamatsu and Noda 1971; Zhang 1991; Yamashita and Tamura 2001). Within this group, diploid diploid, tetraploid, and hexaploid species exist only in Ophiopogon. Ophiopogon species have both 2B and 2C type chromosomes, with subtelocentric (st) chromosomes occurring rarely. Liriope shares the basic chromosome number of \( x = 18 \), but has only 2B chromosomes, with a few species possessing st chromosomes. In contrast, Peliosanthes species have a basic chromosome number of \( x = 17 \) or 18, possess 2C chromosomes, and have st chromosomes in all species (Hsu 1971; Yang et al. 1990; Zhang 1991; Wang et al. 2013).

**Phylogenetic relationships within Ophiopogon**—Two major clades were revealed in Ophiopogon by the ITS sequence data (Fig. 2). Clade A included species from both sect. Ophiopogon and sect. Peliosanthes. Clade B consisted of species from sect. Ophiopogon as classified by Wang and Tang (1978). The cpDNA data failed to provide enough variation to resolve the relationships within Ophiopogon (Fig. 3). Therefore, the following discussion of Ophiopogon is largely based on phylogenetic reconstructions from the ITS dataset.

Two well-defined, moderately to well-supported subclades, A1 and A2, were resolved within clade A (Fig. 2). All species in subclade A1 belonged to sect. Peliosanthes except for *O. platyphyllus*, *O. latifolius*, *O. mairei*, and *O. tsii*, which belonged to sect. Ophiopogon according to Wang and Tang (1978). In sect. Peliosanthes, most species grow on karst landforms. Ophiopogon *tsii* also grows on karst landforms but grouped within sect. Peliosanthes. Except for *O. tonkinensis* L. Rodr. and *O. dracenaenoides*, species placed in subclade A1 have petiolate, oblong-ob lanceolate leaves, solitary flowers, short filaments, and slender styles. Ophiopogon *tonkinensis* and *O. dracenaenoides* have leathery leaves, clustered flowers, and obvious fila-

- **Subclade A2 was sister to subclade A1 but with low support** (PP 0.59, BS 52%, Fig. 2). All taxa included in subclade A2 belong to sect. Ophiopogon except for *O. sylvicola* F. T. Wang et Tang and *O. marmoratus* Pierre ex L. Rodr., which were assigned to sect. Peliosanthes by Wang and Tang (1978). Except for *O. zingiberaceus*, which has distinctive Zingiber-like rhizomes and deltoid-ovate tepals, all species of this subclade possess slender roots. Tanaka (1999) reduced *O. revolutus* to synonymy with *O. griffithii* D. Don on the basis of leaf shape and width. Given the broad morphological diversity exhibited by *O. intermedius*, numerous related species were synonymized by Tanaka (2001a, b). Ophiopogon *megalanthus* and *O. yunnanensis* formed a moderately supported clade, but the two species show little morphological similarity; *O. megalanthus* has rhizomes, basal leaves, and lanceolate bracts, with flowers in clusters of 2–4; *O. yunnanensis* has elongate stems, distant tufted leaves, paired flowers, and lanceolate to linear-lanceolate bracts. We classify *O. yunnanensis* in sect. Ophiopogon based on the possession of grass-like, sessile leaves.

Ophiopogon *clarkei* is distributed in Tibet and can be distinguished by its slender underground rhizome, obvious midvein leaves, white, ovate to lanceolate tepals, and long filaments. Although this species was placed in sect. Ophiopogon by Wang and Tang (1978), the present results indicate that *O. clarkei* occupies an isolated position in clade A as a sister species to subclades A1 and A2 with high support (PP 1.00, BS 99%, Fig. 2).

Species in the weakly supported clade B in Ophiopogon are characterized by grass-like leaves, nodding flowers, and short filaments (PP 0.52, Fig. 2). Three moderately to robustly supported subclades (B1, B2, and B3) also were recovered (Fig. 2).

Although the nrDNA and cpDNA phylogenies resolved similar higher-level relationships, the phylogenetic placement of several individual species differed. The most notable difference was in the position of subclade B1 (comprising *O. multiflorus* and *O. umbraticola*), which is sister to subclades B2 and B3 in the nrDNA tree but sister to all other Ophiopogon species in the cpDNA tree. Subclade B1 was moderately supported (PP 0.95, BS 70%, Fig. 2; PP 0.94, Fig. 3). Ophiopogon *multiflorus* and *O. umbraticola* share bluish flowers and three internal tepals wider than the external ones. Ophiopogon *multiflorus* has grass-like and sessile leaves and is morphologically similar to *O. bockianus* (sect. Ophiopogon) in flower structure (Wan 1988). After considering both morphology and our molecular data, we elected to move *O. multiflorus* to sect. Ophiopogon.

In the nrDNA tree, subclade B2 was highly supported (PP 1.00, BS 100%, Fig. 2) and was sister to subclades B1 and B3. Two well-defined groups were resolved within subclade B2. One group comprised only accessions of the polymorphic species *O. japonicus*. Different forms of *O. japonicus* have been
distinguished as distinct species by many authors. However, the flower structure is relatively stable, especially in Ophiopogon longibracteatus and O. reversus are morphologically distinct, but co-occur in moist environments in Qingyuan County, Guangdong Province. The most striking difference between the two species is that O. longibracteatus has longer bracts and tepals than O. reversus. In light of their grass-like, sessile leaves, we placed the two species into sect. Ophiopogon. Species of O. lancangensis and O. bockianus formed a well-supported clade (PP 1.00, Fig. 2). Their close relationship was supported by their lanceolate bracts, which articulate below the middle. Furthermore, they occur in similar subtropical, humid climates. Combined with their possession of grass-like and sessile leaves, we classified O. lancangensis in sect. Ophiopogon.

The final group resolved within clade B was subclade B3 (PP 1.00, BS 100%, Fig. 2), where the presence of lanceolate tepals, lanceolate bracts, conspicuous filaments, and central articulate are potential synapomorphies. All species belong to sect. Ophiopogon. Ophiopogon chinensis is morphologically distinct from the other members of this subclade by its possession of prostrate stems, scattered leaves, and membranous bracts. Ophiopogon bodinieri and O. grandis resolved as sister species (PP 1.00, BS 91%, Fig. 2; PP 1.00, BS 53%, Fig. 3). The main difference between these two species is that O. bodinieri has underground rhizomes and O. grandis has conspicuous filaments and articulation above the middle. In addition, O. bodinieri constitutes a polyploid complex of diploids, tetraploids, and hexaploids.

Previously, O. marmoratus, O. sylvicola, and O. dracaenoides were classified in sect. Peliosanthaoides, and O. tsaii was placed into sect. Ophiopogon (Wang and Tang 1978). However, our analyses of nrDNA sequence data indicate that O. marmoratus, O. sylvicola, and O. dracaenoides belong to sect. Ophiopogon, and O. tsaii to sect. Peliosanthaoides. Our field observations have revealed that O. marmoratus, O. sylvicola, and O. dracaenoides produce grass-like leaves under drought conditions but cauline leaves in humid environments. The sectional placement of O. tsaii (which has grass-like leaves and erect stems in the field) also has been problematic. When the grass-like leaves are emphasized, it is better classified in sect. Ophiopogon, but when the stem erect is emphasized, it is better classified in sect. Peliosanthaoides. A detailed examination of morphological characters revealed that the erect stem is of primary taxonomic importance in O. tsaii, which is consistent with our molecular results.

Yang et al. (1990) divided Ophiopogon into two sections with five series based on inflorescence position and leaf shape. However, our present results show that taxa classified in these series were not resolved as clades, but were dispersed throughout the trees. This finding indicates that inflorescence position and leaf shape may not represent suitable characters for use in an infrageneric classification of Ophiopogon. Compared to the classification of Yang et al. (1990), molecular evidence better supports the classification proposed by Wang and Tang (1978) with respect to sects. Ophiopogon and Peliosanthaoides.

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