FLORAL MORPHOLOGY RESOLVES THE TAXONOMY OF CAMELLIA L. (THEACEAE) SECT. OLEIFERA AND SECT. PARACAMELLIA

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Abstract

Numerical taxonomy and cladistic analysis of 19 species of *Camellia* L. were performed using floral morphology containing continuous and discrete units. The current study mostly supports the classifications of 19 species as proposed in previous works. In addition, it also agrees with combining the following species together: *C. oleifera* and *C. vietnamensis*; *C. sasanqua* and *C. hiemalis*; *C. brevistyla* and *C. puniceiflora*; and *C. grijsii* and *C. shensiensis*. Further, we propose that *C. maliflora* be recognized as a variety of *C. sasanqua*, and *C. phaeoclada* is best placed in sect. *Paracamellia*. Moreover, we conclude that these species can be combined: *C. tenii* and *C. miyagii*; and *C. confusa* and *C. fluviatilis*. Our study indicates that the numerical taxonomy and cladistic analysis based on morphological characters of floral organ is useful in species classification, and this technique appreciated in sect. *Oleifera* and sect. *Paracamellia* can be used for identification and classification of other taxa.

Introduction

The genus *Camellia* L. (Theaceae) is endemic to southeastern Asia, and 80% species are native to China (Lu *et al.*, 2008). Some species of the plant are used to produce green tea, to cultivate as ornamental plants, and the seeds of others are used to produce edible oils (Lu *et al.*, 2012). More than three million hectares of agricultural land is used to grow *Camellia* to produce in excess of 164 thousand tons of edible cooking oil (Vijayan *et al.*, 2009). Obviously, the economic value of *Camellia* is significant. However, the taxonomic relationships between various *Camellia* species are still unclear. Since Linnaeus (1753) assigned *Camellia japonica* L. and *Thea sinensis* L. [=*C. sinensis* (L.) O. Kuntze] in his first edition of *Species Plantarum*, there is little consensus upon the combination and species number that should be recognized. Estimates vary from about 82 to 119 or 280 species, depending on the taxonomic authority (Chang, 1998; Ming, 2000).

Camellia is regarded as morphologically, anatomically and molecularly heterogeneous genus based on studies of its various sections (Lin *et al.*, 2008; Pi *et al.*, 2009; Pi *et al.*, 2011). Different taxonomic questions in relation to many of its sections remain unresolved, for example, sect. *Oleifera* and sect. *Paracamellia*. Sealy (1958) listed six species and two varieties in the sect. *Paracamellia*. Chang (1998) divided Sealy's system into sect. *Oleifera* and sect. *Paracamellia*, because species of sect. *Oleifera* have longer styles and androecium, and higher seed oil content (Table 1). There are total 21 species in Chang's taxonomic classification. Ming (2000) concluded that there was no essential difference between sect. *Oleifera* and sect. *Paracamellia*, which was largely on the basis of a structural framework proposed by Sealy (1958). By combination of species, Ming (2000) reduced the section to seven species. Since Chang's classification of *Camellia* provided as part of a comprehensive taxonomic revision, we used it as the primary context for assessing the results of the present study.

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During the last 10 years, efforts to resolve classification issues in sect. Oleifera and sect. Paracamellia have involved data on leaf morphology (Lin et al., 2008), chemical composition analysis (Shen et al., 2008) and molecular marker (Vijayan et al., 2009). However, the taxonomic position of a few species is still ambiguous. It is necessary to seek other information for reassessing the classification of these two sections. The construction of classifications with their positive features depended upon a careful comparison of attributes of the organisms (Stuessy, 2009). Stuessy (2009) reported that although many vegetative characters have also been used to good effect, in a general sense, floral features have been most useful in angiosperm taxonomy. The androecium in flowers was obviously of high taxonomic value within angiosperms, stamens were important in classification of plants, and the anthers occurred in many different sizes and shapes (Hufford and Endress, 1989). Many studies have employed comparisons of several different floral structures (Kocyan and Endress, 2001; Matthews and Endress, 2005). Liston (2003) showed the importance of careful observations of floral morphology to infer homology of ovary position. These all indicate that types of floral characters are more distinguished according to its variability of characters and states. Thus, a detailed analysis of floral morphology can be regarded as a significant method to identify the disputed species among these two sections. The aims of our study were: (1) to explore if numerical taxonomy and cladistic analyses based on morphological characters of floral organ is of value in classification, (2) to investigate the distinction between sect. Oleifera and sect. Paracamellia of the genus Camellia, and (3) to assess the phylogenetic relationships among the 19 species in these two sections.

Material and Methods

Plant materials

Five species from sect. *Oleifera* and 14 species from sect. *Paracamellia* were examined (Table 1). As a outgroup, closely related species of sect. *Camellia* such as *C. chekiangoleosa* and *C. japonica* were chosen. The research was based on the investigation of living collections, which were obtained from the International *Camellia* Species Garden of Jinhua city (ICSG, 29°7'N, 119°35'S, 40 m in altitude). At least three different individual plants per species were selected in this study. Voucher specimens were deposited in the Chemistry and Life Science College of Zhejiang Normal University (ZJNU).

Morphological characters

For each plant, floral morphological attribute observations were obtained mainly from living plants and partly from the literature. The salient informative morphological variations were selected principally from petal, perule, filament, stamen, style, stigma, ovary, receptacle, pollen and anther (Table 2). A total of five binary characters were coded as 0 and 1, such as petal colour and number of perule. Pollen microscopic observations and measurements were made by using a Zeiss microscope. Other remaining 17 characters were studied by measuring the photographic images (Fig. 1) through the ImageJ software. Seven other variables were added (derived variables, Table 2): petal width-length ratio = petal width / petal length; petal form coefficient = $16 \times \text{petal}$ perimeter/ (petal area)²; style height-stamen height ratio = height of style / height of stamen; divided style ratio = length of divided style / height of style; ovary diameter-height ratio = diameter of ovary/ height of ovary; ovary diameter- receptacle diameter ratio = diameter of ovary/ diameter of receptacle; anther width-length ratio = anther width / anther length. The floral morphological characters we selected in this study were followed by earlier researches (Takahata and Hinta, 1986).

Table 1. Species level differences between the classifications of sect. *Oleifera* and sect. *Paracamellia* of *Camellia* L. by Chang (1998) and Ming (2000).

Chang (1998)	Ming (2000)					
Framework for classifications						
Composed 5 species in sect. Oleifera H. T. Chang	Composed 7 species in section sect. Oleifera H.T.					
and 14 species in sect. Paracamellia Sealy	Chang					
Sections with similar members						
1. C. gauchowensis H.T. Chang	1. C. gauchowensis H. T. Chang					
2. C. oleifera Abel	2. C. oleifera Abel					
3. C. vietnamensis T.C. Huang ex Hu						
4. C. lanceoleosa H.T. Chang & J.S. Chiu	3. C. fluviatilis HandMazz. var. fluviatilis					
5. C. fluviatilis HandMazz.	var. megalantha					
6. C. sasanqua Thunb.	4. C. sasanqua Thunb.					
7. C. hiemalis Nakai						
8. C. miyagii (Koidz.) Makino & Nemoto						
9. C. brevistyla (Hayata) Coh. Stuart	5. C. brevistyla (Hayata) Coh. Stuart					
10. C. obtusifolia H.T. Chang	var. brevistyla					
11. C. puniceiflora H.T. Chang	var. microphylla Merr.					
12. C. confusa Craib	6. C. kissi Wall. var. kissi					
13. C. kissi Wall.	var. vonfusa (Craib.) Ming					
14. C. grijsii Hance	7. C. grijsii Hance var. grijsii					
15. C. yuhsienensis Hu	var. shensiensis (H.T. Chang) Ming					
16. C. shensiensis H.T. Chang						
17. C. maliflora Lindl.	hybrid					
18. C. phaeoclada H.T. Chang	C. saluenensis Stapf ex Bean					
19. C. tenii Sealy	Sect. Heterogenea Sealy					

Numerical taxonomy

A total of 76 variables for each species were used: 5 binary and 71 continuous (including Max, Ave and Mix). Mix values of diameter of pollen, width of anther, length of anther and anther width-length ratio were ultimately excluded considering dysplasia and abortion of anther and pollen. Gower's (1971) general similarity coefficient (SC) was used to measure pairwise similarities for mixed datasets. Both cluster analysis and principal coordinate analysis were conducted using MVSP software (Version 3.13n, Kovach Computing Services).

Cladistic analysis

Cladistic analysis was based on a set of 30 characters consisting of five discrete and 25 continuous characters. Discrete characters were scored and entered directly into a data matrix. Continuous characters were converted to discrete characters following Otalora *et al.* (2008), and modifications were made for our study. Firstly, an ANOVA was analyzed on each character by using SPSS version 17.0 (SPSS Inc., Chicago, IL). If the null hypothesis (H₀= mean of each group is equal) was rejected for a given character, a pairwise mean comparison using the Duncans Multiple Range Test (P < 0.05) was performed. Means for groups in homogeneous subsets were obtained. The values with different number were significantly different to convert morphological continuous characters into discrete characters.

Morphological characters	Morphometrical analysis				
1. petal	a. colour white (0), pink or red (1)				
	f. area (cm)				
	g. perimeter (cm)				
	h. width (cm)				
	i. length (cm)				
	j. number of petal				
2. perule	b. number of perule: 6-9 (0), 10-12 (1)				
	k. number of full petaloid perule				
3. filament and stamen	c. stamen fusion: connate near the base (0), connate in the lower half (1)				
	d. stamen fusion: even (0), uneven (1)				
	e. filament: vertical (0), curved (1)				
	l. number of stamen				
	m. number of stamens layer				
	n. height of stamen				
4. style and stigma	o. number of stigma				
	p. height of style (mm)				
	q. length of divided style (mm)				
5. ovary and receptacle	r. diameter of ovary (mm)				
	s. height of ovary (mm)				
	t. diameter of receptacle (mm)				
6. pollen and anther	u. diameter of pollen (mm)				
	v. width of anther (mm)				
	w. length of anther (mm)				
7. derived variables	x. petal width-length ration = h/i				
	y. petal form coefficient = $16*g/f^2$				
	z. style height- stamen height ratio = p/n				
	ab. divided style ration = q/p				
	ac. ovary diameter-height ratio = r/s				
	ad. ovary diameter- receptacle diameter ratio = r/t				
	ae. anther width-length ration = v/w				

Table 2. Salient morphological characters	s of Camellia	species used	for the	morphometrical	analysis.
Binary characters are coded as 0 and 1	•				

*See 'Morphological characters' section under Materials and Methods for explanation.

The cladistic analysis was performed using Mrbayes 3.1 (Ronquist and Huelsenbeck, 2003) as an alternative for likelihood analysis. A Bayesian Analysis (BA) was performed on standard data set, using a General Time Reversible (GTR) model (nst=6; rates=invgamma) with gamma-distribution rate variation and a proportion of invariant sites, and one million generations of the Markov Chain Monte Carlo (MCMC) chains in two independent runs (Yuan *et al.*, 2010). All other trees sampled were used to calculate a strict consensus tree, thus yielding the Posterior Probability (PP). The tree was rooted with two species of sect. *Camellia: C. chekiangoleosa* and *C. japonica*.

Results

Cluster analysis and principal coordinate analysis

The results of cluster analysis and principal coordinate analysis are shown in Figure 2 and Fig. 3, respectively. Fig. 2 shows that the dendrogram divided the 21 species into three clear-cut clusters, Outgroup, Clade 1, and Clade 2. *C. chekiangoleosa* and *C. japonica* as outgroup were separated at the top level (SC = 0.715) from the other species of sect. *Oleifera* and sect. *Paracamellia*. Clade 1 included 6 species. Except *C. hiemalis* and *C. maliflora*; *C. gauchowensis*, *C. vietnamensis*, *C. oleifera* and *C. sasanqua* belonged to sect. *Oleifera*. Clade 2 comprised the remaining 12 species of sect. *Paracamellia* except *C. lanceoleosa*. On closer inspection, the largest similarity coefficient was recorded within *C. tenii* and *C. miyagii* (SC = 0.924), which indicates their close relationship. While *C. grijsii* and *C. shensiensis* (SC = 0.885), *C. puniceiflora* and *C. oleifera* (SC = 0.864), *C. fluviatilis* and *C. confusa* (SC=0.849), respectively, formed a group distinct from the other species. Principle coordinate analysis (PCO) is displayed in a two-dimensional plot using the first two principal coordinates (Fig. 3). It permitted a visualization of the degree of affinity among these species. Figure 3 also shows that all the species formed three groups, which were consistent with results obtained from cluster analysis.



Fig. 1. Petal shape variability of *Camellia* species. Petals from A) *C. fluviatilis*; B) *C. kissi*; C) *C. confusa*;
D) *C. miyagii*; E) *C. tenii*; F) *C. puniceiflora*; G) *C. obtusifolia*; H) *C. phaeoclada*; I) *C. brevistyla*; J) *C. lanceoleosa*; K) *C. oleifera*; L) *C. gauchowensis*; M) *C. vietnamensis*; N) *C. yuhsienensis*; O) *C. shensiensis*; P) *C. grijsii*; Q) *C. maliflora*; R) *C. hiemalis*; and S) *C. sasanqua*.

Cladistic analysis

The phylogenetic relationship deduced from the morphology characters using Bayesian Analysis (BA) (Fig. 4) was largely consistent with cluster analysis (Fig. 2). The species included

in Clade 1 and Clade 2 was exactly the same in both trees. The monophyly of Clade 1 was supported with posterior probabilities (PP = 0.66). The difference being that the BA majority rule tree was somewhat worse resolved than the cluster tree: 1) C. hiemalis, C. sasanqua and C. maliflora diverged at the same time in the BA tree, while C. hiemalis and C. sasanaua were more closely related to each other than to C. maliflora in the cluster tree; and 2) in the BA tree, C. gauchowensis was separated as the most distant from two subclades comprising C. oleifera and C. vietnamensis, and C. hiemalis, C. sasangua and C. maliflora. While C. oleifera and C. vietnamensis were more closely related to each other than C. gauchowensis. The Clade 1 consisted of these three species, and together with another subclade including C. hiemalis, C. sasanqua and C. maliflora. In Clade 2, BA tree and cluster tree were consistent with one another in two places: 1) C. puniceiflora and C. brevistyla were clustered in subclade at the same level (PP = 0.65), and C. lanceoleosa was closely related to these two species (PP = 0.67); and 2) C. phaeoclada was separated as a distant from two subclades comprising C. obtusifolia, and C. tenii and C. miyagii (PP = 0.80). However, the distinct interrelationships among Clade 2 remained: 1) C. shensiensis, C. grijsii and C. vuhsienensis diverged earlier on in Clade 2 in BA tree, while C. shensiensis and C. grijsii were closer to each other than C. yuhsienensis in cluster tree; and 2) C. kissi had distinct systematic position in both trees.



Fig. 2. UPGMA dendrogram based on Gower's general similarity coefficient for the sect. *Oleifera* and sect. *Paracamellia* species of *Camellia*.

Discussion

Relationship between sect. Oleifera and sect. Paracamellia

The present study is the first attempt to reconstruct the phylogenetic relationships between sect. *Oleifera* and sect. *Paracamellia* based on floral organ morphology. A comparison between topologies obtained by cluster tree and BA majority rule tree indicated that both methods provided highly similar estimates of phylogeny (Figs 2-4). Based on the present taxon samples, the relationships among the species in sect. *Oleifera* and sect. *Paracamellia* are closer. Our results suggest that sect. *Oleifera* is closely related to sect. *Paracamellia*. Sealy (1958) and Ming (2000)

combined the species into one section because they are according to their common morphological characteristics, like the colour of flowers, bloom and perules drop time. However, Chang (1998) divided them into sect. Oleifera and sect. Paracamellia because he found plants of sect. Oleifera have longer styles and androecium and higher seed oil content than of sect. *Paracamellia*. Shen et al. (2008) also demonstrated that the mergence of the two sections is quite unnatural based on macro-morphology, micro-morphology and chemical characteristics. In this study, we selected various morphological characters of floral organs combined numerical taxonomy to investigate the distinction between sect. Oleifera and sect. Paracamellia, our results agree on Chang's system and Shen et al. (2008). Thus, two clusters are supported with low PP value. In addition, there were several morphological traits that demonstrated the discrimination within sect. Oleifera and sect. Paracamellia. The most prominent ones are that sect. Oleifera species had bigger floral organs, more stamen series, longer androecium, longer styles, bigger ovary, and even bigger anthers and pollens than sect. Paracamellia. The analysis of leaf anatomy revealed that sect. Oleifera may differ from sect. Paracamellia. The sect. Oleifera shared the same pattern of anticlinal cells and the same size between adaxial and abaxial epidermal cells, and long ovate stomatal shape (Lin et al., 2008). Shen et al. (2008) reported that sect. Oleifera was distinguishable from sect. Paracamellia in FTIR (Fourier Transform Infrared) fingerprint-like spectra, and they concluded that the merger of the two sections was quite surprising. The formation of sect. *Oleifera* by Chang (1998) was also confirmed by molecular phylogeny (Vijayan et al., 2009). Differences among the classifications based on leaf anatomy (Lin et al., 2008) and leaf FTIR (Lu et al., 2008), and our results are highlighted in Figure 5. The problems with classification of C. hiemalis, C. maliflora, C. lanceoleosa and other species are discussed below separately.





Interspecies relationships in sect. Oleifera and sect. Paracamellia

Classification of C. sasanqua, C. hiemalis and C. maliflora

Floral morphology data has certainly improved the comprehension of evolutionary relationships between sect. *Oleifera* and sect. *Paracamellia*, but some questions remain. Sealy

(1958) noted that there was a little doubt that *C. hiemalis* was a form of *C. sasanqua*. Parks *et al.* (1981) later reported that *C. hiemalis* was well known as a group of *C. sasanqua*. However, Chang and Bartholomew (1984) considered *C. hiemalis* a distinct species based on short and thick androecium and styles, and free petals at the base, and placed it in sect. *Paracamellia*.

Recently, Lin *et al.* (2008) showed that characteristics of the leaf anatomy in *C. sasanqua* and *C. hiemalis*, such as stomata, size of adaxial and abaxial epidermal cells, and the thickness ratio of palisade parenchyma, were different. Nonetheless their close relationships were demonstrated in the dendrogram of FTIR data, which showed Ming's (2000) combination of the two species is reasonable (Shen *et al.*, 2008). In the trees we constructed, *C. hiemalis* had closest relationship with *C. sasanqua*. Thus, the merge of *C. hiemalis* and *C. sasanqua* seems to be natural.

Sealy (1958) considered *C. maliflora* a hybrid of unknown origin and placed it in sect. *Theopsis.* Chang (1998) also believed that *C. maliflora* was a hybrid according to the floral characteristics, but leaved its status as a distinct species in sect. *Paracamellia.* Subsequently, Ming (2000) revealed the *C. maliflora* was a cultivar and should not be recognized at the species level. Leaf micro-morphology characteristics displayed *C. maliflora* had closer relationship with sect. *Oleifera* which convincing evidenced that it was a cultivar of sect. *Oleifera* species. Our data suggested that it is quite reasonable to place it in sect. *Oleifera.* It had closest relationships with a clade of *C. hiemalis* and *C. sasanqua.* From a morphological point of view, these three species had similar petal colour and shape (Fig. 1Q-S), stamen fusion connate near the base, longer androecium and styles than sect. *Paracamellia* species. Therefore, based on these above evidence, we cannot dismiss the correctness of results and should do more work to demonstrate that *C. maliflora* may be regarded as a hybrid of *C. sasanqua.*



Fig. 4. Phylogram from Bayesian phylogenetic analysis of 30 morphological characters of 21 *Camellia* species. Number behind branches indicate Bayesian clade-credibility values (posterior probability).

Classification of C. sasanqua, C. oleifera and C. vietnamensis

Chang and Bartholomew (1984) believed that *C. vietnamensis* was closely related to *C. sasanqua* and *C. oleifera*. Ming (2000) merged it into *C. oleifera*, although there were significant differences between *C. oleifera* and *C. vietnamensis*. The combination of *C. oleifera* and *C. vietnamensis* by Ming (2000) also had good support from our results, and cluster analysis based on leaf anatomy characters. Although, from our observation, *C. vietnamensis* had larger petals, they

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had similar petal form coefficient, height of style, number of stamen, and diameter of ovary. Further, they had unique curved filament, while the others had vertical filament. Based on the above features we support the Ming's (2000) combination of *C. oleifera* and *C. vietnamensis*.

Classification of C. grijsii, C. shensiensis and C. yuhsienensis

When reconsidering *C. grijsii, C. shensiensis* and *C. yuhsienensis*, Ming (2000) treated *C. shensiensis* and *C. yuhsienensis* as a variety of *C. grijsii*. According to our floral morphological observations, *C. grijsii, C. shensiensis* and *C. yuhsienensis* had the most similar petal shape - broadly obcordate (Fig. 1), whereas other species had obcordate petals. However, *C. yuhsienensis* had larger flowers than *C. grijsii* and *C. shensiensis*. Our results support the combination of *C. grijsii* and *C. shensiensis* (Fig. 2). Lin *et al.* (2008) showed area of adaxial and abaxial epidermal cell of *C. grijsii* and *C. shensiensis* nearly match, but *C. yuhsienensis* was larger. Pollen exine sculpture characteristics (Ao *et al.*, 2001) and molecular taxonomy from nrITS sequence (Vijayan *et al.*, 2009) also support the combination of *C. shensiensis* into *C. grijsii*. Close relations of these two species was also demonstrated in the dendrogram of FTIR data (Shen *et al.*, 2008). Therefore, combining of *C. grijsii* and *C. shensiensis* should be treated as a variety of *C. grijsii*.



Fig. 5. Differences among species classifications based on leaf anatomy, leaf FTIR and our results from sect. *Oleifera* and sect. *Paracamellia* of *Camellia* species.

Classification of C. puniceiflora, C. brevistyla and C. obtusifolia

Camellia puniceiflora and *C. obtusifolia* were designated as the varieties of *C. brevistyla* by Ming (2000). In our study, *C. puniceiflora* and *C. brevistyla* formed a clade with PP = 0.65 and SC = 0.88, which is in accordance with the results of studies on leaf anatomy (Lin *et al.*, 2008) and FTIR (Shen *et al.*, 2008). However, the position of *C. obtusifolia* was unclear, because it was considered to belong to *C. brevistyla* by leaf anatomy (Lin *et al.*, 2008), but not supported by FTIR (Shen *et al.*, 2008) and our results. These results indicate that *C. puniceiflora* should be treated as a variety of *C. brevistyla*, but their relationship with *C. obtusifolia* requires more evidence.

Classification of C. phaeoclada, C. tenii, C. miyagii

Ming (2000) combined *C. phaeoclada* into *C. saluenensis* (belong to sect. *Camellia*), and placed *C. tenii* in sect. *Heterogenea*. Our analyses, combined with FTIR (Shen *et al.*, 2008), leaf anatomy (Lin *et al.*, 2008), strongly suggest that *C. phaeoclada* is the best placed in sect. *Paracamellia*. This species is morphologically distinct from other *C. saluenensis* as well as the remainder of sect. *Camellia*. We also propose combining *C. miyagii* and *C. tenii*, which is congruent with results of leaf FTIR (Shen *et al.*, 2008), but disagrees with results of leaf anatomy (Lin *et al.*, 2008).

On the basis of present study in conjunction with other studies based on leaf anatomy (Lin *et al.*, 2008), FTIR (Shen *et al.*, 2008; Lu *et al.*, 2008) and molecular data (Vijayan *et al.*, 2009), we propose a bifurcation of sect. *Paracamellia* supporting Chang's (1998) creation of sect. *Oleifera* from sect. *Paracamellia*. This proves that there is consistency of the information from different organs of a plant. It also highlights that every new technical development offers promise for improving the description of relationships among species.

The present study shows that sect. *Oleifera* included six species: *C. oleifera*, *C. vietnamensis*, *C. gauchowensis*, *C. sasanqua*, *C. hiemalis* and *C. maliflora*. The sect. *Paracamellia* comprised the remaining 13 species. Moreover, we extended this observation for interspecies relationship confirming Ming's (2000) combination of *C. oleifera* - *C. vietnamensis*, *C. sasanqua* - *C. hiemalis*, *C. brevistyla* - *C. puniceiflora*, and *C. grijsii* - *C. shensiensis*. Further, we suggest combining *C. tenii* - *C. miyagii*, and *C. confusa* - *C. fluviatilis*. Moreover, we recognize *C. maliflora* as a variety of *C. sasanqua*, and have assigned *C. phaeoclada* to sect. *Paracamellia*. Numerical and cladistic analyses based on the floral morphometric data employed in this study had enough discriminating power to classify a group of species at section level. Additional floral information is needed to classify individuals at the species level. This technique appears to have taxonomic value and can be widely used for identification and classification of other taxa when the species are closely related.

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