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Original Article

Monochoria angustifolia (G. X. Wang) Boonkerd & Tungmunnithum, the new species of the genus *Monochoria* from Thailand

Duangjai Tungmunnithum[1, 2*], Norio Tanaka[2], and Thaweesakdi Boonkerd[3]

[1] *Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Ratchathewi, Bangkok, 10400 Thailand*

[2] *Department of Botany, Tsukuba Botanical Garden, National Museum of Nature and Science, Amakubo, Tsukuba, 305-0005 Japan*

[3] *Department of Botany, Faculty of Science, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand*

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Abstract

Monochoria C. Presl is a genus member of the aquatic plant family Pontederiaceae which exhibits high morphological variations. The populations of *Monochoria vaginalis* exhibit extreme variations in their morphology. Previous morphological and phenetic studies suggested that *M. vaginalis* s.l. in Thailand contained cryptic species. In this recent study, the molecular data from DNA sequencing and phylogenetic analysis of 50 living plants from 10 populations of *M. vaginalis* with 3 outgroups using 5 regions from both cpDNA and nuclear DNA were intensely investigated. The results showed a similar trend as in previous studies that *M. vaginalis* populations should be separated into 2 species. The new species, *M. angustifolia* (G. X. Wang) Boonkerd & Tungmunnithum, is also described in this work.

Keywords: *Monochoria*, new species, molecular data, Pontederiaceae, Thailand

1. Introduction

According to the World Checklist of Selected Plant Families (http://wcsp.science.kew.org/home.do), the genus *Monochoria* consists of 7 species worldwide: *M. africana* (Solms) N.E.Br. and *M. brevipetiolata* Verdc. distributed in Africa, *M. australasica* Ridl. distributed in Australia, *M. brevipetiolata* Verdc. and *M. cyanea* (F.Muell.) F.Muell disctributed in Australia and Indochina, as well as *M. korsakowii* Regel & Maack distributed mainly in Japan. The last three species are distributed in Thailand and some countries of Southeast Asia (Chayamarit, 2005).

*Corresponding author
Email address: duangjai.tun@mahidol.ac.th

M. vaginalis (Burm.f.) C.Presl is an aquatic flowering plant in the Pontederiaceae family occurring in standing water bodies of tropical and subtropical regions (Chayamarit, 2005; Solms, 1883; Tungmunnithum, Boonkerd, Zungsontiporn, & Tanaka, 2016; Wang, Li, Wan, & Itoh, 2004). The taxa was also reported as a morphologically variable species (Chayamarit, 2005; Tungmunnithum *et al.*, 2016; Tungmunnithum, Boonkerd, Zungsontiporn, & Tanaka, 2017; Wang *et al.*, 2004; Wang, Li, Wan, & Ito, 2003). Previously, many studies were based mainly on the herbarium specimens (Backer, 1951, 1968; Chayamarit, 2005; Cook, 1989; Kunth, 1843; Solms, 1883; Wang *et al.*, 2004). Therefore, some characteristics that could be observed easily on living specimens became unclear in dried herbarium specimens. Many taxonomists still consider this taxa as a monotypic species (Chayamarit, 2005; Lansdown, 2013), but a decade

ago a new variety, *M. vaginalis* var. *angustifolia* G. X. Wang, was described (Wang *et al.,* 2003) using morphological characters on a few specimens that were examined. Currently, the results from the morphological and phenetic analysis (cluster and canonical discriminant analyses) demonstrated that the population members of *M. vaginalis* s.l. in Thailand should not be a single species (Tungmunnithum *et al.,* 2016).

DNA sequencing and phylogenetic data of *M. vaginalis* were examined in this research using both cpDNA and nuclear DNA regions to determine the taxonomic status of this plant group. Fifty living *M. vaginalis* plants from 10 populations of *M. vaginalis* with three outgroups, *Pontederia cordata* var. *lancifolia* (Muhl.) Torr., *Eichhornia crassipes* (Martius) Solms, and *E. azurea* (Sw.) Kunth, were employed in this analysis.

2. Materials and Methods

2.1 Specimen collection

Plant specimens were collected from the previous records on herbarium specimens and all water bodies within Thailand. All of the water bodies were located using Google Earth. The target provinces were selected to cover all floristic regions of the country that included these regions and provinces: (1) Northern (Chiang Mai, Lampang, Nakhon Sawan, Nan, Phitsanulok; (2) North-eastern (Kalasin, Khon Kaen, Loei, Maha Sarakham, Mukdahan, Nong Bua Lam Phu, and Udon Thani); (3) Eastern (Chaiyaphum, Nakhon Ratchasima, Roi Et, and Si Sa Ket); (4) South-western (Kanchanaburi, Prachuap Khiri Khan, Phetchaburi, and Ratchaburi); (5) Central (Angthong, Bangkok, Chai Nat, Nakhon Nayok, Nakhon Pathom and Nonthaburi, Pathum Thani, Phra Nakhon Si Ayuthaya, Samut Prakan, Samut Songkhram, Sing Buri, and Suphan Buri); (6) South-eastern (Chonburi, Chachoengsao, and Prachin Buri); and (7) Peninsular (Chumphon, Nakhon Si Thammarat, Surat Thani, and Phatthalung). Each population was named according to the province where it was collected (Table 1). The collected plants were identified using the key-to-species and description in the existing Floras (Backer, 1951; Chayamarit, 2005; Guofang & Horn, 2000; Ridley, 1924; Yang, 1976). The herbarium abbreviations are used according to Thiers which is continuously updated.

Table 1. Ten populations of *M. vaginalis* s.l. in this analysis.

Population No.	Population names	Number of samples
1	Kanchanaburi	5
2	Chiang Mai	5
3	Ratchaburi	5
4	Suphan Buri	5
5	Angthong	5
6	Nan	5
7	Prachin Buri	5
8	Chachoengsao	5
9	Phatthalung	5
10	Chumphon	5
outgroup	*Pontederia cordata* var. *lancifolia*	1
outgroup	*Eichhornia crassipes*	1
outgroup	*Eichhornia azurea*	1

Note: The population names come from the provinces where they were collected.

2.2 DNA extraction, polymerase chain reaction amplification and sequencing

The leaves of 5 specimens per population from all of the *M. vaginalis* populations were used to extract the total genomic DNA. Furthermore, three closely related species of *Monochoria*, *Pontederia cordata* var. *lancifolia*, *Eichhornia crassipes*, and *E. azurea*, were analyzed as the outgroups (Table 1). DNA was extracted from the dry leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1992). The total genomic DNA of each sample was checked by gel electrophoresis.

Polymerase chain reaction (PCR) was conducted by iCycler (Bio-Rad, Hercules, CA, USA) using 5 primer pairs (Table 2). The amplifications were performed using Takara *Taq* polymerase (Takara, Otsu, Japan) and Ampdirect Plus buffer (Shimadzu, Kyoto, Japan). The components of the PCR mixture are shown in Table 3. Then, the PCR product from each DNA region was checked by gel electrophoresis before purification with ExoSAP-IT (USB Corp., Cleveland, OH, USA). The cycle sequencing was carried out with a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) using the same PCR primers

Table 2. PCR regions of amplification, annealing temperature, and PCR product size.

No.	Regions	Primer pairs	References	Annealing temperature (^{O}C)	PCR product size (bp)
1	*rpl32-trnL*	rpL32-F: 5'-CAGTTCCAAAA AAACGTACTT C-3'	(Shaw et al., 2007)	55.7	890
		trnL: 5'-CTGCTTCCTAAGAGCAGCGT-3'			
2	*Agt1*	PeF1: 5'-ACCGAACCATYTCATTCTTGATT-3'	(Naumann et al, 2011)	54.6	1570
		PeR1: 5'-ATCTTTCTWACTGTTGCYAA-3'			
3	*ndhJ-TabE*	ndhJ: 5'-ATGCCYGAAAGTTGGATAGG-3'	(Taberlet et al., 1991)	56.8	1200
		TabE: 5'-GGTTCAAGTCCCTCTATCCC-3'			
4	*ITS4-ITS5*	RITS4: 5'-TCCTCCGCTTATTGATATGC-3'	(White et al, 1990)	51.6	780
		FITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3'			
5	*trnL–trnF*	tmLF: 5'-AAAATCGTGAGGGTTCAAGTC-3'	(Shinozaki et al., 1986;	52.7	1150
		tmFR: 5'-GATTTGAACTGGTGACACGAG-3'	Burns et al., 2011)		

Table 3. Components of the PCR mixture for PCR amplification.

Reagent	Volume (µL)
Distilled water	3.95
Taq polymerase (5 units/µl)	0.05
SHIMAZU Ampdirect plus	5.00
DNA template (10 ng/µl)	1.00
Forward primer (10 µM)	2.50
Reverse primer (10 µM)	2.50
Total	15.00

shown in Table 2. The components of the mixture for DNA sequencing analysis are given in Table 4 and were purified by ethanol precipitation. Automated sequencing was carried out with an Applied Biosystems 3130xl Genetic Analyzer. The electropherograms were assembled using ATGC ver. 6 (GENETYX, Tokyo, Japan).

Table 4. Components of the PCR mixture for sequencing.

Reagent	Volume (µL)
Distilled water	6.30
Sequencing buffer	3.00
PCR product template (10 ng/µl)	3.00
Primer (10 µM)	2.40
BigDye premixed (included *Taq* polymerase)	0.30
Total	15.00

2.3 Phylogenetic trees construction

Following manual alignment, DNA sequencing using the auto-alignment option was performed using BioEdit (Hall, 1999). Phylogenetic analyses were conducted by the Bayesian approach using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and a maximum parsimony (MP) criterion using PAUP* version 4.0b10 (Swofford, 2002).

In the Bayesian phylogenetic analysis, the hierarchical likelihood ratio test implemented in MrModeltest 2.2 (Nylander, 2004) was used to estimate the appropriate evolutionary model of nucleotide substitutions. Based on the model selected, two separate runs of Metropolis coupled Markov chain Monte Carlo (MCMCMC) analyses were performed with a random starting tree and four chains (one cold and three hot). The MCMCMC was 10 million generations long, and the chain was sampled every one-hundredth generation from the cold chain. The first 2,500 sample trees (25% of 10,000 sample trees) were discarded as burn-in after confirming that the value of the average 15standard deviation of split frequency was less than 0.01. As a guide to convergence, the potential scale reduction factors were determined to be close to 1.0 for all parameters of the output. The 50% majority-rule consensus tree of all post-burn-in trees was generated using Tree ver. 1.3.1.

For the MP phylogenetic analysis indels, the insertion or deletion of bases in the DNA sequence was treated as missing data. The characters were treated as unordered, and character transformations were weighted equally. The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony search was performed with 200 replicates of random additions of sequences with ACCTRAN character optimization, tree bisection-reconnection (TBR) branch swapping, and the MULTREES and STEEPEST DESCENT options switched on. Statistical support for each clade was assessed based on bootstrap analysis (Felsenstein, 1985). A total of 10,000 replicates of heuristic searches with TBR branch swapping switched on and the MULTREES options switched off were performed to calculate bootstrap values.

3. Results and Discussion

The Bayesian and a maximum parsimony analysis of *M. vaginalis* (Figure 1) using 5 regions from both 3 cpDNA and 2 nuclear DNA regions revealed 2 clades in the ingroup. The upper clade contained 6 populations (populations 1−6 in Table 1) which were recognized as *M. vaginalis* in morphological and phenetic works (Tungmunnithum *et al.*, 2016). The second one consisted of 4 populations (populations 7−10 in Table 1) which were proposed as a cryptic species of *M. vaginalis* (Tungmunnithum *et al.*, 2016).

Both of the two clades were separated at the maximum value (i.e., 1.00 of Bayesian posterior probabilities and 100% bootstrap percentages) (Figure 1). According to the Flora of Thailand (Chayamarit, 2005), the 10 populations were recognized as *M. vaginalis* (Burm.f.) C. Presl ex Kunth, a monotypic species. Conversely, the molecular analysis indicated that the two clades should be divided into 2 different species. Interestingly, the molecular results suggested that *M. vaginalis* in Thailand should be recognized as *M. vaginalis*

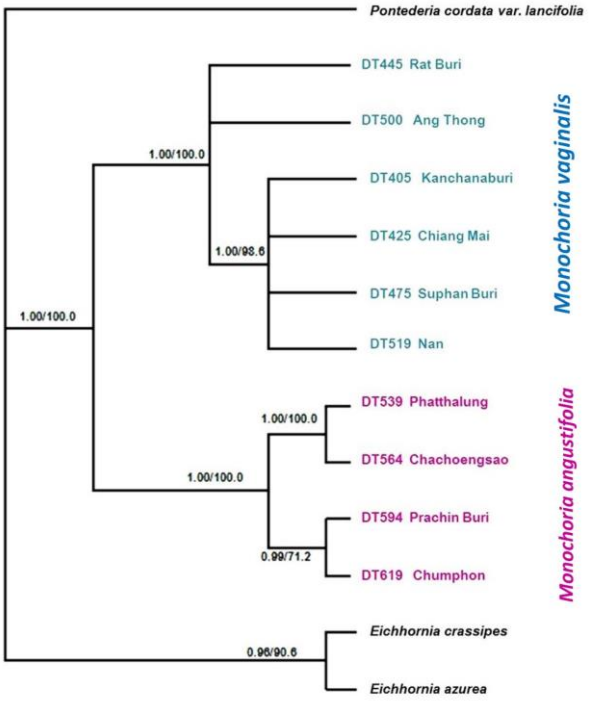

Figure 1. The Bayesian 50% majority rule consensus tree of 10 *M. vaginalis* s.l. populations. The topology of the maximum parsimony strict consensus tree was compatible with the Bayesian tree. Numerals above branches indicate Bayesian posterior probabilities (left) from Bayesian analysis and bootstrap percentages (right) from the maximum parsimony analysis.

and a closely related species. It is clearly seen that phylogenetic analysis using both cpDNA and nuclear DNA regions are helpful for classification of *M. vaginalis* populations. This approach is also beneficial for the classification of many plant groups, such as providing better classification of *Helwingia japonica* subsp. *japonica* at the variety level (Umemoto, Nakamura, Maeda, Yokota, & Kokubugata, 2014), *Sedum* populations (Crassulaceae) in Japan, Taiwan, and the Philippines (Ito *et al.*, 2014) and clarifying the taxonomic status of the *Portulaca okinawensis*'s populations (Portula caceae) in Japan (Kokubugata *et al.*, 2013).

Furthermore, the holotypes of *M. vaginalis* (Burm. f.) C.Presl ex Kunth var. *angustifolia* G.X.Wang (Maha Sarakham [Koksung, 18 September 1984, *Fukuoka 36166* (KYO!)]) and all the specimens examined including Bangkok (Bang Khen, 14 November 1965, *Tagawa and Iwatsuki T280* [KYO, BKF]), Ubon Ratchathani (between Nong Khon and Nam Yeun, 11 October 1984, *Murata et al. T-52165* [KYO]), and Si Sa Ket (Si Sa Ket City, 8 October 1984, *Murata et al. T-49700* [KYO, BKF]) of this new variety in Japan and Thailand were also intensely investigated. However, Chinese researchers separated *M. vaginalis* into two varieties, *M. vaginalis* (Burm.f.) C. Presl ex Kunth var. *vaginalis* and var. *angustifolia* G.X.Wang, by studying 4 herbarium specimens and using basal lobe length, average fruit weight, seed number per fruit, seed weight per fruit, leaf shape, and size as the diagnostic characters (Wang *et al.*, 2003, 2004). However, almost all the diagnostic characters which were used are quantitative characters without any statistical test. These characters may not be sufficiently suitable to classify the new taxa because most of them have a high probability to be affected by the environment (Backer, 1951; Hill, 1988; Tungmunnithum, Kidyoo, & Khunwasi, 2011). Moreover, the authors described the new variation using a few specimens and only four herbarium specimens were examined. Furthermore, all of the four specimens, including the holotypes, were collected from Thailand.

It was found that the characters of *M. vaginalis* var. *angustifolia* G.X. Wang were similar to a cryptic species of *M. vaginalis* in this research. Nevertheless, the populations 7−10 listed in Table 1 from this cryptic species exhibited greater variations than the reported new variety in leaf apex and base, leaf length and width, perianth characters, seed shape and the number of longitudinal ridges on seed surface. Thus, this variety should be placed as a sub-set of this new cryptic species.

3.1 Taxonomic treatment

Monochoria angustifolia (G.X. Wang) Boonkerd & Tungmunnithum stat. nov. (Figure 2)

Basionym: *Monochoria vaginalis* var. *angustifolia* G. X. Wang.

Monochoria angustifolia is distinguished from *M. vaginalis* by lax flowers of its inflorescence, barrel-shaped seeds, distinct longitudinal ridges on seed surface and obtuse leaf base.

Type: THAILAND. Maha Sarakham [Koksung, 18 September 1984, *Fukuoka 36166* ***** (holotype KYO!, BKF!, L!)].

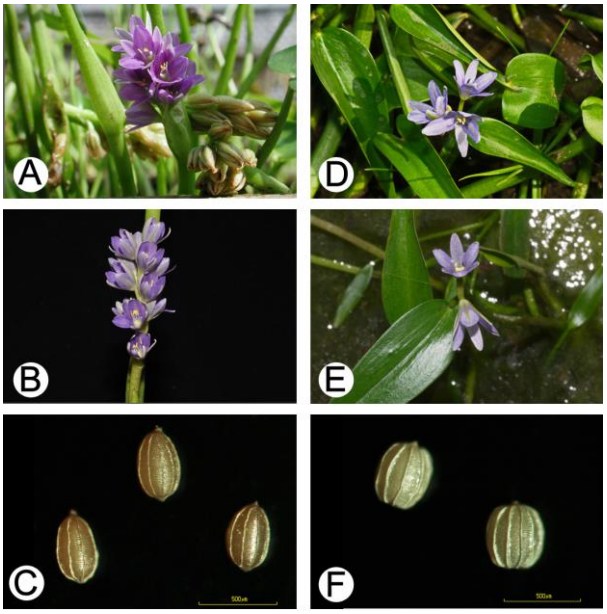

Figure 2. Vegetative and reproductive structures of the morphology of *M. vaginalis* (A−C) and *M. angustifolia* (D−F): A−B inflorescence; C oval-shaped seeds and obscure longitudinal ridges (Bar=500 µm), D−E inflorescence; F barrel-shaped seeds and distinct longitudinal ridges (Bar=500 µm).

Leaves stipulate, simple with broad leaf sheath, glabrous; *petiole* curved or erect, groove along the length of petioles absent, leaf blade and its petiole forming acute or right angle to each other; *leaf blade* lanceolate-linear, lanceolate or ovate-lanceolate, base obtuse, apex abruptly-acuminate, 6.0−7.4 cm long, 1.4−2.0 cm wide, midrib on the adaxial surface groove, abaxial surface smooth. *Inflorescence* racemose, bearing 2−6 flowers, lax; *flowering stem* 10.4−11.0 cm long, smooth; *peduncle* 2.8−3.0 cm long; *rachis* 5.2−5.4 cm long; *spathe* 2.6−2.8 cm long, terminal appendage 0.3 cm long; *floral leaf blade* lanceolate, midrib groove, adaxial and abaxial surfaces smooth, apex acute, base obtuse, 6.3−6.7 cm long, 1.7−1.8 cm wide; *floral leaf petiole* 4.1−4.5 cm long, level of inflorescence tip higher than floral leaf tip and mature leaf tip; *pedicel* green, glabrous, 1.3−1.4 cm long; *outer perianth* 3, glabrous, lanceolate, purple, middle of abaxial outer perianth green, 0.7−0.8 cm long, 0.3 cm wide; *inner perianth* 3, glabrous, ovate or elliptic, apex obtuse, purple, middle of abaxial inner perianth green, 0.8−0.9 cm long, 0.3 cm wide; *largest stamen* 1, filament dark purple with appendage, 0.2 cm long, anther basifixed, dark purple, 0.4−0.5 cm long, 0.1 cm wide; *normal stamen* 5, filament white without appendage, 0.7−0.8 cm long, anther basifixed, yellow, 0.4−0.5 cm long; *ovary* superior, style light purple, 0.4−0.5 cm long. *Fruits* capsule, glabrous. *Seeds* numerous, barrel, 373−429 µm long, longitudinal ridges distinct, 7−10 veins.

Recent distribution: Peninsular Thailand and South-eastern Thailand at 100−400 m elevation.

Ecology: Rice field, flowering from early April to May and from early July to August

Specimens examined: NORTH-EASTERN: Nong Khai [Se Ka, 28 August 2001, *Pooma et al. 2874* (BKF); Se Ka, 28 August 2001, *Pooma et al. 2891* (BKF)]; Maha Sarakham [Koksung, 18 September 1984, *Fukuoka 36166* * (holotype KYO!, BKF!, L!)]; Mukdahan [Dong Luang, 24 August 2001, *Pooma et al. 2471* (BKF)]; EASTERN: Chaiyaphum [Dat Don, *Larsen et al. 31774* (KYO!, AAU)]; Nakhon Ratchasima [Ban Chum Saeng, 24 May 1929, *Hoe 243* (BK)]; Buri Ram [22 November 1976 *Phengklai et al. 3364* (BKF); Huai Thalaeng, 20 December 2005, *Pooma et al. 5942* (BKF)]; Si Sa Ket [Si Sa Ket City, 8 October 1984, *Murata et al. T-49700****** (KYO, BKF)]; Ubon Ratchathani [between Nong Khon and Nam Yeun, 11 October 1984, *Murata et al. T-52165****** (KYO); Koodkhaopoon, 26 September 2003, *Wongprasert 039* (BKF)]; CENTRAL: Bangkok [Bang Khen, 14 November 1965, *Tagawa and Iwatsuki T 280****** (KYO, BKF)]; SOUTH-EASTERN: Prachin Buri [Muang, 17 August 2013, *Tungmunnithum 594, 599, 618* (BCU); 5 August 1920, *Phengklai et al.* 3702 (BKF)]; Chachoengsao [Bang Khla, 16 August 2013, *Tungmunnithum 564, 583*, 593 (BCU)]; Chon Buri [Sattahip, 4 December 1972, *Maxwell 600* (BK)]; Chanthaburi [Makham, 20 August 1997, *Boonma 175* (BKF); Muang, 17 October 1971, *Maxwell 553* (BK)]; Trat [Ko Chang, 22 February 1955, *Smitinand 2266* (BKF)]; PENINSULAR: Chumphon [Muang, 31 May 2013, *Tungmunnithum 619, 623, 648* (BCU)]; Ranong [Ban Kam Phuran, 7 December 1979, *Shimizu et al. 26330* (KYO)]; Phuket [Ban Bo Han, 8 October 1970, *Charoenphol et al. 3422* (BKF)]; Krabi [Klongtom, 29 November 1986, *Maxwell 991* (BKF)]; Phatthalung [Khuan Khanun, 31 May 2013, *Tungmunnithum 539, 553, 563* (BCU); Thale Noi, *Larsen 625* (BKF)]; Songkhla [Had Yai, 24 April 1985, *Maxwell 553* (BKF)]; Narathiwat [Tak Bai, 17 February 1988, *Niyomdham 1698* (KYO, BKF)].

Note: *Specimens examined of *M. vaginalis* var. *angustifolia* are recognized as synonym of *M. angustifolia* in this recent study.

3.2 Key to species of the genus *Monochoria* in Thailand

1. Indeterminate inflorescence
    2) Leaf base cordate. Raceme bearing 9−23 flowers, dense. Seed oval, longitudinal ridges obscure ..…… *M. vaginalis*
    2) Leaf base obtuse. Raceme bearing 2−6 flowers, lax. Seed barrel, longitudinal ridges distinct ……..... *M. angustifolia*
1. Determinate inflorescence
    3) Inner perianth obovate, Seed oval, longitudinal ridges obscure …….…….. *M. hastata*
    3) Inner perianth elliptic, Seed barrel, longitudinal ridges distinct …….... *M. elata*

4. Conclusions

According to the morphological and phenetic evidence from previous work and molecular evidence from this study, it became obvious that *M. vaginalis* s.l. should be distinguished as *M. vaginalis* and a new species as *M. angustifolia*. Moreover, the morphological, phenetic, and molecular investigations were also useful to determine the taxonomic status of this plant group.

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