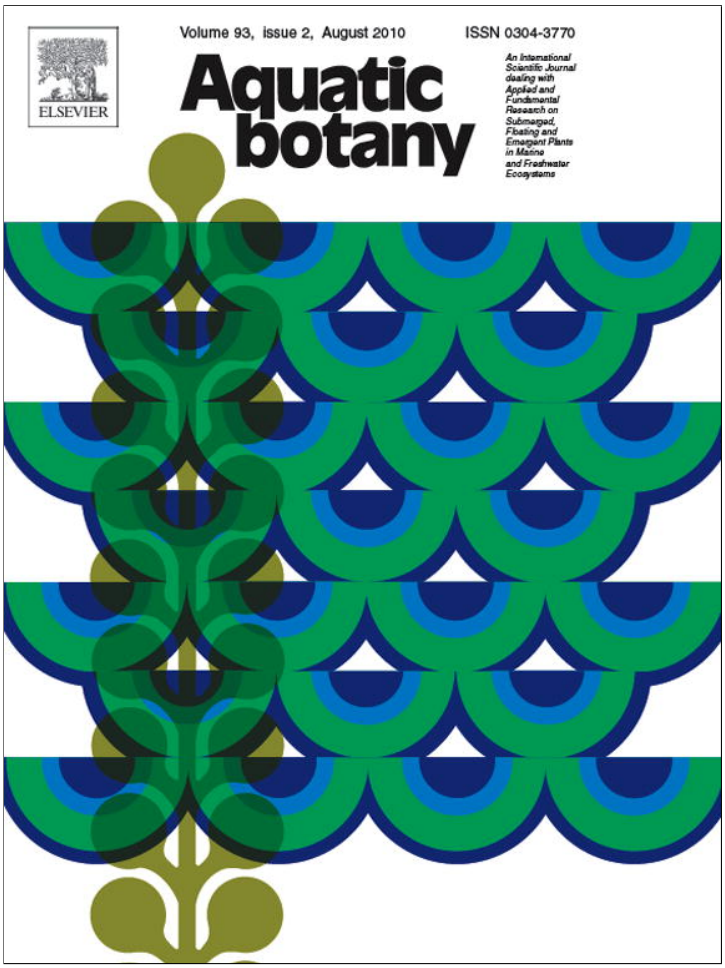

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Short communication

Molecular phylogenetics and taxonomic reassessment of four Indian representatives of the genus *Nymphaea*

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ABSTRACT

Because the classification of *Nymphaea* in India has been reported to be confusing, molecular taxonomic revision of four Indian representatives of the genus namely *N. nouchali*, *N. pubescens*, *N. rubra* and *N. tetragona* based on ITS, *trnK* intron and *matK* gene is presented and discussed. Molecular evidence provided here is in disagreement about the taxonomic identity of one specimen of *N. nouchali* and indicated a probable misidentification of *N. tetragona*. Interestingly, sequence analysis revealed lack of or low sequence divergence between *N. pubescens* and *N. rubra*. Phylogenetic relationship among members of *Nymphaea* subg. *Lotos*, represented by all known species viz. *N. lotus*, *N. petersiana*, *N. pubescens* and *N. rubra* was also conducted. Maximum parsimony analysis of the combined data matrix depicted two clades with *N. petersiana* and *N. lotus* forming one, *N. pubescens* and *N. rubra* representing the other. Bayesian inference showed *N. petersiana* as first branching, followed by *N. lotus* with *N. pubescens* and *N. rubra* emerging as a separate clade. The results indicated no close association between *N. petersiana* and *N. nouchali*, thereby, contradicting the morphology-based treatment of placing *N. petersiana* in synonymy under *N. capensis* and *N. nouchali*, respectively.

1. Introduction

Nymphaea is the most diverse genus in the order Nymphaeales and is well represented globally. The genus, comprising 45–50 species, has been classified into five subgenera viz. *Anecphya*, *Brachyceras*, *Hydrocallis*, *Lotos* and *Nymphaea* with each subgenus exhibiting distinct distribution. In India, ten species of *Nymphaea*, both wild (*N. alba*, *N. candida*, *N. nouchali*, *N. pubescens*, *N. rubra* and *N. tetragona*) and cultivated (*N. caerulea*, *N.* × *marliacea*, *N. micrantha* and *N. alba* var. *rubra*), are reported (Mitra, 1990). *N. alba* and *N. candida* are restricted to the state of Jammu and Kashmir whereas *N. tetragona* is confined to the state of Meghalaya. However, the classification of *Nymphaea* in India has been reported to be confusing with some names inaccurately used (Cook, 1996).

Borsch et al. (2007) conducted the first molecular phylogenetic analysis of *Nymphaea* based on chloroplast *trnT-trnF* region. In their study, three well-supported major lineages within *Nymphaea* are resolved with subg. *Nymphaea* emerging as the first branch followed by a clade comprising subg. *Hydrocallis* and subg. *Lotos*, and another clade comprising subg. *Anecphya* and *Brachyceras*. *Nymphaea* subg. *Lotos* is the smallest among all five subgenera of *Nymphaea*, consisting of four species namely *N. pubescens*, *N. lotus*, *N. rubra* and the recently added *N. petersiana*. The *trnT-trnF* based study of Borsch et al. (2007) depicts *N. pubescens* as sister to *N. lotus* and provides a clear distinction between these two species. The inclusion of *N. petersiana*, earlier treated in synonymy under *N. capensis* and *N. nouchali* (Conard, 1905; Verdcourt, 1989), revealed a close relationship between this taxon and members of subg. *Lotos*. However, genetic relatedness among these species is yet to be investigated. Furthermore, *N. rubra* was not included in any of the previous studies and its relationship with other members of the group is still unclear.

The main aim of the study is to reassess the taxonomical identity of four Indian wild species namely *N. nouchali*, *N. pubescens*, *N. rubra* and *N. tetragona* by adopting a phylogenetic-based approach. We also intend to establish the interspecific relationship among members of *N.* subg. *Lotos* and confirm the close association between *N. petersiana* and this subgenus with the inclusion of *N. nouchali*.

2. Materials and methods

2.1. Plant material and taxon sampling

Exploratory trips were conducted to survey and collect plants of the genus *Nymphaea* from the states of Meghalaya and Assam (North-East India). Out of ten species reported from India, seven

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Table 1
Specimen voucher, place of collection and GenBank accession numbers of deposited sequences of all the four Indian *Nymphaea* species investigated. Sequence data retrieved from GenBank are also listed.

Species	Specimen voucher[a]	Place of collection	GenBank Accession no.	
			trnK and *matK*	ITS
N. nouchali Burm.f.	JD 02	Guwahati, Kamrup District, Assam	FJ597752	FJ597740
N. nouchali Burm.f.	JD 06	Paikan, Goalpara District, Assam	FJ597751	FJ597742
N. pubescens Willd.	JD 09	Guwahati, Kamrup District, Assam	FJ597753	FJ597743
N. rubra Roxb. ex Andrews	JD 10	Nongpoh, Ri-Bhoi District, Meghalaya	FJ597754	FJ597744
N. tetragona Georgi	JD 01	Nongkrem, East Khasi Hills District, Meghalaya	FJ597755	FJ597745
N. tetragona Georgi			NA	EU428056
N. tetragona Georgi			NA	AY707899
N. amazonum Mart. & Zucc.			DQ185543	FM242149
N. jamesoniana Planch.			DQ185544	FM242152
N. lotus Linn.			DQ185547	FM242153
N. petersiana Klotzsch			DQ185548	FM242156
Nuphar advena (Aiton) W.T. Aiton			DQ185531	FM242145

[a] Specimen vouchers deposited at the Herbarium, Department of Botany, North Eastern Hill University, Shillong. NA: not available.

Nymphaea species viz. *N. alba* var. *rubra*, *N. caerulea*, *N.* × *marliacea*, *N. nouchali*, *N. pubescens*, *N. rubra* and *N. tetragona* were found. Two morphologically distinct plants of *N. nouchali* (*N. nouchali* JD 02 and *N. nouchali* JD 06) found at two different locations: Paikan, Goalpara District, Assam (26°02′N–90°38′E) and Guwahati, Kamrup District, Assam (26°10′N–91°46′E) were identified. The seven *Nymphaea* species were identified at the Botanical Survey of India, Eastern Circle, Shillong. Although all the molecular sequence data (ITS, *matK* and *trnK*) are available for the seven species, we intend to include only four natural species of the genus *Nymphaea*, i.e. *N. nouchali*, *N. pubescens*, *N. rubra* and *N. tetragona* in the present study, excluding the cultivated forms. Information pertaining to the four *Nymphaea* species is summarized in Table 1. To meet the objectives of the study, we have retrieved sequence data of relevant species from GenBank (Table 1).

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was isolated from fresh leaves after thorough cleansing in running tap water. The Doyle and Doyle (1987) method of DNA extraction was used with the addition of the saturated phenol extraction step prior to ethanol precipitation. Polymerase chain reaction was used to amplify the *trnK* intron (including the entire *matK* gene) and ITS region. Universal PCR primers trnK3914F and trnK2R of Johnson and Soltis (1995) were used to amplify the *trnK* region; sequencing was done utilizing these and other internal primers (NytrnKJD689-R 5′-GGGGAGGATT TCTTGGGTTA-3′; NymatKJD1853-F 5′-CCTCTGATTGGATCGTTGGT-3′; NymatKJD1995-R 5′-CACCCGAATCGAGCAATAAT-3′; NytrnKJD525-F 5′-TCGGGTTGCAAAAATAAAGG-3′). The PCR primers ITS 4 and ITS 5 of White et al. (1990) were used to amplify the ITS region (ITS 1, 5.8S, ITS 2) utilizing these same primers for sequencing. DNA amplification was performed in an Applied Biosystems Gene Amp® PCR System 2700. Amplified PCR products were purified using QIAQuick gel extraction kit (QIAGEN, Germany) and sequenced at Bangalore Genei, India and Axygen Scientific Pvt. Ltd., India.

2.3. Sequence alignment and indel coding

The boundaries of the ITS region, *matK* gene and *trnK* intron for all four species of *Nymphaea* were determined by comparison with published sequences (Goremykin et al., 2004; Woods et al., 2005; Löhne et al., 2007). Sequences, thus obtained, including those retrieved from GenBank were subjected to multiple sequence alignment using Clustal X program (Thompson et al., 1997) with default settings. A separate alignment matrix for each genomic region was produced. Clustal X generated alignments were further re-aligned manually. Gaps were included into analysis and coded automatically in a binary matrix using SeqState v.1.21 (Müller, 2005) applying the simple indel coding strategy (Simmons and Ochoterena, 2000). Alignments of all genomic regions were then combined to a single Phylip/nexus file comprising several data partitions.

In addition, genetic closeness between the generated ITS sequence of *N. tetragona* and those retrieved from GenBank (Accession no. AY707899, presumed to be a specimen from China; Accession no. EU428056, a specimen from Russia) was evaluated.

2.4. Phylogenetic analyses

Prior to phylogenetic analyses, sequence characteristics of all genomic regions were calculated using MEGA version 4 (Tamura et al., 2007).

Maximum parsimony (MP) method was used to analyze the aligned sequence data matrix. MP trees were constructed using Phylip (Felsentein, 1989). Bootstrap analysis was carried out with 999 random seed and 1000 replicates to examine the relative level of support for individual clades on the cladograms of each search. Several MP analyses were conducted to compare nodal support provided by separate and combined dataset.

Besides MP analysis, Bayesian inference (BI) of phylogeny was conducted for the combined dataset using MRBAYES v.3.1.2 (Ronquist and Huelsenbeck, 2003). BI analyses were performed for 1,000,000 generations applying the default settings (MCMC, two runs with four chains each, heating temperature 0.2, saving one tree every 100 generations). The best model of molecular evolution for each datasets was determined using jModelTest 0.1 (Posada, 2008). The GTR model of molecular evolution with gamma-distributed rate variation across sites was assigned to the ITS and *trnK* data, respectively, whereas *matK* was assigned the GTR model. The binary (restriction site) model was applied to the indel partition. All trees were viewed with the program Tree View 1.5 (Page, 1996).

2.5. Outgroup selection

The morphology-based classification of *N. petersiana* has received unconvincing treatments. Conard (1905) and Verdcourt (1989) has placed this taxon in synonymy under *N. capensis* and *N. nouchali* of subg. *Brachyceras*, respectively. In view of these treatments, we have included *N. nouchali* of subg. *Brachyceras*. *Nymphaea amazonum* and *N. jamesoniana* of subg. *Hydrocallis* have been included as ingroup members. *Nuphar advena*, reported as a

first branch of family Nymphaeaceae (Les et al., 1999), has been chosen as outgroup.

3. Results

The nucleotide sequences for all the genomic regions of the four species of *Nymphaea* have been submitted to the GenBank databases (www.ncbi.nlm.nih.gov) and can be accessed under the Accession nos. FJ597740, FJ597742–FJ597745, FJ597751–FJ597755.

3.1. Sequence alignment and statistics

Sequence alignment for elucidating the relationship among members of *N.* subg. *Lotos* produced 707, 1530 and 1045 characters for ITS, *matK* and *trnK*, respectively. Among all the molecular markers, the *matK* gene is the most conserved as is evident from the number of conserved sites (1460 out of 1530, 95.42%) shown in Table 2. The ITS region, as is reported earlier (Borsch et al., 2008), is highly variable (29.70%) and records the highest percentage (17.82%) of parsimony informative sites. Unlike ITS, *matK* and *trnK* recorded more number of autapomorphic sites as compared to synapomorphic sites. All markers recorded frequent transitions rather than transversions. The highest number of indels was recorded in the ITS region (81) followed by *trnK* (18) and *matK* (3). The *matK* and *trnK* intron recorded similar average number of base substitution per site whereas the ITS region recorded the highest. The ITS region showed the highest GC content (53.2%) and a rather similar GC percentage of 36.4 and 37.0 was recorded for *matK* and *trnK*, respectively.

Comparison of the genetic relatedness among the ITS sequences of *N. tetragona* revealed a close relationship between the Indian and the Chinese material with 97.89% sequence homology. Sequence homology of 96.54% was recorded between the Russian and the Indian material.

3.2. Maximum parsimony analysis

MP analysis of individual datasets yielded one most parsimonious tree for ITS (677 steps) and *trnK* (149 steps), respectively. *MatK* resulted in two equally parsimonious trees of 93 steps. A strict consensus tree was constructed that matched one of the two most parsimonious trees. All trees showed close association between *N. petersiana* and other members of *N.* subg. *Lotos*. Both trees of the chloroplast markers depict *N. petersiana* as first branching with *N. lotus* emerging as sister to a clade comprising of *N. pubescens* and *N. rubra*. However, the ITS tree could resolve *N.* subg. *Lotos* into two clades: one clade comprises *N. petersiana* and *N. lotus*, and a second clade comprises *N. pubescens* and *N. rubra*. The combined data matrix resulted in one most parsimonious tree (927 steps) similar in topology as that revealed by the nrDNA ITS marker (Fig. 1). The inclusion of *N. nouchali* did not show any closeness to *N. petersiana*.

3.3. Bayesian inference

The Bayesian inference of phylogeny resulted in a tree topology depicting *N. petersiana* as sister to all members of *N.* subg. *Lotos* (Fig. 2). The exclusion of gaps from analysis did not alter the tree topology.

4. Discussion

4.1. Taxonomic revision

The present study vindicated Cook's remarks for at least one individual of *N. nouchali* among all the species investigated. The

Table 2
Comparison of sequence characteristics for all genomic regions calculated using MEGA 4.

Genomic Region	Character	Number of								Avg no. base substitutions per sites (d)	GC (%)
		Conserved sites (%)	Variable sites (%)	Parsimony informative sites (%)	Autapomorphic Sites	Synapomorphic Sites	Transitions	Transversions	Multiple subsites	Indels		
matK	1530	1460 (95.42)	68 (4.44)	21 (1.37)	48	20	38	30	0	3	0.0125	36.4
trnK	1045	906 (86.70)	46 (4.40)	19 (1.82)	26	17	25	18	3	18	0.0153	37.0
ITS	707	289 (40.88)	210 (29.70)	126 (17.82)	68	80	104	44	62	81	0.2207	53.2

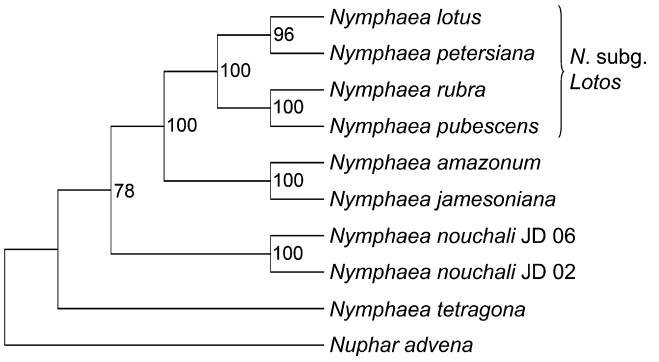

Fig. 1. Single most parsimonious tree (927 steps) obtained from maximum parsimony analysis of combined dataset (ITS, *matK* and *trnK*). Numbers at nodes indicate bootstrap values.

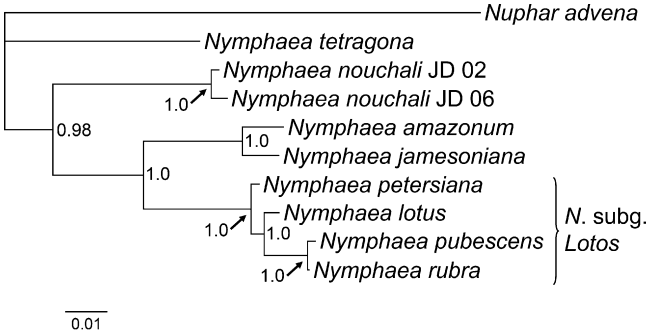

Fig. 2. 50% majority-rule consensus of 3 trees obtained from four runs of Bayesian analysis of combined dataset (substitution + indels) implementing the GTR + G model for the ITS and *trnK* dataset and the GTR model for *matK*. Posterior probabilities are given at the nodes. Branch lengths represent the number of changes per site.

name *N. nouchali* was initially applied to two specimens' viz. *N. nouchali* JD 02 and *N. nouchali* JD 06. The two specimens are morphological dissimilar and showed variability in texture (thick/thin), mottling and veins (impressed/raised) of the leaf, and number of petals and stamens. In the present study, sequence analysis of *matK* and *trnK* altogether revealed 7 substitutions among the two samples and a repeat motif GGGC was found in the *trnK* sequence of *N. nouchali* JD 06. Analysis of complete ITS sequences recorded 5 substitutions between them and 2 deletions (CAA and A) in *N. nouchali* JD 06. Such variations suggest distinctness between the two specimens and a probable identity of *N. nouchali* JD 02 as *N. capensis* (nucleotide BLAST of the ITS region of *N. nouchali* JD 02 showed 99.13% sequence identity with *N. capensis*, Accession no. AY707898). However, *N. capensis* has never been reported from India.

The taxonomic identity of *N. rubra* has been problematic with De Candolle (1821, 1824), Wight and Walker-Arnott (1834), Prain (1903, 1905) and Conard (1905) treating it as a distinct species. Hooker and Thompson (1855), however, considered *N. rubra* in synonymy under *N. lotus*. Because of its failure to set fruits/seeds in nature, Mitra and Subramanyam (1982) questioned the treatment of *N. rubra* as a taxonomic species at par with other sexually reproducing species. Molecular evidence provided here suggests that *N. rubra* is a natural hybrid with *N. pubescens* as the maternal parent. Molecular cloning techniques of the ITS region are being carried out to confirm this assumption and also identify the other putative parental species.

The identity of *N. tetragona* is however ambiguous. Hooker (1882) treated this species as *N. pygmaea*, so did Chauhan (1983) whose attempt to locate the species proved unsuccessful. The origin of *N. pygmaea*, although given by Aiton (1811), is attributed to Salisbury (1807) who referred to it as *Castalia pygmaea* based on two cultivations studied, one in China and the other at Kew, England. Caspary (1866), however, treated *N. pygmaea* in synonymy under *N. tetragona* and has since been adopted. Salisbury's description of *C. pygmaea* matches *N. tetragona* in several aspects except his depiction of the receptacle as slightly quadrangular and the stigma as yellow colored (Wiersema, 1996). Wiersema's (1996) specimens of *N. tetragona* showed purplish stigma. Morphological observation of *N. tetragona* found in India indicated a deviation from what was reported by Wiersema (1996). The studied samples showed yellow colored stigma matching Salisbury's account. Could our specimen be *N. pygmaea*? Comparison of the genetic relatedness between the generated ITS sequences of *N. tetragona* and those found in the GenBank database revealed close association with specimen from China rather than from Russia. Moreover, morphological similarities shared between the Indian and the Chinese materials are evident as was reported in the Flora of China (eFloras, 2008), indicating that *N. tetragona* from China have flowers with yellow stigma and slightly tetragonous receptacle. To confirm this further investigation and comparisons with *N. tetragona* from China may be envisaged.

4.2. Relationship among members of N. subg. Lotos

Prior molecular phylogenetic study based on the chloroplast *trnT-trnF* region depicted *N. pubescens* as sister to *N. lotus* (*N. rubra* was not included) and provided clear distinction between these two species (Borsch et al., 2007). The present investigation based on the nuclear ITS region and two chloroplast markers, *matK* gene and *trnK* intron, showed close association between *N. pubescens* and *N. rubra*. Mitra (1990) did consider *N. rubra* as being very similar to *N. pubescens* differing only in color of leaves and flowers and in being completely sterile. Interestingly, sequence analysis of *matK* and *trnK* did not reveal any nucleotide substitution between *N. pubescens* and *N. rubra*. Also, additional signals were depicted in the sequencing chromatogram of the ITS region of *N. rubra*, hinting at the presence of divergent ITS paralogues. Such deviating paralogues of the ITS region have been reported in several species of *Nymphaea* (Löhne et al., 2008). The lack of or low sequence divergence between *N. pubescens* and *N. rubra* might have been influenced by recent hybridization and introgression. Such events have been reported between two species of *Callicarpa* (Tsukaya et al., 2003), *Rheum* species (Wang et al., 2005), *Vasconcellea* species (Van Droogenbroeck et al., 2006), and between *Silene latifolia* and *Silene dioica* (Minder et al., 2007).

Morphological characteristics of *N. petersiana* revealed similarities in leaf margins and veins to those of subg. *Lotos* (Borsch et al., 2007) but it strongly resembles *N.* subg. *Brachyceras* in terms of floral morphology (Mendonca, 1960). This was even commented by Conard (1905) who treated *N. petersiana* as synonymous to *N. capensis* of subg. *Brachyceras*. Another morphology-based treatment of *N. petersiana* placed this taxon in synonymy under *N. nouchali* of subg. *Brachyceras* (Verdcourt, 1989). Our study did not reveal any association between *N. petersiana* and *N. nouchali* but rather substantiated the earlier findings that depicted *N. petersiana* as sister to all members of *N.* subg. *Lotos* (Borsch et al., 2007; Löhne et al., 2007). Although, the position of *N. petersiana* within the subgenus is ambiguous (as revealed in both MP and BI trees), its closeness to *N. lotus* is indicated.

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