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Nymphaea alba var. *rubra* is a hybrid of *N. alba* and *N. odorata* as evidenced by molecular analysis

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Sequencing signals of the biparentally inherited ITS marker and sequence matching of the chloroplast *trn*K intron, *mat*K and *rbc*L gene of an Indian plant identified as *Nymphaea alba* var. *rubra* contradict its identity. Additional signals depicted in chromatograms of the ITS region and the exact match of the maternally inherited chloroplast DNA sequences suggest that the Indian material is a hybrid of *N. alba* and *N. odorata*. Molecular cloning techniques resulted in the isolation of ITS alleles from the putative hybrid with DNA sequences not exclusive of either of the parental species. Such allelic DNA sequences confirmed that hybridization between *N. alba* and *N. odorata* has occurred. Our DNA sequence analyses indicate this plant is an interspecific hybrid involving *N. odorata* as the maternal parent, and *N. alba* as the paternal parent.

Introduction

Anomalies in the identification of waterlily hybrids and cultivars have instigated the International Waterlily and Water Gardening Society (www.iwgs.org) to initiate a programme directed at providing a complete account of cultivated *Nymphaea*. However, the difficulty that exists in conclusively documenting waterlily hybrids and the reluctance of hybridizers to divulge explicit data on the methodology and parentages (Conard 1905, Swindells 1983, Les & Philbrick 1993) would be a setback in the successful completion of this project. In certain cases, some plant hybridizers provide precise documented manipulations relying exclusively on morphological features in determining parental characteristics in suspected hybrid plants (e.g. Pring 1934a, 1934b). More recently, plant researchers (Moody & Les 2002, Les *et al*. 2004) presented explicit documentation of hybrids using molecular markers. The approach of identifying hybrids morphologically is troublesome because hybrids can exhibit features that are purely a parental mixture, intermediary or novel (Conard 1905, Rieseberg 1995, Les *et al*. 2004). However, with the advent of genetic markers, it has become feasible for demonstrating genetic contributions of different parental genotypes in suspected hybrid plants. Co-existence of the nrDNA repeats from both parents in the putative hybrids provides useful means of identifying parental species (Peterson *et al*. 2004, Saito *et al*. 2007), whereas chloroplast DNA can assist in recognizing the direction of hybridization (Albarouki & Peterson 2007). Evidence of hybridization employing both nuclear and chloroplast markers have been reported in species of *Nuphar* (Padgett *et al*.

1998, Padgett *et al*. 2002), *Carthamus* (Vilatersana *et al*. 2007), *Crataegus* (Albarouki & Peterson 2007), *Doellingeria* (Saito *et al*. 2007), *Paeonia* (Pan *et al*. 2007), *Dubautia* (Friar *et al*. 2008) and *Gagea* (Peterson *et al*. 2009). In addition, a waterlily hybrid *Nymphaea* 'William Phillips', produced for the first time from parents representing two different subgenera of *Nymphaea*, was documented using nrDNA ITS and chloroplast *trn*K 5´ intron (Les *et al*. 2004). The approach of combining nuclear and chloroplast markers for documenting hybrids is valuable because it validates the presence of different alleles (coming from each parent) and identifies the maternal parent (Les *et al*. 2004).

"*Nymphaea alba* var. *rubra*" represents one of the cultivated species reported from India for Ward's Lake, Shillong, Meghalaya and elsewhere (Mitra 1990). Our attempts to locate the plant in states other than Meghalaya have failed. Moreover, a study conducted by Ansari *et al*. (2005) on the pollen morphology of the *Nymphaea* species found in India includes this taxon, collected from Ward's Lake. The origin of Ward's Lake dates back to the 1860s when it was known as the Hopkinson Tank, and further beautification (enlargement and planting of trees and flowers) of the Lake was done during 1892–1893 by Sir William E. Ward, hence its name (Hussain 2005). So, "*N. alba* var. *rubra*" could have been introduced in Ward's Lake during this era thereby coinciding with the period (1885–1890) when Joseph Bory Latour-Marliac (1830–1911) produced some of his magnificent hybrids, a few decades after the discovery of 'The Swedish Red Waterlily' from central Sweden. In this regard, the present paper does not warrant a comparison between the Indian and the Swedish variety because it is quite possible that the Indian material studied may represent a taxon entirely distinct from the actual *N. alba* var. *rubra*. Moreover, the Indian representative of *N. alba* var. *rubra* does not set seed, circumstantial evidence suggesting that it could be a hybrid. However, the similarities shared between the Indian and the Swedish waterlily are evident. The morphological resemblance could have convinced Mitra (1990) to identify the plant as *N. alba* var. *rubra*. But without authentic material of *N. alba* var. *rubra* available for evaluation, molecular observations made from the present study are not applicable to the Swedish red waterlily. The present investigation aims at providing molecular authentication of an Indian taxon, identified as *N. alba* var. *rubra*, employing an approach based on direct sequencing and cloning of the ITS region followed by sequence matching of three chloroplast markers. To avoid confusion, it may be mentioned here that the present material investigated does not represent *N. rubra*, another cultivated taxon found in India that does not develop seeds (Cook 1996).

Material and methods

Plant material and taxon sampling

Exploratory trips were made to survey and collect plants of "*N. alba* var. *rubra*" from the state of Meghalaya (NE India). Three populations were identified: (1) Ward's Lake, Shillong, East Khasi Hills District (25°34´N, 91°53´E), (2) pond at Mawmluh Village, Smit, East Khasi Hills District (25°30´N, 91°53´E), and (3) pond at Mukhla, Jaiñtia Hills District (25°30´N, 92°10´E). Six individuals, two from each population, were analyzed. The plant has a horizontal rhizome, the leaf margin is entire and thick with impressed veins and without mottling. The number of central air canals of the peduncle and petiole are five and four, respectively. Average numbers of 26 (23–28), 85 (75–100) and 19 (17–22) were recorded for the petals, stamens and carpels respectively. Nucleotide sequence data of *N. alba* (Borsch *et al*. 2008), *N. mexicana* (Woods *et al*. 2005) and *N. odorata* (Borsch *et al*. 2008) representing some of the putative parental species were retrieved from GenBank. In addition, ITS sequence data of *N. tetragona* (probable identity *N. pygmaea*, based on morphological and molecular evidence) was taken from our own previous studies (Dkhar *et al*. 2010).

DNA extraction, amplification and direct sequencing

Total genomic DNA was extracted from fresh or frozen leaves by following the method of

Doyle and Doyle (1987) with the addition of the saturated phenol extraction step prior to ethanol precipitation. A polymerase chain reaction (PCR) was used to amplify the ITS region, *trn*K intron (including the entire *mat*K gene) and *rbc*L gene. The PCR primers ITS-4 and ITS-5 (White *et al*. 1990) were used to amplify the ITS region (ITS-1, 5.8S, ITS-2) utilizing same primers and an internal primer (ITS-3, 5´-GCATCGATGAAGAACGCAGC-3´) for sequencing. For *trn*K intron amplification, primers trnK3914F and trnK2R as described by Johnson and Soltis (1995) were used; sequencing was done by utilizing these and other four internal primers (NytrnKJD689-R 5´-GGGGAGGATTTCTTGGGTTA-3´; NymatKJD1853-F 5´-CCTCTGATTGGATCGTTGGT-3´; NymatKJD1995-R 5´-CACCCGAATCGAGCAATAAT-3´; NytrnKJD525-F 5´-TCGGGTTGCAAAAATAAAGG-3´). Amplification of the *rbc*L gene was done using the primers 1-F and 1460-R (Fay *et al*. 1998) employing these and an internal primer NyrbcLJD361-F (5´-GTGGGTAATGTATTTGGGTTC-3´) for sequencing. DNA amplification was performed in an Applied Biosystems Gene Amp® PCR System 2700, Rotkreuz, Switzerland. Amplified PCR products were purified using QIAQuick gel extraction kit (QIAGEN, Germany) and sequenced at Bangalore Genei, India and Axygen Scientific Pvt. Ltd, India. Sequencing chromatograms were used to evaluate the additional signals in the ITS region.

Cloning of the ITS region

The purified PCR-amplified product from the ITS region of "*N. alba* var. *rubra*" was cloned into plasmids using CloneJET™ PCR Cloning Kit (Fermentas, Canada). The cloned ITS regions were amplified using primers pJET1.2 F and R supplied along with the kit. Twenty-six clones were screened by sequencing the entire ITS region utilizing the above-mentioned primers. Sequencing was carried out at the Central Instrumentation Facility, School of Life Sciences, North Eastern Hill University, Shillong, using a 3130 Genetic Analyzer, Applied Biosystems (California, USA). Sequencing signals of each individual allele of the ITS region isolated through molecular cloning techniques were evaluated for singular signals in their respective chromatograms.

Sequence alignment

Published sequences of *Nymphaea* were used to compare and determine the boundaries of the *trn*K intron including *mat*K (Löhne *et al*. 2007), *rbc*L (Goremykin *et al*. 2004) and ITS region (Woods *et al*. 2005) of "*N. alba* var. *rubra*". The *trn*K intron is incomplete at the 5´ and 3´ ends because these regions correspond to the position where the sequencing primers are placed. The *trn*K, *mat*K, *rbc*L and ITS nucleotide sequences of "*N. alba* var. *rubra*" and those retrieved from GenBank were subjected to multiple sequence alignment using ClustalX program (Thompson *et al*. 1997) with the default settings. ClustalX-generated alignments were further re-aligned manually. Sequencing chromatograms were used to evaluate the additional signals in the ITS region.

Maximum parsimony analysis

A maximum parsimony based method using Phylip 3.69 was utilised to evaluate the genetic relatedness between the DNA sequences of the individual clones and the putative parental species. Bootstrap analysis was carried out with 999 random seeds and 1000 replicates (Felsentein 1985). The MP tree created by Phylip was viewed with the program Tree View 1.5 (Page 1996).

Results

The nucleotide sequences of "*N. alba* var. *rubra*" were submitted to the GenBank databases (www.ncbi.nlm.nih.gov) and can be accessed under the accession numbers GQ358629, GQ358631 and GQ358632 (*trn*K intron and *mat*K); GQ358627 (*rbc*L); GQ358633–GQ358642, GQ477274 and FJ597737 (partial ITS-1, complete 5.8S and partial ITS-2) and GU222350–GU222364 (complete and partial ITS alleles sequences).

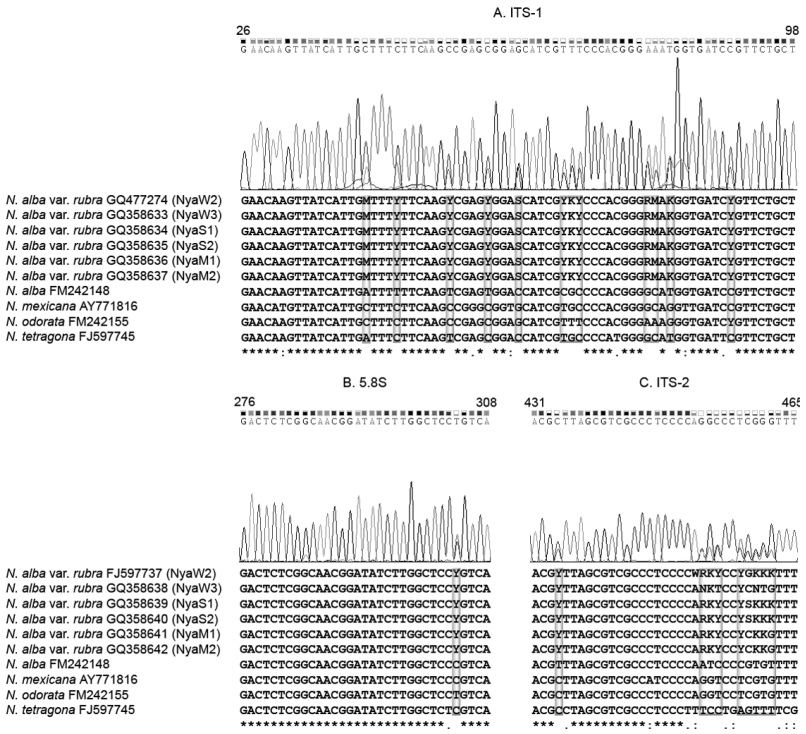

Fig. 1. Sequencing signals of the ITS region (ITS-1, 5.8S, ITS-2) of "*Nymphaea alba* var. *rubra*" along with the corresponding nucleotide sequence data of the suspected hybrid (i.e., "*N. alba* var. *rubra*") and the putative parental species *viz*., *N. alba*, *N. mexicana*, *N. odorata* and *N. tetragona*. Numbers at the ends above each chromatogram refer to the nucleotide positions; asterisk (*) indicates exact match; colon (:) and dot (.) indicate nucleotide sites where substitutions have occurred in only one parental species; 'shaded' nucleotide sites correspond to position in the chromatogram where additional signals are depicted. Nucleotides in the sequence data of "*N. alba* var. *rubra*", corresponding to the position where double peaks are represented in the chromatogram, are replaced by ambiguity codes. The letters and numbers in parentheses following the GenBank Accession numbers indicate the DNA sample number.

Sequencing signals in ITS

The ITS region of all samples of "*N. alba* var. *rubra*" revealed additional signals in their respective chromatograms, hinting at the presence of divergent ITS paralogues (Fig. 1). When compared with sequence data of the putative parents, the double peaks corresponds to nucleotide positions differentiating the suspected parental species (Fig. 1). At nucleotide site number 42 (Fig. 1A), the chromatogram depicted two peaks corresponding to nucleotides A and C, which are represented in the sequence data of the putative parental species, with C characterizing *N. mexicana* and *N. odorata* and A representing *N. alba* and *N. tetragona*. Similar patterns were observed at nucleotide position number 434, where double peaks corresponding to T and C are depicted in the chromatogram, with T representing *N. alba* and *N. tetragona*, C signifying *N. mexicana* and *N. odorata*, in their respective sequence data (Fig. 1C). The sequencing chromatograms do not show additional signals in some nucleotide positions (position numbers 31, 56, 61, 75, 84, 89 of Fig. 1A, nucleotide sites number 303 of Fig. 1B and nine nucleotide positions of Fig. 1C), where substitutions have occurred among the suspected parental species. Single peaks were observed throughout the 5.8S region except at position number 304 where an additional signal is depicted corresponding to nucleotides C and T (Fig. 1B).

Cloning of the ITS region of "*N. alba* var. *rubra*" followed by sequencing resulted in the isolation of distinct ITS alleles (Fig. 2). Barring

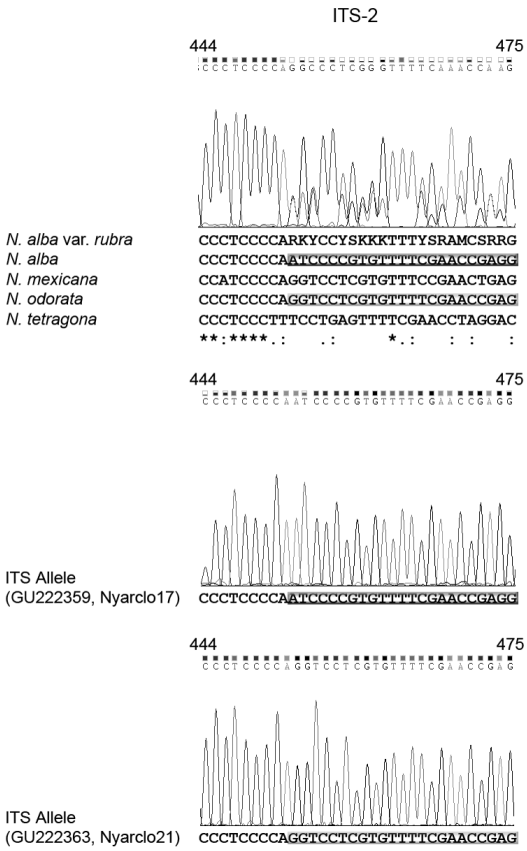

Fig. 2. Sequencing signals of the ITS alleles of "*N. alba* var. *rubra*" isolated through molecular cloning techniques. The DNA sequence of allele Nyarclo17 (shaded) is identical to that of *N. alba*, whereas allele Nyarclo21 (shaded) has a nucleotide sequence corresponding to that of *N. odorata.* Numbers at the ends above each chromatogram refer to the nucleotide positions; asterisk (*) indicates exact match. The letters and numbers in parentheses following the GenBank Accession numbers indicate the ITS allele number.

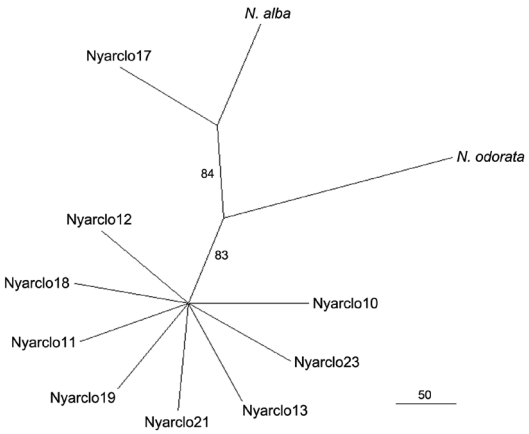

Fig. 3. The single most parsimonious tree of 81 steps obtained from analysis of the isolated ITS alleles of "*N. alba* var. *rubra*" and the ITS nucleotide sequences of the putative parental species. Allele Nyarclo17 is closely related to *N. alba*, whereas the remaining alleles formed a separate clade as a result of genetic recombination between the parental ITS alleles. Numbers denote bootstrap values. Branch length is indicated below the tree.

allele Nyarclo17, whose nucleotide sequence is identical to that of *N. alba*, the remaining alleles contained sequences indicative of genetic recombination between the two parental ITS alleles. For example, allele Nyarclo21 showed nucleotide sequences of *N. odorata* at position number 453–493 whereas the remaining portion of the ITS sequence belong to *N. alba*.

MP tree

A maximum parsimony analysis of the ITS data matrix, comprising the isolated ITS alleles from "*N. alba* var. *rubra*" and the ITS nucleotide sequences of the putative parental species, resulted in one most parsimonious tree of 81 steps (Fig. 3). The unrooted MP tree depicted allele Nyarclo17 as having an identical sequence with *N. alba*. The remaining alleles, however, formed a separate clade.

Sequence matching of the *trn*K intron, *mat*K and *rbc*L genes

Nucleotide sequences of the chloroplast *trn*K intron, *mat*K and *rbc*L gene were synthesized for "*N. alba* var. *rubra*". Sequence alignment of these along with those (*N. alba*, accession no. NC_006050; *N. odorata*, accession no. DQ185549 and M77034) retrieved from GenBank yielded 1013, 1530 and 1183 characters for *trn*K, *mat*K and *rbc*L respectively. All markers recorded low sequence divergence (substitution only) with 0.327% for *mat*K, 0.423% for *rbc*L and a relatively higher percentage of 1.086 for *trn*K. Exact sequence matches between "*N. alba* var. *rubra*" and *N. odorata* were indicated

by comparing sites, where nucleotide substitutions have occurred among the suspected parental species (Table 1). Furthermore, among the simple sequence repeats recorded, the motifs TTAATGA and TACTTCA were repeated once in "*N. alba* var. *rubra*" and *N. odorata*, whereas the repeat motifs TAAAA, ACAA and CTAGGGA were recorded for *N. alba* only.

Discussion

For biparentally inherited nuclear markers the approach of employing molecular cloning techniques, wherein individual copies of the nuclear sequences are isolated from the putative hybrid, is preferable for identifying alleles unique to the parental species (Moody & Les 2002, Les *et al*. 2004, Pan *et al*. 2007). Direct sequencing of the amplified nuclear DNA sequences to detect nucleotide polymorphism among samples has been proven to be effective (Aguilar & Feliner 2003, Lorenz-Lemke *et al*. 2005, Kaplan & Fehrer 2007, Löhne *et al*. 2008). Recently, Peterson *et al*. (2009) utilized both direct sequencing and cloning for hybrid detection in *Gagea* species (Liliaceae). In the present investigation, we adopted the direct sequencing method and compared the nucleotide polymorphisms manifested as additional signals in the chromatograms with sequence data of putative parents followed by molecular cloning of the ITS region. Sequencing signals of the ITS region of all samples of "*N. alba* var. *rubra*" revealed double peaks in their respective chromatograms, 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 additional signals detected in the chromatogram may be due to random mutations occurring in some of the ITS paralogues (Baldwin *et al*. 1995). However, this explanation seems unlikely, because on comparison with sequence data of the putative parents (Fig. 1), the additional signals occur at sites differentiating the suspected parental species, i.e. *N. alba* and *N. odorata*. Genetic contribution from *N. tetragona* is improbable because of lack of additional

Table 1. Comparison of nucleotide positions where substitutions have occurred among the suspected parental species. Note the exact nucleotide matches between the putative hybrid "*Nymphaea alba* var. *rubra*" and *N. odorata*.

Genomic region	Position number	Species		
		"*N. alba* var. *rubra*"	*N. odorata*	*N. alba*
	207	A	A	T
	400	A	A	T
	423	A	A	T
*trn*K 5´ intron	433	T	T	G
	482	A	A	T
	503	T	T	C
	541	A	A	G
	6	A	A	C
	28	A	A	C
*trn*K 3´ intron	150	T	T	C
	207	A	A	G
	6	T	T	A
	358	C	C	T
*mat*K	561	T	T	C
	837	A	A	G
	1031	T	T	C
	253	A	A	G
	418	C	C	T
*rbc*L	647	C	C	A
	727	C	C	G
	956	T	T	G

signals at nucleotide sites (e.g. sites number 75 and 435 in Fig. 1A and C) differentiating that plant taxon. This was further confirmed by means of molecular cloning techniques, whereby distinct alleles were isolated from the putative hybrid. Although ITS alleles corresponding to the nucleotide sequence of *N. odorata* were not obtained, genetic recombination between the two ITS alleles belonging to each parent were observed in most of the alleles isolated from "*N. alba* var. *rubra*" (data not shown). Such allelic DNA sequences confirmed that hybridization between *N. alba* and *N. odorata* had occurred. Representation of these allelic DNA sequences in the context of genetic relatedness revealed close association between *N. alba* and Nyarclo17 (Fig. 3). However, the remaining alleles diverged from either of the parental species, an outcome possibly brought about through genetic recombination. Another technique of hybrid detection is the sequence matching of the chloroplast DNA that can assist in identifying the direction of hybridization. Les *et al*. (2004) utilized the *trn*K 5´ intron sequence to identify the maternal parent of the hybrid *Nymphaea* 'William Phillips'. Similarly, the chloroplast *trn*K intron, *mat*K and *rbc*L gene sequence of "*N. alba* var. *rubra*" were compared with those of the parental species for exact sequence match. Comparing polymorphic nucleotide sites of all chloroplast markers that differentiate the two parental species indicates an exact sequence match between *N. odorata* and the suspected hybrid. Based on the evidence provided by the nrDNA ITS and the chloroplast *trn*K intron, *mat*K and *rbc*L gene, we suggest that the Indian material, which has been identified as *N. alba* var. *rubra*, represents a hybrid originating from *N. alba* and *N. odorata*.

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