Confirming the identity of newly recorded Nymphaea rubra Roxb. ex Andrews discerning from Nymphaea pubescens Willd. using morphometrics and molecular sequence analyses

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CONFIRMING THE IDENTITY OF NEWLY RECORDED *NYMPHAEA RUBRA* ROXB. *EX* ANDREWS DISCERNING FROM *NYMPHAEA PUBESCENS* WILLD. USING MORPHOMETRICS AND MOLECULAR SEQUENCE ANALYSES

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Keywords: *mat*K; Morphometric Analysis; Nymphaeaceae; *psb*A-*trn*H; Water-lilies; Sri Lanka.

Abstract

A multivariate statistical analysis was carried out to evaluate the morphological variation between *Nymphaea pubescens* Willd., and a deep purplish red flowered *Nymphaea* that occur in Sri Lanka. The plant resembles *N. rubra* Roxb. ex Andrews, a species that had been sometimes circumscribed as a variety under *N. pubescens* Willd. DNA sequences data of *matK* and *psbA-trnH* regions were used to obtain further support. Morphological data were scored from collected samples and analyzed using PAST software. Extracted DNA were amplified for *matK* and *psbA-trnH* gene regions. Obtained sequences were matched with the related accessions deposited in the GenBank. Multivariate analysis supported the recognition of deep purplish red flowered *Nymphaea* as a different species from *N. pubescens*, and was identified as *N. rubra* based on literature. GenBank accessions for the *matK* region of *N. rubra* showed 99% similarity while it gave only a 96% similarity for *N. pubescens* with query coverage of 97% and 96% respectively, corroborating with the morphological analysis. Comparison of the sequence divergence between *N. pubescens* and *N. rubra* sequences indicated a 95% similarity for *matK* gene region while 92% similarity for *psbA-trnH* gene region. The sequences generated during the present study would provide additional reference sequences for the two taxa.

Introduction

The genus *Nymphaea* L. (*Nymphaeaceae* Salisb.) or Water-lilies comprise of about 40-50 species and is widespread in tropical and temperate regions covering vast extents of natural water-bodies. All are aquatics with perennial or annual rhizomes (Jaime *et al.*, 2000). Species of *Nymphaea* show a high morphological plasticity where the size of leaves and flowers are thought to be strongly dependent on hydrological and edaphic conditions (Polina and Alexy, 2007). They grow in open waters of large swamps, lakes, ponds, shallow ditches, and also in marshes. The species of *Nymphaea* may be either day- blooming or night-blooming. The flowers are showy and born solitarily, containing numerous petals, stamens, and many carpels. The genus *Nymphaea* is a taxonomically difficult group; many species are believed to have numerous subspecies, chromosomal races & forms of hybrids and of artificial origin (Polina and Alexy, 2007). The plants are very popular as ornamental aquatics in the landscape industry.

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Among the diverse members of the family, *N. nouchali* Burm.f., *N. rubra* Roxb. ex Andrews, *N. pubescens* Willd, and *N. alba* L. are some of the most widely spread species in Asia (La-ongsri *et al*, 2009). *Nymphaea rubra* is common throughout the temperate and tropical Asia, such as in Bangladesh, India, Taiwan and Thailand, especially in shallow lakes and ponds. The species have somewhat big flowers compared to many other *Nymphaea* species, and it prefers to grow in non-acidic water above 15ºC (Hossain *et al*., 2007).According to the Revised Handbook to the Flora of Ceylon, the family Nymphaeaceae is represented in Sri Lanka only by the genus *Nymphaea*, with two species, *N. nouchali* Burm. f., and *N. pubescens* Willd. (Dassanayake, 1996). Other than these native species, during field visits, a deep purplish red flowered *Nymphaea* species with a morphological resemblance to *N. pubescens* was also encountered in natural water bodies in the dry lowland of the country. Many morphological features that are described under the *N. pubescens* (Dassanayake, 1996) overlap with this *Nymphaea* species. The plant has been referred to as *N. pubescens* variety *rubra* by de Vlas and de Vlas-de Jong in 2008. According to The Revised Handbook to the Flora of Ceylon (Dassanayake, 1996) the flower petals of *N. pubescens* are white, purplish pink or red, where inner petals are smaller. Leaf upper surface is glossy dark green and dark purplish green, velvety on leaf lower surface with very prominent veins. The petiole is red-brown, while the pedicel bears short prickles. However the presence of short prickles on the pedicels is a mis-conception as *N. pubescens* never poses prickles but *Nelumbo nucifera*, a species belonging to the family Nelumbonaceae, instead. The filament colour is described as yellowish white becoming deeper yellow distally, or pale purplish pink to crimson. Although the above description is accommodating many characters of *N. pubescens*, the description seems to include some characters of those of the deep purplish red flower species of *Nymphaea* as well. Characters such as red- brown petiole, dark purplish green lower surface with velvety appearance and highly prominent veins are more towards the plants with deep purplish red flowered *Nymphaea* rather than *N. pubescens*. On the other hand, according to literature, this deep purplish red flowered *Nymphaea* species share morphological similarities with *N. rubra*, a species that had not been recognized as occurring in the island during the revision of the Flora. According to Conard (1905), *N. rubra* possess deep purplish red coloured flowers with cinnabar red stamens, and the reddish leaves becoming greenish with the age, and rarely producing fruits or seeds. Mitra and Subramanyam (1982), questioned the treatment of *N. rubra* as a true species at par with other sexually reproducing species because of its failure to set fruits/seeds in nature. According to Gupta (1980), *N. rubra* has two cytotypes, one which is highly fertile and another nearly sterile. Further, La-ongsri *et al.* (2009), describes *N. rubra* as, leaf dark reddish above and below, nine pairs of prominent and angular veins below, petiole green or reddish-brown, and a deep purplish red flower bearing orange or cinnabar-red stamens, becoming brownish with age. *Nymphaea pubescens* and *N. rubra* are two closely related taxa (Jeremy *et al*., 2010).

Hence, a multivariate statistical analysis was carried out to evaluate the morphological variation between the ambiguous taxa and described *N. pubescens*, and further DNA sequences data of *matK* and *psbA-trnH* regions were used in verification of the identity between *N. pubescens* and the deep purplish red flowered *Nymphaea* species occurring in Sri Lanka. The *matK* is one of the rapidly evolving coding region in the plastid genome, while Chloroplast non-coding intergenic *psbA-trnH* spacer has recently become a popular tool in plant molecular phylogenetic studies at low taxonomic levels (Biswal *et al*., 2012).

Materials and Methods

Sample collection

Live plant material of the two *Nymphaea* species, including populations with both white and pink flowered *N. pubescens*, and deep purplish red flowered *Nymphaea* species, were collected

from 50 different locations covering all the three major climatic zones of the island. The map showing the field localities are given in Fig. 1. From each locality, a minimal of five specimens were collected. All the collected populations were treated separately with a different acronym; DPRN (Deep Purplish Red flowered *Nymphaea* species), NPW (*N. pubescens* White) and NPR (*N. pubescens* Pink), for easy references. The collected specimens were examined in detail in the laboratory for different morphological characters.

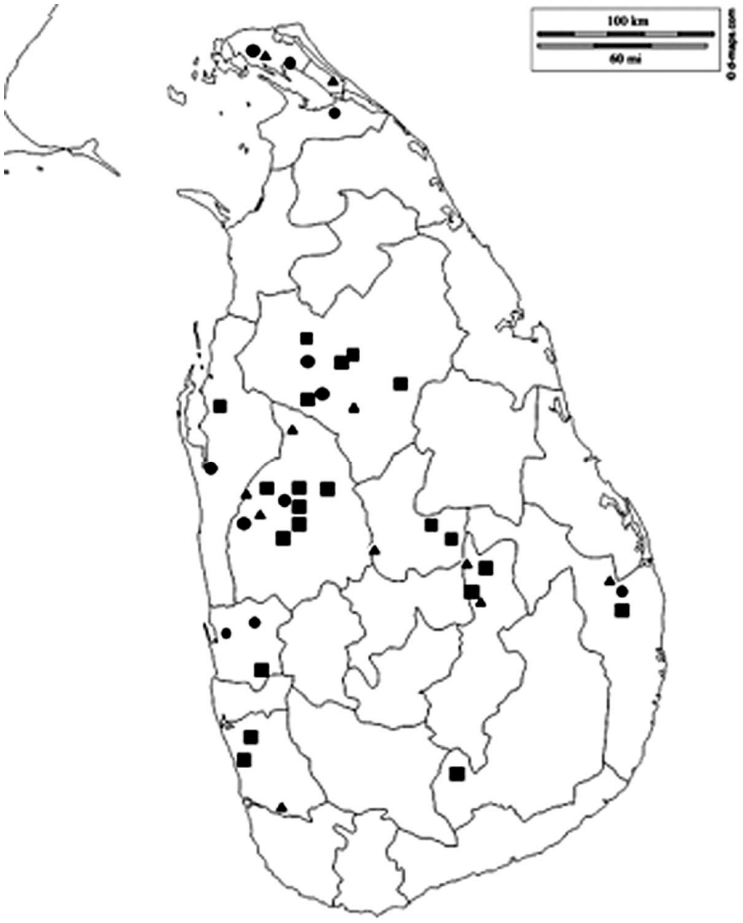

Fig. 1. The map depicting the locations where specimens were collected for the present study. ● *N. pubescens*-white (11 populations), ■ *N. pubescens*- pink (21 populations), ▲ Deep purplish red flowered *Nymphaea* (11 populations)

Morphological studies

Vegetative characters such as leaf shape, length, petiole diameter and reproductive characters such as flower size, petal and sepal length, number of stamens were studied either with the naked eye, under a dissecting microscope or under a stereo microscope (Leica, 10446322, 2X WD). Five individuals from each population were studied in detail where the measurement was averaged. The mean value for up to three measurements of each character was recorded for each specimen. Special attention was paid to characters with distinct variations. Colour of the lower and upper

surfaces of the leaf and petal, stamens, stigmatic segments, and petiole were determined using the Royal Horticulture Society Colour Chart (RHS Colour Chart 2001). Nineteen quantitative and 21 qualitative characters were coded for 50 representatives of *N. pubescens* including both white and pink flowered populations and deep purplish red flowered *Nymphaea* species. All qualitative characters were expressed quantitatively by giving a coding value, to avoid misrepresenting the possible range of variation (Stevens, 1991).

Morphometric analyses

The multivariate statistical analysis was carried out using the PAST - Paleontological Statistics program version 2.17 (Hammer *et al.*, 2001). Cluster analysis, Principal Component Analysis (PCA) and Principal Coordination Analysis (PCoA) were performed. The cluster solution was selected from the best suitable similarity measure method and the algorithm; Gower similarity measure and 'Paired group' option (UPGMA), which produced the highest Co-phenetic correlation value of 0.933 over the other similarity distance methods and algorithms. Similarity Percentage Analysis (SIMPER) was performed to obtain overall average dissimilarity levels of the groups. Other than the SIMPER, PCA loadings were also used to rank characters regarding their contribution for the separation of clusters, and thereby to find the best vegetative characters to differentiate between the two species.

DNA studies

Total genomic DNA was extracted using a Qiagen DNAeasy Plant Mini kit, from fresh leaf materials from three selected samples representing deep purplish flowered *Nymphaea* , white flowered and pink flowered *N. pubescence*. *matK* (matK-390f 5'-CGATCTATTCATTCAA TATTTC-3', and matk-1326r 5' –TCTAGCACACGAAAGTCGAAGT- 3') (Cuenoud *et al*., 2002) and *psbA-trnH* [*psbA*-F 5' –GTTATGCATGAACGTAATGCTC- 3' (Sang *et al*., 1997), *trnH-R* 5'- CGCGCATGGTGGATTCACAATCC-3' (Tate & Simpson, 2003)] regions were amplified using Polymerase Chain Reaction (PCR) technique. Amplifications were carried out in 50 µL reaction solutions that contained 1× PCR reaction buffer, 2.5mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate (dNTPs), 0.2 µM each forward and reverse primer, 1 U of Taq DNA polymerase and 0.75–1.5 µL unquantified DNA extract. The PCR program was run on a Techne- Flexigene Thermal Cycler. The program consisted of 3 min of initial denaturation at 94°C, 35 cycles of 30 S denaturation at 94°C, 30 S annealing at 48°C/ 57°C for matK and *psbA-trnH* respectively, 1 min primer extension at 72°C, followed by a final extension for 10 min at 72°C. PCR products were run on a 1% agarose gel stained with ethidium bromide, and visualized on a UV table. The molecular mass of the resulted bands were estimated with a 1kb DNA ladder and confirmed the amplification of the primer. Obtained PCR products were submitted for sequencing reactions using Applied Biosystems, 3500 genetic analyzer.

Consensus for resulted sequences of forward and reverse primers was compiled using Bioedit version 7.1.11 and edited visually. Sequences deposited in the GenBank, for the *matK* gene region for the two taxa by other literatures were extracted using a BLAST (Basic Local Alignment Search Tool). ClustalW multiple sequence alignment was also used for Sequences alignment and comparison other than the BLAST.

Results and Discussion

The list of characters that were studied in detail together with their character states is given in the Table 1. The UPGMA dendrogram (co-phenetic correlation coefficient = 0.933) resolved two discrete clusters (denoted as cluster A and B), which separated respectively at approximately 0.35

Table 1. List of characters together with their character states.

Character	Character states
Diameter of the receptacle	mm
Receptacle height	cm
Flower colour (inner colour of petal)	White/Yellow/ Pink/ Deep purplish red
Flower colour (outer colour of petal)	White/ Yellow/ Pink/ Deep purplish red
Number of petals	
Petal length (outer petals)	cm
Petal width (maximum) (outer petals)	At the broadest point in cm
Petal shape	Linear-lanceolate/ Ob-lanceolate
Number of veins per petals	
Petal base	More or less widen into rectangular shape
Petal apex-shape and angle	Acute/ Obtuse
Number of stigmatic segments	
Number of sepals	Always 4 in number
Sepal length	cm
Sepal width (maximum)	cm
Sepal shape	Linear-lanceolate/ Ob-lanceolate
Sepal apex – shape and angle	Acute/ Obtuse
Number of stamens	
Stamen colour	Yellow/Red
Stigmatic segments colour	Yellow/ Crimson-red colour
Pedicel diameter	At the end of the receptacle end in cm
Pedicel shape	Round/Slightly flat/Oval
Pedicel shape in cross section	No. of lacunae
Petiole – cross section	No. of lacunae
Leaf size	Length/Width in cm
Leaf shape	Round/ Ellipsoid
Leaf length	Apex to base in cm
Leaf width	Across the mid rob in cm
Length/Width ratio	
Lamina colour (upper)	Dark green/Light green/Green
Lamina colour (lower)	Brownish-red/ Purple/Green
Leaf margin	Dentate/ Strongly dentate
Leaf venation (lower) number of veins	14 or less / Over 14
Leaf venation (lower) pattern	Prominent/ Not prominent
Leaf hairs (lower)	Long hairs/ Short hairs
Leaf apex	Division present/ Division absent
Petiole diameter	cm
Shape of the petiole	Round/Oval/ Irregular shape
Petiole colour	Yellowish-white/ Reddish-brown/ Green
Pedicel colour	Dark-green/ Brown/ Brownish red
Hairs on the petiole	Present/ Absent

distance units. The OTUs within each cluster grouped together closely, with none of them exceeding a distance of more than 0.7 units within any given cluster (Fig. 2). The scatter plot that resulted from the PCoA is given in Fig. 3 (Transformation component, C = 2). The first four (principal) eigenvalues recovered from the PCoA (1.3985, 0.2106, 0.1268, and 0.0878) accounted for 71.17% of the total variance (54.53%, 8.27%, 4.95%, and 3.43% respectively). A plot of the first and second coordinates (which provided the greatest separation of OTUs) returned a result similar to that obtained by the Cluster Analysis. Here the PCoA also resolved two discrete clusters, with each corresponding exactly to one of the clusters indicated by the UPGMA dendrogram.

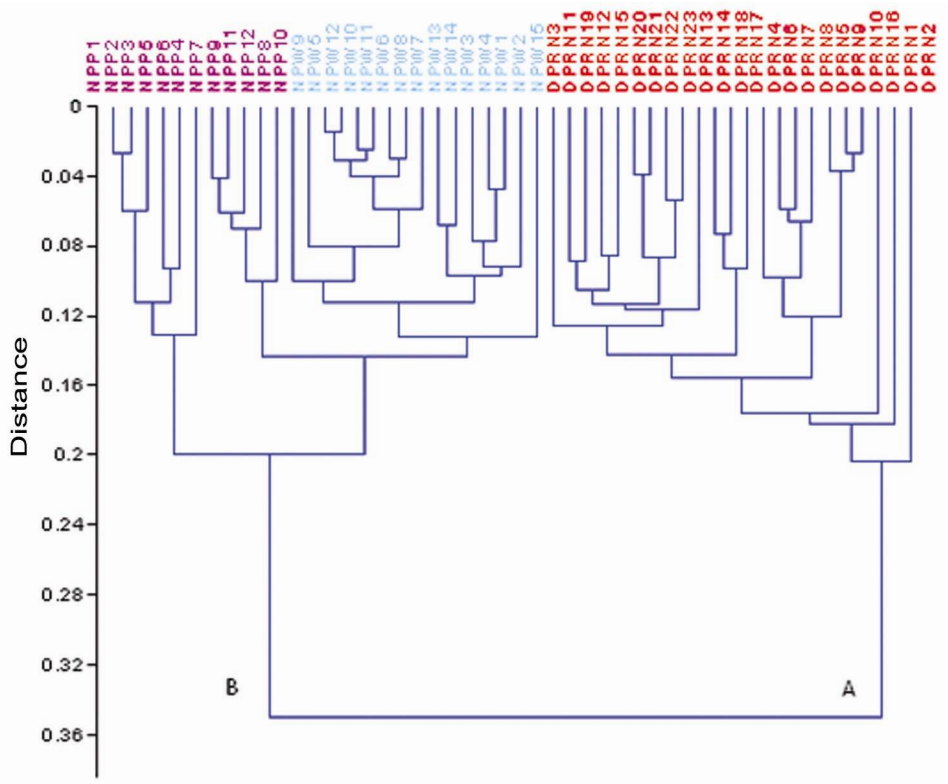

Fig. 2. Dendrogram that resulted from morphometric analysis showing the clearly separated groups of *N. pubescens* and deep purplish red flowered *Nymphaea* species. Deep purplish red flowered *Nymphaea* species – DPRN, *N. pubescens* (white) – NPW and *N. pubescens* (pink) – NPP.

According to the results of PCA loading and SIMPER analysis, the number of stamens, leaf length, leaf width, number of petals and number of stigmatic segments are the highly contributed quantitative characters while lamina colour (both upper and lower), leaf venation pattern, petiole colour and stamen colour are the highly contributed qualitative characters for the separation. The character variation of the highly contributing six quantitative characters (number of stamens, leaf width, leaf length, number of stigmatic segments, number of petals and leaf size) between the two major groups; identified in analysis is given in Fig. 4 as box plots.

The morphometric analysis identifies two main phenetic groups A and B that corresponds to the deep purplish red flowered *Nymphaea* congregated in the cluster A while the cluster B

corresponds to *N. pubescens*. Cluster B further branched at a distance of 0.2, where one group encompassed only pink flowered *N. pubescens* while the larger group consisted of both the white and pink flowered *N. pubescens*. Similarly the scatter plot obtained by PCoA clearly supports to the clustering of the populations into two major phenetic groups as A and B as recognized by the cluster analysis with non-overlapping distribution and the overlapped scattering of the members in group B. The detailed study of the characters of the members of the deep purplish red flowered *Nymphaea* and the character comparison with literature, Conard (1905) and La-ongsri *et al.* (2009), confirmed the identity of the group as *N. rubra* Roxb. ex Andrews, a species that has not been recorded before as occurring in the island.

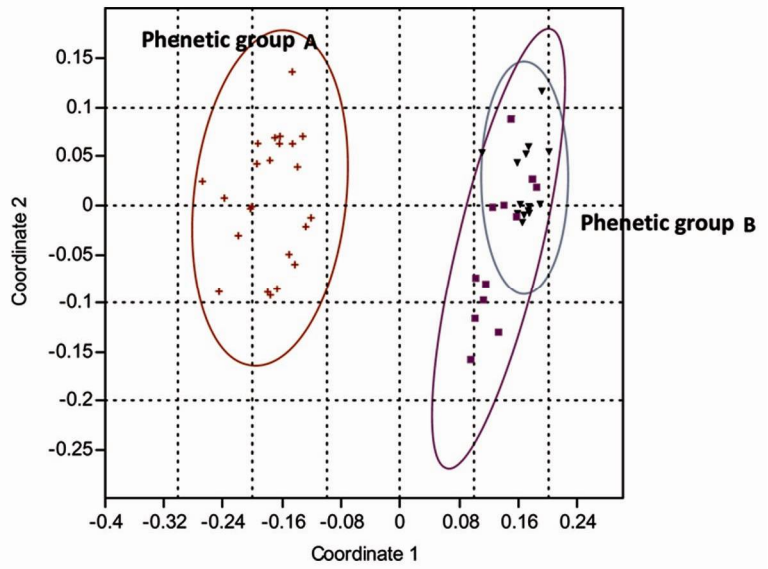

Fig. 3. Scatter plot at 95% ellipse level with eigenvalue scale obtained from PCoA.

DNA sequence analysis

The obtained sequence length of the *matK* and *psbA-trnH* gene regions were between 916-917 bp and 541-555 bp for deep purplish red flowered *Nymphaea* and *N. pubescens* respectively. The similarity percentage comparison of the obtained sequences of both *N. pubescens* (white and pink), with deep purplish red flowered *Nymphaea* with alignment scores obtained from BLAST search are given in Table 2. There were no sequence data deposited in the GenBank for both taxa for the *psbA-trnH* gene region.

Comparison of the obtained sequences with the GenBank (*N. rubra* - Acc. No. HQ592335.1) (Jeremy *et al.*, 2010) gave a 99% similarity for the deep purplish red flowered *Nymphaea* for the *matK* gene region with *N. rubra* while it gave a 96% similarity for *N. pubescens* (Acc. No. FJ597753.1) (Jeremy *et al.*, 2010). Comparison of the BLAST sequence divergence between *N. pubescens* and deep purplish red flowered *Nymphaea* for the sequences that were obtained in the study indicated only a 95% similarity for *matK* gene region existed between the two, while only 92% similarity for *psbA-trnH* gene region was indicated. Further, the comparison of both white and pink flowered *N. pubescens* sequences with the GenBank gave a 99% similarity match with *N. pubescens* (Acc. No. FJ597753.1).

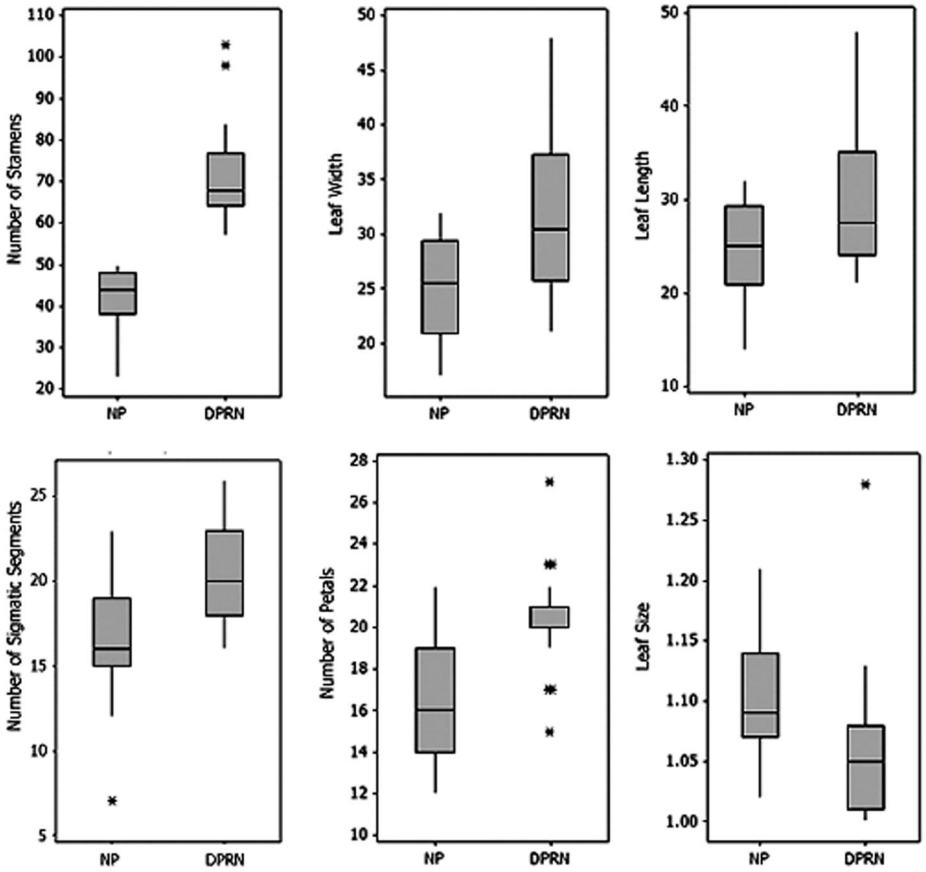

Fig. 4. Box-plots of the six highly contributing quantitative characters between the two major groups identified in analysis [*N. pubescens* (NP), and Deep purplish red flowered *Nymphaea* (DPRN) species].

Table 2. Percentage similarity obtained from the comparisons of studied sequences and the GenBank accession, using BLAST.

Compared sequences	Blast score	
	matK	*trnH-psbA*
Deep purplish red *Nymphaea* (DPRN) *vs. N. pubescens* white (NPW)	95%	92%
Deep purplish red *Nymphaea* (DPRN) *vs. N. pubescens* pink (NPP)	98%	92%
N. pubescens white (NPW) vs. *N. pubescens* pink (NPP)	99%	99%
Deep purplish red *Nymphaea* (DPRN) *vs. N. rubra* (HQ592335.1)	99%	NA
N. pubescens white (NPW) *vs N. rubra* (HQ592335.1)	96%	NA
N. pubescens pink (NPP) *vs N. rubra* (HQ592335.1)	98%	NA

BLAST search results, while indicating that the deep purplish red flowered *Nymphaea* is a different taxa from the native *N. pubescens*, further confirms its identity as *N. rubra*. Comparison of the sequences using ClustalW, mismatches accounted for 6 point mutations and 17 insertion/

deletion (INDELS) events (single base pair) observed in the alignment for the two sequences of *matK* for the two species, *N. rubra* and white *N. pubescens* obtained in the present study while 4 point mutations and 3 INDELS (4, 5, and 17 bp length) were encountered for *psbA-trnH* sequence alignment. When compare between pink and white *N. pubescens*, there were only 2 gaps observed for *matK* and 5 gaps observed for *psbA-trnH* sequence. INDELS occurred in both *matK* and *psbA-trnH* gene regions, where the most informative was in the *psbA-trnH* region.

The results of both multivariate statistical analyses and the molecular sequences comparison have supported the recognition of the deep purplish red *Nymphaea* as a different species from *N. pubescens.* The detailed comparison of morphological characters has identified the species as *N. rubra* Roxb. ex Andrews while the molecular sequence comparison has further confirmed its identity.

According to the literature *N. rubra* could be easily separated from *N. pubescens* from the close examination of floral and leaf characters. Average dissimilarity value for the separation of *N. rubra* (DPRN) from white flowered *N. pubescens* (NPW) and pink flowered *N. pubescens* (NPP) is 15.31. According to the results, characters such as number of stamens, leaf length and width, and number of petals are good quantitative characters for delimitation of these two species while lamina colour (both upper and lower), leaf venation pattern, petiole and stamen colour are the highly contributed qualitative characters. Even though the cluster encompassing *N. pubescens* (Cluster B) initially separates a few individuals with pink flowers in a separate cluster, the remaining group includes both individuals with pink and white flowers once again separating the pink flowered into a sub-cluster. The overall dissimilarity value between the members of the two pink and white flowered groups within *N. pubescens* is 10.14 according to the SIMPER analysis while the gap was less than 0.2 in distance units (Fig. 2). Further in both BLAST and ClustalW sequence alignments for both pink and white flowered *N. pubescens*, the two sequences for both gene regions, showed a very high sequence identities with zero or very few mismatches, implying that they are just two color variations of the same species.

Table 3. Comparison of distinct morphological characters between *Nymphaea rubra* and *Nymphaea pubescens*.

Characters	*Nymphaea rubra*	*Nymphaea pubescens*
Flower size	Large (35 - 44 cm)	Small (28 - 36 cm)
Petal colour	Deep purplish red	White or light pink
	The colour intensity from apex to base on both adaxial and abaxial surfaces is uniform	The colour intensity is not uniform on both sides of the petal, showing a gradual fading from apex to base
Stamen - colour	Cinnabar red	Yellow
Stigmatic segments - colour	Crimson red	Yellow
Leaf size	Large (25 - 48 cm)	Small (25 - 30 cm)
Leaf shape	Orbicular	Ovate- orbicular
Leaf colour -adaxial	Bronzy red while young, turning dark green with age	Green
Leaf colour -abaxial	Dark purple colour	Brown
Venation	Over 9 pairs of very prominent secondary veins	7-9 prominent secondary veins

A comparison of the characters between the two species is given in Table 3 and Fig. 5, and an identification key for the Sri Lankan *Nymphaea* is given below.

1. Leaves pubescent beneath with many short hairs, margin sharply dentate-mucronate; stamens without a tongue-shaped appendage beyond the anther or appendage very short 2
- Leaves glabrous, margin entire to dentate with blunt teeth; stamens with a tongue-shaped appendage beyond the anther to 5 mm long ***N. nouchali***
2. Leaf abaxial surface brown, venation pattern less prominent, petiole light green; flowers white, yellowish white or pink; stamens short, yellow; stigmatic surface yellow ***N. pubescens***
- Leaf abaxial surface dark purple, venation pattern very prominent, petiole reddish; flowers deep purplish red; stamen long, cinnabar red; stigmatic surface crimson red ***N. rubra***

All Water-lily species occur together in large water bodies in both dry and wet zones of the country. However, in many instances *N. rubra* occurs towards the center of the deep waters in isolation.

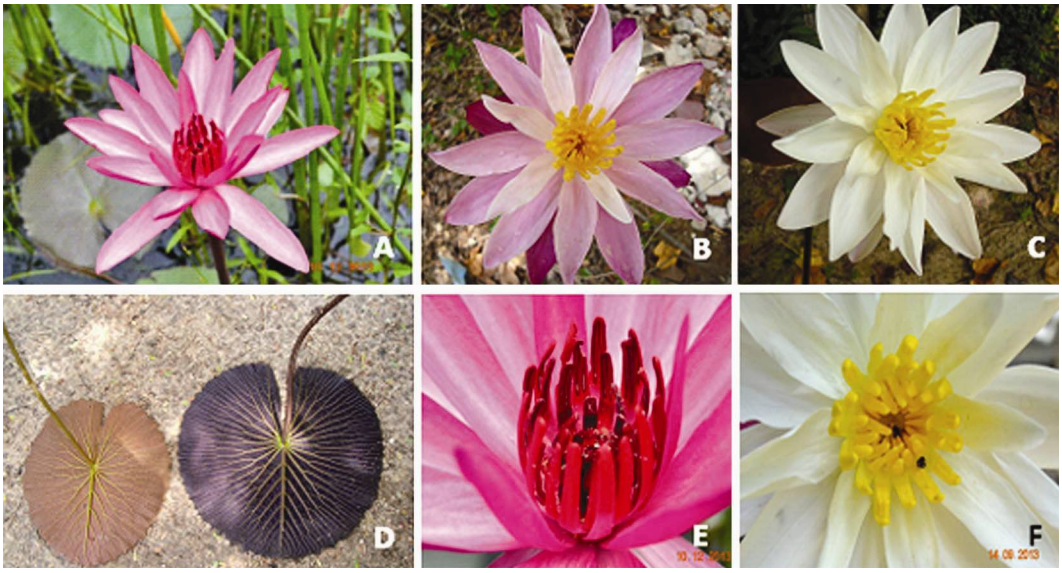

Fig. 5. Deep purplish red flowered *Nymphaea* species identified as *N. rubra* during the present study (A). Flowers of *N. pubescens*, pink (B) and white (C). Leaf upper surfaces of *N. pubescens* (left) and *N. rubra* (right) (D) Cinnabar red colour stamens of *N. rubra* (E) and yellow colour stamens of *N. pubescens* (F).

The study has resulted in adding a new member to the genus *Nymphaea* in Sri Lanka enriching the islands biodiversity. *Nymphaea* is a taxonomically difficult group with many natural and man-made hybrids occurring in the nature and therefore, morphological features alone are not sufficient in confirming the identity. The present study provides additional reference sequences for

both *N. pubescens* (Pink and white flowered groups) and *N. rubra* as well as a new gene region, *psbA-trnH* for reference.

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