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MORPHOLOGICAL AND GENOTYPIC CHARACTERIZATION OF DIFFERENT LOTUS (*NELUMBO NUCIFERA* GAERTN.) SAMPLES AVAILABLE IN BANGLADESH

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Abstract

Asian lotus (*Nelumbo nucifera* Gaertn.), commonly known as sacred lotus is a basal eudicot. It has been grown and cultivated as food, medicine and for cultural, and religious activities. In the current study, samples were collected from six different locations to evaluate the variation among different lotus germplasm based on external morphological characteristics, as well as, to study the genetic variation and the molecular characterization. Analysis of variance showed a higher level of variations among the germplasm for all the morphological features. Based on the morphological features, a dendrogram was constructed to assess the linkage among the germplasm. The yellow lotus of Cumilla was considered superior among the germplasm studied. To assess the genetic diversity and the correct identification of lotus germplasm, molecular method "Barcoding" was performed. To achieve the goal, two plastidial regions: *rpoB* and *rpoC1* were employed. The germplasm showing successful PCR were subjected to sequence analysis of their barcode genes. All the selected barcode genes showed successful identification of all the germplasm as *N. nucifera* in multilocus identification based on their sequences except for the germplasm of Rajshahi and also confirmed the yellowish lotus of Cumilla considered as a new cultivar *N. nucifera* 'Gomoti', newly found in Bangladesh. Genetic sequences obtained in the context of DNA barcoding had also been used to create a phylogenetic tree in which the germplasm were clustered into five main clades. The current study was successful in establishing an efficient protocol for the correct identification of lotus germplasm and was capable of establishing an elite gene source. Moreover, future studies are warranted to see the identifying capability and diverging power of the barcodes.

Introduction

Lotus (*Nelumbo nucifera* Gaertn.) is an aquatic plant that is ecologically, medicinally, economically and ornamentally very important due to its several uses. Historically, the lotus has been grown for 5000–7000 years in the Far East (Wong, 1987) and has been cultivated more than 3000 years ago as food, medicine and for cultural and religious activities (Shen-Miller *et al.*, 2002). Significantly, the longevity of lotus seed is phenomenal, with the world's record for long-term seed viability (Shen-Miller *et al.*, 2002). The importance of exceptionally long-term seed viability is the secret of their ability to resist ageing for hundred years – a trait reflected in their possible capability to mend cellular damage (US-DHEW, 1974; Huang, 1987). This may be important for future research with regard to senescence and ageing. Lotus is native to East Asia, South Asia and Southeast Asia and is better-known. So, Indo-malayan center is considered to be one of the centers of the origin of lotus. Regardless of its origin, many lotus variants grow in different areas of Bangladesh with great variability.

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The lotus diversity can be used for medicinal, economical and aesthetic purposes. Lotus is used to treat sunstroke, diarrhoea, dysentery, hemorrhoids, dizziness, blood vomiting, uterine bleeding disorders, promoting conception, improving the skin condition, controlling burning sensation, against infections, cough, hypertension, fever, urinary problems, hematemesis, epistaxis, hemoptysis, hematuria and metrorrhagia etc. (Sridhar and Rajeev, 2007; Ou, 1989) and because of strong antipyretic, cooling, astringent, antioxidant activity, anti-HIV effect and demulcent properties, it is also used as a source of herbal medicine (Han *et al*., 2007a,b; Hu and Skibsted, 2002; Kashiwada *et al.*, 2005; Lee *et al*., 2005; Ling *et al.*, 2005; An *et al.*, 2009). Lotus can be used in waste water treatment. It is particularly noted for its exceptional water repellency, known as the lotus effect. Despite its significance, less and limited information on the genetic characteristics and genomic variation of lotus are available in Bangladesh. Therefore, an attempt was made to understand the presence of variations in their morphology and molecular level.

The published sequencing data of lotus genome showed that varieties of lotus have great variability among them and carry a number of beneficial traits. However, until now, very less or limited information on the morphological characteristics and genomic variation of lotus in Bangladesh are available. Morphological traits play a vital role in selecting the important characters, variability and genetic relationship among the genotypes (Osei *et al.*, 2014). The genus *Nelumbo* consists of two species, *Nelumbo nucifera* Gaertn. and *Nelumbo lutea* Willd (Les *et al*., 1991; Huang *et al*., 1992; Borsch and Barthlott, 1994). *N. lutea* is distributing in South-eastern Asia and America and the species *N. nucifera* is called the Indian lotus. Appendage of Asian lotus is milky white, shape oval, whereas appendage of American lotus is bright yellow, shape boat like (Zhang *et al*., 2019). As the collected germplasm of Cumilla morphologically partially appeared like *N. lutea*, an attempt was taken to molecular identification of the germplasm.

The advent of molecular marker based technique which utilized short fragment of DNA and correctly assign plant taxa to their taxonomic group, called as DNA barcoding. A DNA barcode is an aid to taxonomic identification which uses a standard short genomic region that is universally present in target lineages and has sufficient sequence diversity to discriminate among species (Herbert *et al.*, 2003, 2004; Savolainen *et al*., 2005; Hajibabaei *et al*., 2007). It refers to a sequence-based identification system that be constructed of one locus or several loci used together as a complementary unit (Kress and Erickson, 2007). DNA barcoding is a relatively new concept that has been developed for providing rapid, accurate and automatable species identification.

Markers used for DNA barcoding are called barcodes and the most important characteristic features of a DNA barcode are its universality, specificity on variation and easiness on employment. A good DNA barcode should have low intra-specific and high inter-specific variability (Herbert *et al.*, 2003) and possess conserved flanking sites for developing universal PCR primers for wide taxonomic application. The purpose of this study was to identify lotus germplasm and to test the utility of DNA barcoding for the identification of closely related lotus variants. To assess the identification, two plastidal regions: *rpoB* and *rpoC1* were employed. In the present study an attempt was made to confirm morphological variation of lotus available in Bangladesh and to identify the germplasm at molecular level by barcoding marker analysis and study genetic linkage among the germplasm.

Materials and methods

To evaluate morpho-molecular diversity, twenty-four germplasm of lotus variants were collected from six different locations throughout Bangladesh (Table 1, Fig. 1). Four replica from each landrace were randomly selected and data were recorded on these germplasm for the fourteen morphological traits (Table 2). The raw data were purveyed by taking the means for all the replica

for different traits in the experiment. The mean, standard deviation, and minimum and maximum values were calculated for each character in each landrace. Analysis of variance was performed to determine morphological variations among germplasm using JMP 4.0 software tool. The data of morphological trails were analyzed by JMP 4.0 software and a dendrogram was constructed based on squared Euclidean distance by Ward's method.

Table 1. List of the germplasm employed for the current study.

Sampling no.	Location	GPS	Name of Germplasm
1	Norait Beel, Vikertek, Barishab Union, Kapasia Upazila, Gazipur	24.2029072, 90.6645243	Kap.W
2	Padma Beel, Kalabari Union, Kotalipara Upazila, Gopalgonj	23.0861422, 89.9909445	Go
3	Bhutiar Beel, Terokhada Upazila, Khulna	23.0861422, 89.6965614	Khul
4	Haram Beel, Baksimoil Union, Mohanpur Upazila, Rajshahi	24.5480723, 88.6380534	Mo
5	Sarkerpara, Aahar, Pachandar Union, Tanore Upazila, Rajshahi	24.5902671, 88.4878373	Raj
6	Dakshing Gram, Rajapur Union, Burichang Upazila, Cumilla	23.5755289, 91.1550685	Co.P Co.Y

Fresh young leaves from each germplasm were collected and washed thoroughly with distilled water and ethanol, and wiped off with clean tissue papers. Genomic DNA was extracted from the frozen leaves using a modified cetyltrimethylammonium bromide (CTAB) method as described by Doyle and Doyle (1987). Concentration of isolated DNA was measured through estimating the absorbance of DNA using a spectrophotometer (BioDrop Resolution) at 260 nm.

For molecular identification, two plant DNA barcodes, *rpoB* and *rpoC1* were amplified in 25µl reaction volume, using ½ volume of Go Taq G2 Green Master mix, 1.0 µl each primers and (30-40)ng DNA template. PCR amplification was performed on a Thermal Cycler (Applied Biosystem). The PCR amplified conditions were as follows: Initial denaturation at 95 °C for 3min, 30 cycles of 95 °C for 30s, annealing temperature 50°C for 30s and 72°C for 90s followed by a final extension at 72 °C for 2min reported by Caprari *et al.* (2017). The success of PCR amplification was verified by subjecting 10µl of the PCR product to 1% agarose gel electrophoresis in TAE buffer at 90W for 30 min and visualized under Gel Documentation System (CSL-MDOCUV254/365 1D, Cleaver Scientific LTD, USA). The PCR products were purified using alcohol precipitation method. Purified PCR products were sent to MCLAB (USA) and sequenced in both directions with the same primers used for PCR.

Sequences for each region were viewed and edited using BioEdit. Then, the edited sequences were aligned by ClustalW in MEGA11. Bootstrap values were calculated over 1000 replications. The barcode sequences were queried against GenBank database (NCBI) using Nucleotide BLAST algorithm BOLDSYSTEMS in order to confirm the barcode gene markers along with their locus in lotus germplasm.

Results and Discussion

The mean, standard deviation, maximum and minimum values for different morphological characteristics are presented in Table 2. In case of leaf length, Kap.W showed the highest mean value and Khul showed the lowest. Go showed the highest mean and Khul showed the lowest mean for leaf diameter. In case of petiole length, Raj showed the highest mean and Co.P showed the lowest. Go showed the highest and Khul showed the lowest mean value in case of petiole diameter. In case of petiole pickle's number, Co.Y showed the highest and Go showed the lowest mean. In case of peduncle length, Kap.W showed the highest and Go showed the lowest mean value among all. Kap.W showed the highest mean and Khul showed the lowest in case of peduncle diameter. In case of peduncle pickle's number, Co.Y showed the highest and Kap.W showed the lowest mean value. In case of the number of petals, Co.Y showed the highest and Kap.W showed the lowest mean value. In case of petal length, Kap.W showed the highest mean among all the germplasm and Co.P showed the lowest. Kap.W showed the highest mean value and Co.P showed the lowest in case of petal width. In case of number of stamens Go showed the highest mean value and Kap.W showed the lowest. Go showed the highest mean value and Co.P showed the lowest in case of length of stamen. In case of the number of seeds, Go showed the highest mean value and Raj showed the lowest mean value (Fig. 2).

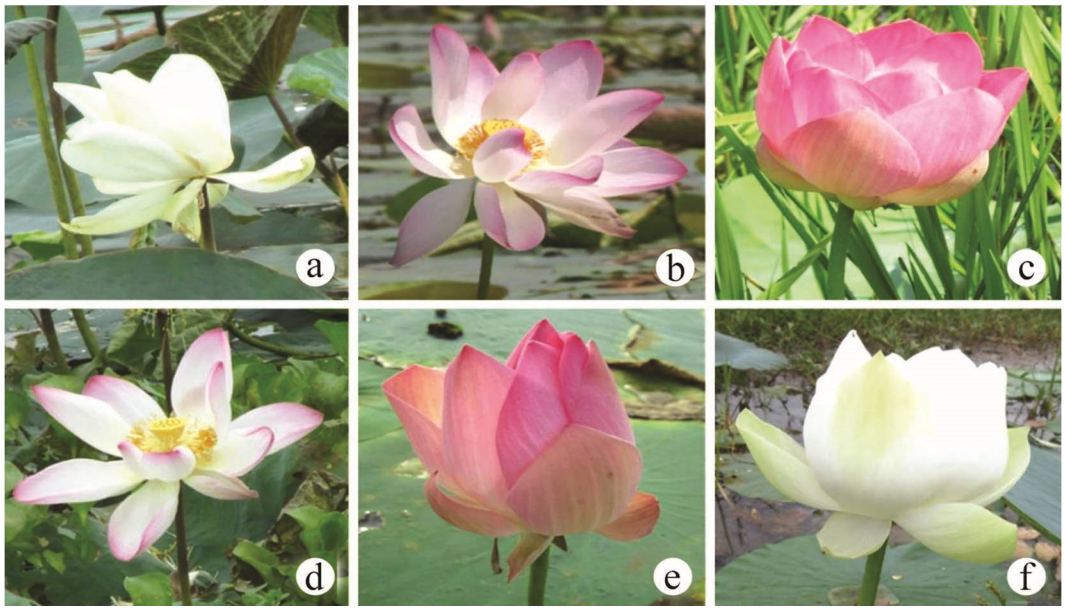

Fig. 1. External morphology (flowering stage) of collected garmplasm, (a) Kap.W (Kapasia-White); (b) Go (Gopalganj); (c) Khul (Khulna); (d) Raj (Rajshahi); (e) Co.P (Cumilla-Pink); and (f) Co.Y (Cumilla-Yellow). Co.Y had exceptional yellowish curvy petals.

The present experiment was conducted on lotus germplasm on fourteen characters for studying morphological variation where significant diversity was found for ten characters and four characters showed no variation among them at both 1% and 5% probability level. Thus, higher level of morphological variations was found among the collected lotus germplasm. Based on the data analyses Co.Y can be considered as superior among the germplasm studied and may be used for producing new variants. Morphological diversity analysis was also done by Guo *et al.* (2010)

on 40 lotus genotypes to see the evolutionary path and reported medium level of variations among the samples.

Table 2. Mean, Standard deviations (S.D.), F value and P value for each characteristics for all collected germplasm.

Parameter	Mean	S.D.	Maximum	Minimum	F value	P value
Leaf length (cm)	39.21	4.71	52.00	29.90	2.2652	0.0918
Leaf breadth (cm)	50.48	6.39	65.00	38.00	2.5097	0.0683
Petiole length (cm)	124.58	16.27	178.00	91.70	1.9041	0.1436
Petiole diameter (cm)	3.63	0.28	4.50	2.20	19.1646	<.0001
Petiole pickles' no.	22.83	4.57	41.00	11.00	5.8474	0.0022
Peduncle length (cm)	147.64	15.76	198.00	105.00	8.3333	0.0003
Peduncle diameter (cm)	3.25	0.26	4.00	2.20	2.7347	0.0523
Peduncle pickles no.	21.21	2.47	37.00	14.00	7.0064	0.0009
Number of petals	22.90	4.40	78.00	10.00	39.7521	<.0001
Petal length (cm)	11.87	0.83	16.00	7.20	23.6417	<.0001
Petal width (cm)	6.78	0.81	8.50	5.50	3.1629	0.0451
Number of stamens	224.25	33.45	365.00	151.00	2.8821	0.0592
Length of stamen (cm)	3.12	0.29	3.90	2.20	6.6776	0.0027
Number of seeds	13.58	1.60	20.00	8.00	17.3831	<.0001

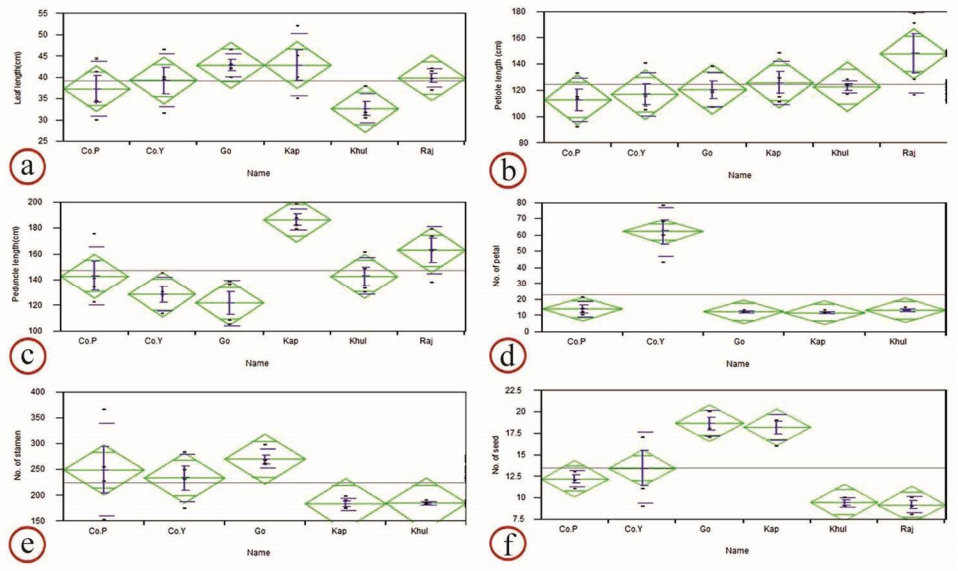

Fig. 2. Graphical representation of analysis of morphological variation of studied genotypes counting four replications in case of- (a) Leaf length; (b) Petiole length; (c) Peduncle length; (d) No. of petals; (e) No. of stamens and (f) No. of seeds. Moderate level of variations was found in all cases.

All the collected lotus germplasm had green to dark-green orbicular leaves except the germplasm collected from Khulna (Khul) which was brownish-green in colour. The petal colour of Kap.W and Go were whitish and Co.P and Khul were pinkish to dark pink. Petal of Co.Y showed distinct yellow colour which is different from other germplasm and also had petals with the shape of boat or curvy whereas other germplasm were elliptical in shape (Fig. 1).Thus Co.Y was considered as distinct from other germplasm. Hassan *et al.* (2020) analyzed the same germplasm collected from the same location and found similar differences and concluded the germplasm had many stamen petaloids, considered as a new cultivar *N. nucifera* 'Gomoti', newly found in Bangladesh.

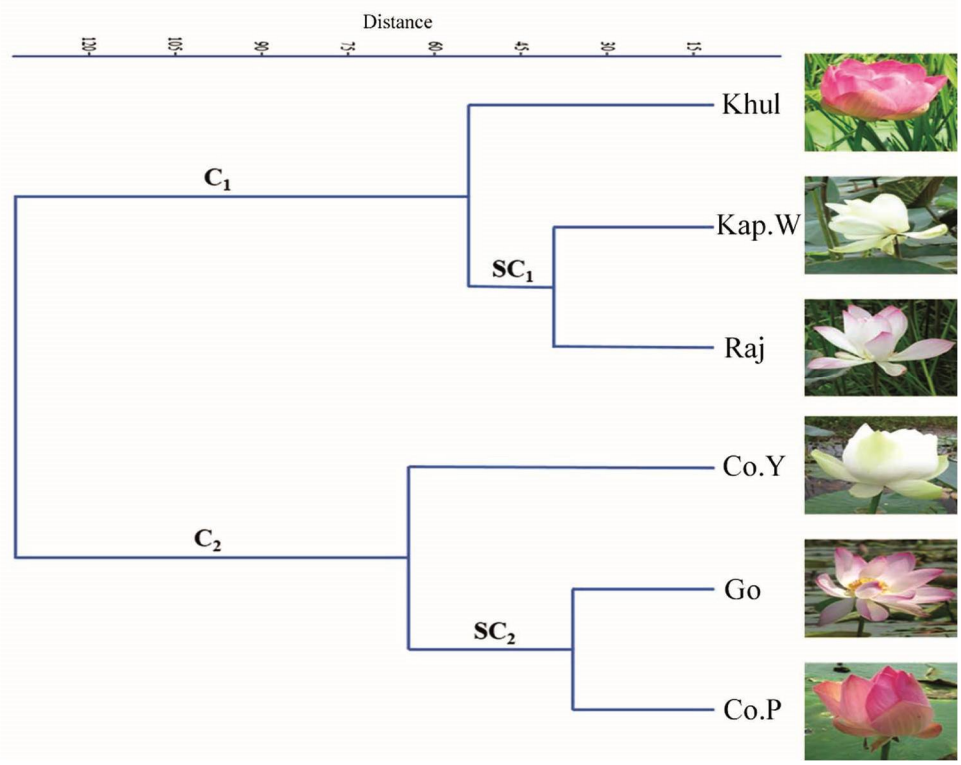

Fig. 3. Dendrogram based on summarized data on whole morphological differentiation among lotus germplasm according to Ward's method. C1 and C2 indicate Cluster 1 and Cluster 2 respectively. SC1 indicates sub-cluster 1 and SC2 indicates sub-cluster 2. Co.Y (Cumilla-Yellow) was most distantly related with others.

For assessing linkage among the germplasm based on their morphological characters, a dendrogram was constructed by Ward's method based on squared Euclidean distance in which the germplasm were grouped into two main clusters. The most closely related germplasm was Go and Co.P and the morphological variations of both the germplasm were minimum among all the germplasm. Co.Y was most distantly related with other germplasm (Fig. 3). So, Co.Y was morphologically most different from others. The same type of dendrogram was constructed by Guo *et al.* (2010) on selected lotus germplasm and found two clusters by cluster analysis.

The lotus is possessing important agronomic traits and the source of important genes which develop through natural selection. For taxonomic identification of all the collected germplasm,

mainly the exceptional yellowish lotus of Cumilla and to observe the phylogenetic relationships among lotus germplasm, the molecular method "Barcoding" was used in the present study. Two barcode genes from plastidial regions: *rpoB* and *rpoC1* were employed for multi-locus identification of the lotus germplasm. All the barcodes are not equally efficient to identify the germplasm. They varied in their rate of PCR amplification (Fig. 4), sequencing success and aligned sequence length (Table 3). PCR success for the barcodes *rpoB* and *rpoC1* were respectively 85.72% and 100%. But all the successfully amplified barcodes had 100% sequence success. Dang *et al.* (2021) did barcode of local lotus germplasm from Thua Thien Hue Province, Vietnam using three barcode genes *rbcL, matK* and *trnH-psbA* and got 100% PCR success. Again Dang *et al.* (2019) did barcode using *ITS4-5* genetic regionand got 100% PCR success. Sharma *et al.* (2012) did a similar experiment on the Mexican sedative and anxiolytic plant *Galphimia glauca* with *matK, rbcL* and *rpoC1,* and succeeded.

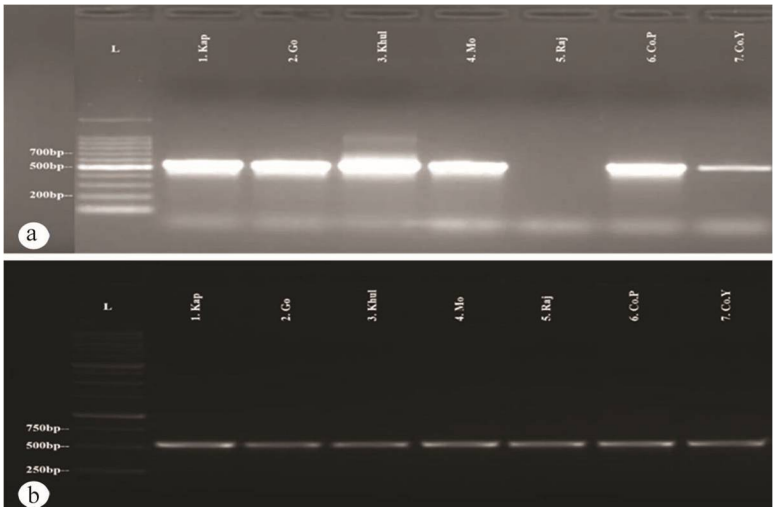

Fig. 4. Results of electrophoresis on 1% agarose gel of PCR products obtained with Barcode genes- *rpoB* and *rpoC1*. Lane L: DNA ladder (1 kb) and lane 1-7: amplified DNA of seven germplasm; (a) PCR products of*rpoB* gene. All except Raj showed positive band at ~550 bp; (b) PCR product of *rpoC1* gene. All lanes showed positive band at ~550 bp.

In multi-locus molecular identification system, Kap.W, Go, Khul, Mo, Co.P and Co.Y were successfully identified as *N. nucifera* with 83.30 to 100% identity. Raj was failed to be identified using multi-locus identification system, as it was only identified with *rpoC1* locus (Table 4). A similar type of study was carried out by Dang *et al.* (2021) for lotus using *rbcL, matK* and *trnH-psbA* in Vietnam. Jannat *et al.* (2020) did similar type multi-locus identification of Tomato with all the six Barcode primers and succeeded. de Vere *et al.* (2012) did DNA barcoding of the native flowering plants and conifers of Wales.

Table 3. Characteristics of each single barcodes.

Marker	PCR Success (%)	Sequencing Success (%)	Average aligned length (bp)
rpoB	85.72%	100%	429.00
rpoC1	100%	100%	366.43

Table 4. Multi-locus identification of the germplasm based on sequence analysis of barcode genes.

Sl. no.	Name of lotus germplasm	Sequence similarity found with the *Nelumbo* sp. (Accession no., % similarity and Query coverage) in NCBI BLAST search		Remarks
		rpoB	*rpoC1*	
1.	Kap.W	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 85.61%, 100%); *N. lutea* (JQ336992.1/FJ754269.1, 85.61%, 100%)	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 83.30%, 100%); *N. lutea* (JQ336992.1/FJ754269.1, 83.30%, 100%)	*N. nucifera*
2.	Go	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 93.59%, 100%); *N. lutea* (JQ336992.1/FJ754269.1, 93.59%, 100%)	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 92.28%, 55%); *N. lutea* (JQ336992.1/FJ754269.1, 92.28%, 55%)	*N. nucifera*
3.	Khul	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 87.42%, 100%); *N. lutea* (JQ336992.1/FJ754269.1, 87.42%, 100%)	*N. nucifera* (KY046359.1/ KY046358.1 and 8 others, 99.30%, 100%); *N. lutea* (JQ336992.1/FJ754269.1, 99.30%, 100%)	*N. nucifera*
4.	Mo	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 100%, 100%); *N. lutea* (JQ336992.1/FJ754269.1, 100%, 100%)	*N. nucifera* (KY046359.1/ KY046358.1 and 8 others, 94.22%, 99%); *N. lutea* (JQ336992.1/FJ754269.1, 94.22%, 99%)	*N. nucifera*
5.	Raj	No PCR amplification	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 84.44%, 54%); *N. lutea* (JQ336992.1/FJ754269.1, 84.44%, 54%)	Molecular identification failed
6.	Co.P	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 100%, 99%); *N. lutea* (JQ336992.1/FJ754269.1, 100%, 99%)	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 96.17%, 73%); *N. lutea* (JQ336992.1/FJ754269.1, 96.17%, 73%)	*N. nucifera*
7.	Co.Y	*N. nucifera* (KM655836.1/ KF009944.1 and 3 others, 99.53%, 100%); *N. lutea* (JQ336992.1/FJ754269.1, 99.53%, 100%)	*N. nucifera* (KY046359.1/ KY046358.1 and 12 others, 100%, 28%); *N. lutea* (JQ336992.1/ FJ754269.1, 100%, 28%)	*N. nucifera*

A Neighbor-joining phylogenetic tree was constructed for assessing the linkage among the germplasm based on the sequences obtained with barcode markers *rpoB* and *rpoC1*. In the neighbor-joining tree, bootstrap values for each node were estimated by 1000 replications. The germplasm made five major clusters regarding their sequences for different markers (Fig. 5). Dang *et al*. (2021) also constructed a dendrogram using *rbcL, matK* and *trnH-psbA* and found two major clusters. Jannat *et al.* (2020) used all the six Barcode primers and found similar type five main clades. Sharma *et al.* (2012) worked with *matK, rpoC1* and *rbcL* and constructed a bootstrap consensus phylogenetic tree based on the sequence obtained with the loci tested for.

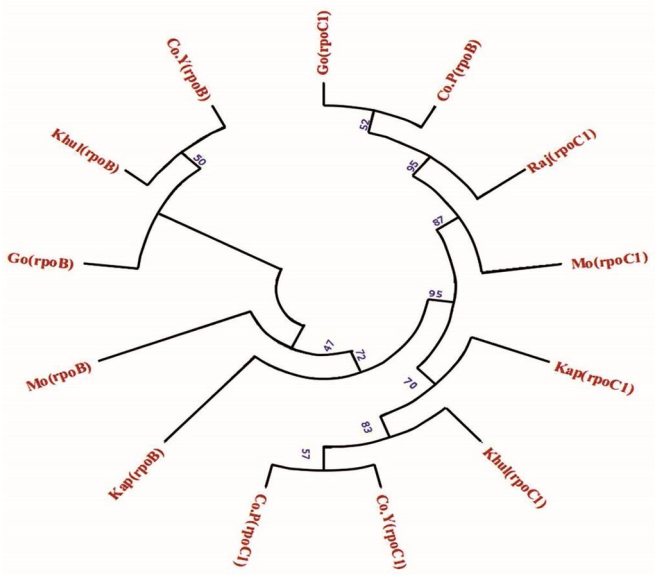

Fig. 5. Bootstrap consensus tree generated by Neighbor-joining method for *rpoB* and *rpoC1* sequences obtained for collected lotus germplasm. Evolutionary analyses were conducted in MEGA 11. Numbers below the branches are bootstrap values expressed as percentage of 1000 replicates.

It can be concluded that, all the germplasm presented a higher morphological variation and molecular analysis proved all the germplasm of pink, white, yellow as *N. nucifera*. Thus, these morphological variations may be occurred due to environmental effects. Again the germplasm Co.Y was identified as *N. nucifera* based on multi-locus molecular identification though it was primarily hypothesized as *N. lutea*. The current study could successfully identify the lotus germplasm based on its barcode sequences. The identified lotus germplasm further could be treated as a donor parent of an elite gene source. In order to conduct research on the lotus, there is an urgent need to enrich the available resources. These research data of genotypic variability can be a useful resource for the construction of high-density genetic maps, improving marker-assisted breeding and transgenic approaches. Nowadays, lotus is an endangered species as the wetland areas are shrinking due to population pressure and water pollution is increasing day by day through various anthropogenic activities, therefore, immediate action needs to be taken to conserve the germplasm of lotus.

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