

## **Genotypic Variability Analysis of Lotus (*Nelumbo nucifera* Gaertn.) from Bangladesh using RAPD and SSR Markers**

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### **Abstract**

Lotus (*Nelumbo nucifera* Gaertn.) is a perennial aquatic plant with high ecological, medicinal, ornamental, and economic value from the prehistoric times. Different types of genotypes of lotus found in Bangladesh which represent a great variability. However, investigations on the variability of the lotus at genetic level are insufficient and limited in Bangladesh. In the current study, fourteen samples of lotus were collected from six different locations of Bangladesh were subjected to molecular analysis using co-dominant Simple Sequence Repeats (SSRs), as well as, dominant Random Amplified Polymorphic DNA (RAPD) markers to estimate the genetic diversity, and to test the genetic basis relationships among the germplasm. Analysis of SSRs showed a moderate level of polymorphism which was 68.12% and Nei's genetic distance was ranging from 0.0328 to 0.7598 showing a significant level of variation among the genotypes. The germplasm collected from Kapasia identified as different and superior among the morphotypes. Moreover, RAPD analysis showed 60.44% polymorphism and Nei's genetic distance was ranging from 0.0600 to 0.5743 indicating a moderate level of variability where yellow lotus was identified as distinct. Two Neighbor-Joining dendrograms were constructed using UPGMA method in which the germplasm were clustered into two main clades with 55% genetic similarity level for both RAPD and SSR markers. The current study was successful in establishing an efficient method for evaluating the variation and the genetic marker system to be used to identify core *N. nucifera* accessions. The method also could further assist in designing of suitable strategic programs to preserve the core lotus gene pool.

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## Introduction

Lotus (*Nelumbo nucifera* Gaertn.), ( $2n = 16$ ) commonly known as sacred lotus is a basal eudicot belonging to family Nelumbonaceae. Due to its edible rhizomes, seeds and leaves, lotus has been cultivated as a vegetable or food for over 7000 years in Asia (Shen-Miller et al. 2002). Seeds of lotus have exceptional longevity, remaining viable for as long as 1300 years (Shen-Miller et al. 2002). Various lotus plant-parts like buds, flowers, anthers, stamens, fruits, leaves, stalks, rhizomes and roots have been used as herbal medicines for the treatment of many diseases including cancer, depression, diarrhoea, heart problems, hypertension and insomnia (Shen-Miller 2002, Duke et al. 2002). *Nelumbo nucifera* exhibits high potential for usage in wastewater treatment removing polluting compounds (Thongchai and Udomphon 2004) and heavy metals (Anawar et al. 2008, Virendra 2009, Gallego et al. 1996).

Indo-Malayan region is considered to be one of the centers of the origin of lotus. Hence, different types of genotypes of lotus found in Bangladesh. Moreover, the population differentiation of lotus is unclear. Although self-pollination is possible, lotus also has cross-pollination, which is usually mediated by insects. The resultant heterozygosity can be maintained as long as lotus undergoes vegetative propagation via rhizomes (Kubo et al. 2009). Previous genetic diversity studies have demonstrated that sacred lotus exhibits moderate polymorphism (Han et al. 2007, Pan et al. 2011). Morphological characters are often hard to distinguish because they can change due to variation within a cultivar or environmental effects. The published sequencing data of *N. nucifera* genome provided great insights for accession improvement through molecular breeding and unique features, including the longevity of its seeds and adaptation to aquatic environments (Ming et al. 2013, Wang et al. 2013). For the effective utilization, conservation and management of germplasm resources, knowledge of the extent of genetic variation among the germplasm and the genetic relatedness among genotypes is important (Kresovich et al. 1995, Davila et al. 1998, Matus and Hayes 2002). Here, some co-dominant and dominant molecular markers are applied including Simple Sequence Repeats (SSRs) (Xue et al. 2006, Han et al. 2007a, Chen et al. 2008, Tian et al. 2008a) and Random Amplification of Polymorphic DNA (RAPD) (Xue et al. 2006, Guo et al. 2007, Han et al. 2007b) to assess the genetic variability of lotus genotypes. The genetic variability of the lotus genome can be utilized to increase biotic and abiotic stress tolerance and to improve agronomic traits, such as quality, maturity and yield potentiality (Rai et al. 2013). The aim and objectives of the current research was to investigate genetic diversity and population differentiation of the prevailing lotus accessions collected from the different regions of Bangladesh through SSR and RAPD markers.

### Materials and methods

Fourteen lotus variants (Table 1) were collected from six different beels (Fig. 1) located throughout Bangladesh. Young, tender leaves from each samples were collected at the mature stage and washed thoroughly with distilled water and ethanol, and wiped off with clean tissue papers to remove spores of microorganisms and any sources of foreign DNA. Genomic DNA was extracted from the frozen leaves using a modified 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.

Ten SSRs and RAPD primer pairs each (Tables 2, 3) were chosen to evaluate the polymorphism among the genotypes. PCR conditions were optimized. The amplifications were performed in 25  $\mu$ l reaction volumes containing  $\frac{1}{2}$  volume of Go Taq G2 Green Master mix, 1.0  $\mu$ l each forward and reverse primers (100 pmoles/ $\mu$ l) and (30-40) ng genomic DNA for SSR markers and  $\frac{1}{2}$  volume of Go Taq G2 Green Master mix, 1.5  $\mu$ l primers (100 pmoles/ $\mu$ l) and same amount of genomic DNA for RAPD markers. PCR amplification was performed on a thermal cyler (Applied Biosystem).

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

Samples	Location	GPS	Lotus variants	pH value of water
1	Norait Beel, Bikertek, Barishab Union, Kapasia Upazila, Gazipur	24.2029072, 90.6645243	Kap.w-1	7.8
			Kap.w-2	7.8
2	Padma Beel, Kalabari Union, Kotalipara Upazila, Gopalganj	23.0861422, 89.9909445	Go-1	7.9
			Go-2	7.9
3	Bhutiari Beel, Terokhada Upazila, Khulna	23.0861422, 89.6965614	Khul-1	7.8
			Khul-2	7.9
4	Haram Beel, Baksimoil Union, Mohanpur Upazila, Rajshahi	24.5480723, 88.6380534	Mo-1	8.0
			Mo-2	8.0
5	Sarkerpara, Aahar, Pachandar Union, Tanore Upazila, Rajshahi	24.5902671, 88.4878373	Raj-1	7.9
			Raj-2	7.9
6	Dakshing Gram, Rajapur Union, Burichang Upazila, Cumilla	23.5755289, 91.1550685	Co.P-3	7.8
			Co.P-4	7.9
			Co.Y-5	7.9
			Co.Y-6	8.0

The PCR amplification conditions were as follows: Initial denaturation at 95°C for 3min, 35 cycles of 94°C for 1min, optimized annealing temperature (58-60°C) for 30s and 72°C for 1min followed by a final extension at 72°C for 7 min for SSRs and initial denaturation at 94°C for 3 min, 38 cycles of 90°C for 30 s, annealing temperature 35°C for 30s and 72°C for 1min followed by a final extension at 72°C for 10min for RAPD. PCR

products were stored at 4°C until use. The PCR amplified DNA was separated on 2% agarose gel at 90W for 60min in case of SSR markers and on 1% at 90W for 35min for RAPD, and stained with ethidium bromide (0.5 µg/ml). After electrophoresis, the gel was visualized in the Gel Documentation System (CSL-MDOCUV254/365 1D, Cleaver Scientific LTD, USA).

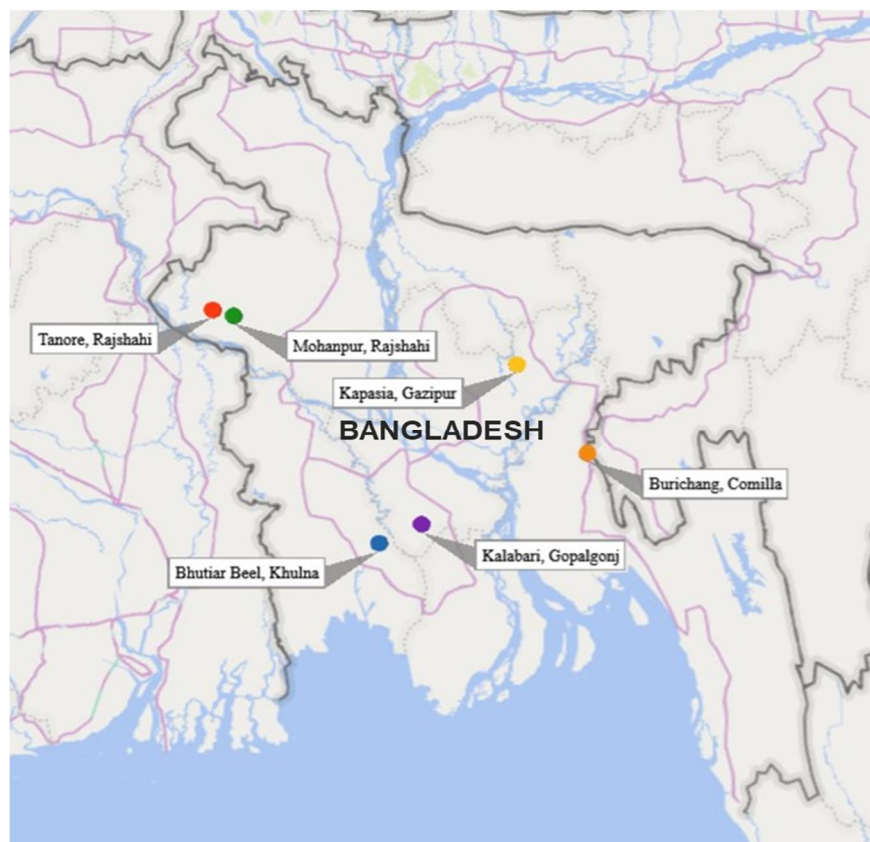


Fig. 1. Map of Bangladesh indicating the locations of *N. nucifera* variants are growing naturally. The experimental sites of sample collections were marked as large colored dots.

The sizes of amplification products were estimated by comparing the migration of each amplified fragment with that of a known size of ladder fragments (New England Biolabs Ltd.). All distinct bands or fragments were scored visually on the basis of their presence (1) or absence (0), separately for each individual sample per primer. The scores obtained using all primers were then combined separately to create a single data matrix for SSRs and RAPD, respectively. This was used for estimating genetic distance (D) and constructing a UPGMA dendrogram for fourteen collected sample of *N. nucifera* variant using a computer program, PopGen (Version 1.32).

## Results and discussion

The measurement of genotypic variability was assessed in 14 *N. nucifera* variant (Table 1), and a total of 483 bands were detected from PCR amplification of genomic DNA with the combinations of 10 SSR primers (Table 2). The number of average alleles per locus was 7.67 which was highly distinct compared to the results reported by Tian et al. (2008b) and Kubo et al. (2009) (3.88 and 3.9, respectively). The amplification produced bands ranged in size from 60bp to 550bp (Fig. 2). The percentage of polymorphic loci (P) was determined by all the ten primers and ranged from 18.84% (NnSSR-49) to 100% (NnSSR-17, NnSSR-64 and NnSSR-68) and the proportion of polymorphism in the collected germplasm was 68.12% which indicated a moderate level of polymorphism (Table 2). Previous work with SSR markers exhibited a low level of polymorphism for the distinction of the lotus accessions (Li et al. 2015, Hu et al. 2012).

**Table 2. No. of PCR amplification products and levels of polymorphism generated with SSR markers.**

Primer code	Sequences (5'→3')	Total bands	Polymorphic bands	Polymorphisms (%)	% of Average Polymorphisms
NnSSR-11	F: ATCCCCCTCCCTTCTCTCTA R: <b>ACAAGAGGGAGAAGAATTACGA</b>	42	14	33.33	68.12%
NnSSR-13	F: CTTAGATTTTCCCGCGCATC R: TGATGCCTTGCATTTGATA	25	11	44	
NnSSR-17	F: CGGGTGGTGATTTTCATTGTT R: GGTCTTCCTCAAACTCTCACG	35	35	100	
NnSSR-21	F: GGGGATTACCGTTAGGCTGT R: CAGTCCAACGTTCAATTGGTT	30	16	53.33	
NnSSR-30	F: TCCCAAGATTACCCCACTTT R: TGAGGGACTTGATAAGATGCAG	56	42	75	
NnSSR-49	F: GATGATTGGACGGACACTCC R: GGAAGTGCGGAACAGACAAT	69	13	18.84	
NnSSR-59	F: TTTGCATTGACAACGAGAGC R: GACATGCTCGGTGACTCGTA	28	14	50	
NnSSR-62	F: AATTGAGGAGGAGGAGGAG R: TGCTGGTAAAGTTGTGGGAAG	48	34	70.83	
NnSSR-64	F: CCGAAAATCCGTCTAGAATCA R: TCATCGGGTCGGTTTAGGTA	66	66	100	
NnSSR-68	F: CCTCTGGCCCTATCGAGAAT R: AGTGGCCAGTGCCACATATC	84	84	100	
Total		483	329		

Again, using 10 combinations of RAPD selective decamer primers, a total of 1097 polymorphic DNA bands were obtained from 14 germplasm. The number of alleles scored per locus was 10.53 and the range of bands size was 100bp to 2kb (Fig. 3). The range of polymorphism found for different primer combinations from 12.50% (OPC-05) to 100% (OPG-10, OPM-02 and OPN-20), with a mean of 60.44% (Table 3). In contrast,

Guo et al. (2007) found 88.72% polymorphism using similar types RAPD markers. Xue et al. (2006) analyzed the genetic diversity of wild lotus in Heilongjiang Province and found 71.68% polymorphism. Han et al. (2007) and Kim et al. (1998) after analyzing lotus also found dissimilar results with the high level of polymorphism.

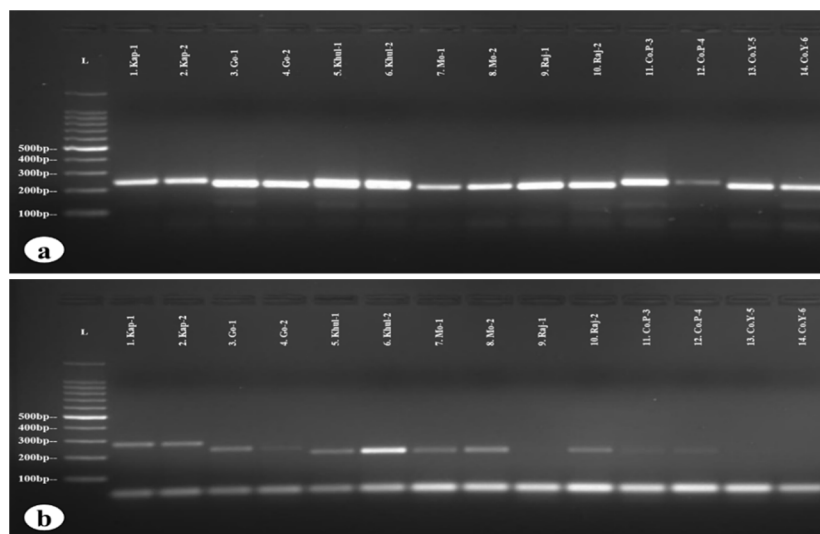


Fig. 2 (a-b). SSRs profile of fourteen lotus variants. (a) Amplification of bands with NnSSR-11 primer. Lane L: DNA ladder (100bp) and lane 1-14: amplified DNA of fourteen lotus samples; same as (a) but with (b) NnSSR-13.

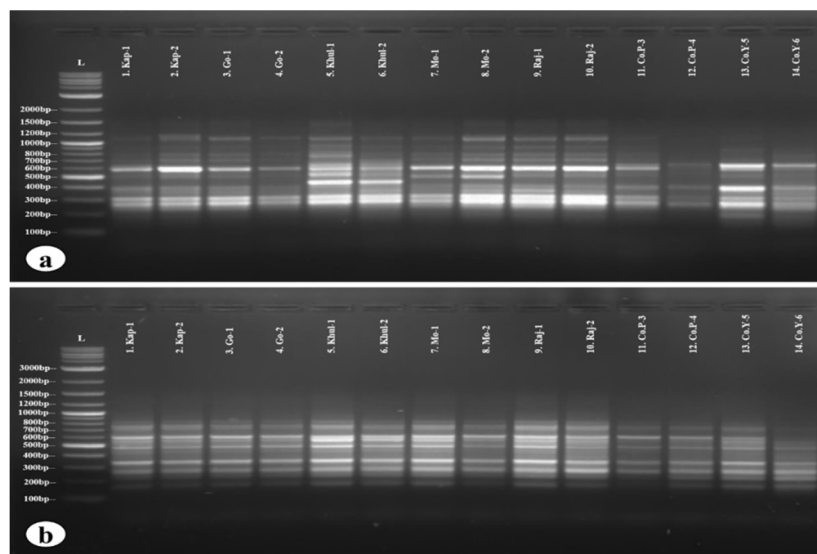


Fig. 3 (a-b). RAPD profile of fourteen collected lotus variants. (a) Amplification of bands with OPA-09 primer. Lane L: DNA ladder (1 kb) and lane 1-14: amplified DNA of fourteen lotus; same as (a) but with (b) OPC-02.

**Table 3. No. of PCR amplification products and level of polymorphism generated with RAPD markers.**

Primer code	Sequence (5'→3')	Total bands	Polymorphic bands	Polymorphisms (%)	% of Average Polymorphisms
OPA-09	GGGTAACGCC	158	102	64.56	60.44%
OPC-02	GTGAGGCGTC	132	34	25.76	
OPC-05	GATGACCGCC	96	12	12.50	
OPG-10	AGGGCCGTCT	117	117	100	
OPH-05	AGTCGTCCCC	85	29	34.12	
OPI-14	TGACGGCGGT	86	58	67.44	
OPM-02	ACAACGCCTC	96	96	100	
OPN-20	GGTGCTCCGT	124	124	100	
OPP-09	GTGGTCCGCA	99	71	71.72	
OPS-17	TGGGGACCAC	104	20	19.23	
<b>Total</b>		1097	663		

Genetic relationships among the 14 germplasm were regulated from an unweighted pair group method of arithmetic averages (UPGMA)-based dendrogram (Fig 4). The genetic similarity coefficient between genotypes varied from 0.0328 to 0.7598. All the collected accessions of *N. nucifera* were divided into 2 major clades present in the dendrogram. Germplasm Kap.w-1 and Kap.w-2 formed a branch (B<sub>1</sub>) which was totally different from others. That means Kap.w-1 and Kap.w-2 were superior in case of SSRs analysis. The rest other 12 genotypes clustered in B<sub>2</sub> which was further divided into two sub-branches where one sub-branch (SB<sub>1</sub>) contained Go-1 and Go-2 and other sub-branch (SB<sub>2</sub>) clustered into remnant ten different lotus samples. Hu et al. (2015) worked with Thailand Wild Sacred Lotus using similar types of SSR markers and found 3 major groups. Pan et al. (2011) analyzed 92 lotus accessions and found two clades in the phylogenetic tree. The genetic distances matrix was prepared from Nei's genetic distance and the highest genetic distance (0.7598) was observed in Kap-2 with Go-2, Mo-1 and Mo-2 where a comparatively lower distance (0.0328) was observed between Khul-1 and Khul-2.

The binary matrix of RAPD data was also used to construct UPGMA tree, and low genetic diversity was revealed in all lotus accessions with the genetic similarity coefficient values varying from 0.0600 to 0.5743. The dendrogram obtained from UPGMA grouped 14 accessions into two clusters (Fig. 5). The germplasm Co.P-3, Co.P-4, Co.Y-5 and Co.Y-6 formed a branch (B<sub>2</sub>) which was totally different from others and sub-divided into 2 sub-branches (SB<sub>1</sub> and SB<sub>2</sub>). SB<sub>1</sub> contained germplasm Co.P-3, Co.P-4 and Co.Y-5. On the other hand, SB<sub>2</sub> totally separated with Co.Y-6. So, the germplasm Co.Y-6 was mostly different from others. Another ten germplasm clustered in B<sub>1</sub> which was divided into 10 different sub-branches. Guo et al. (2007) worked with 65 lotus accessions and found 4 major clades. Han et al. (2007) also worked with lotus and found 2 major groups. Nei's genetic distance showed the highest distance (0.5743) between Raj-2 and Co.Y-6 and lowest distance (0.0600) between Khul-1 and Khul-2. These results indicated that

Co.Y-6 highly genetically diverged from the other germplasm studied in this investigation. The genotype Co.Y-6 showed as distinct type and could be served as special parental lines in breeding program.

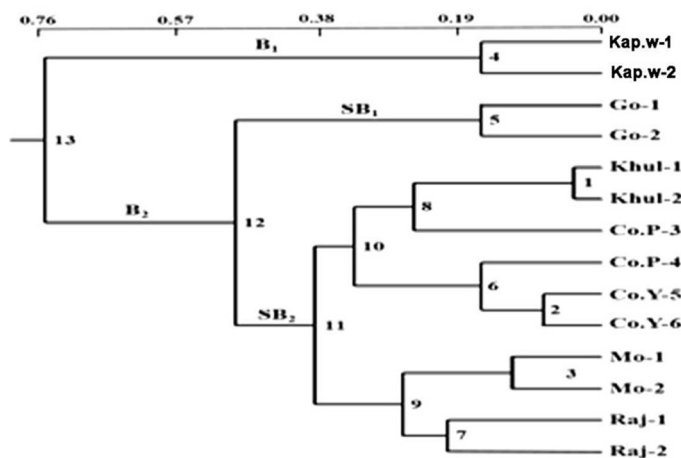


Fig. 4. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among 14 collected lotus variants by SSRs analysis. Kap.w-1 and Kap.w-2 were totally different from other germplasm.

*Nelumbo nucifera* has recently become an endangered species in Bangladesh due to climate changes, artificial insemination, geographical isolation and current fragmentation habitats. Therefore, understanding genetic variation at the population level is necessary. However, morphological variation occurred maximally due to phenotypic plasticity rather than genotypes. It is hard to determine the actual variation from the analysis of morphology. That's why molecular markers is being used to determine the actual genetic diversity. Molecular marker technology provides information that can help to analyze genetic diversity and their phylogenetic position. DNA marker is a new approach based on DNA polymorphism among tested genotypes and thus applicable to biological research. With the development of molecular markers, several recent studies have investigated the genetic variation of lotus using SSR and RAPD markers. In this investigation, both markers represented moderate level of genetic diversity of collected lotus germplasm throughout Bangladesh. The variation may be due to sexual reproduction via flowers as well as for a population bottleneck, a founder effect and a rebirth effect. The extent and pattern of genetic variation is correlated to life history and breeding system (Zeng et al. 2003). The species that were primarily outcrossing and long-lived, especially those with pollen and seed dispersed by bees and wind, have their genetic diversity mainly observed within populations (Newton et al. 1999). In contrast, Tian et al. (2008a) hypothesized that the low level of genetic diversity of lotus cultivars may be related to the 'bottleneck effect' combined with rapid asexual propagation after the Ice Age. Thus, the molecular marker system has the potential to be used in the design



of suitable strategic programs to preserve the core lotus gene pool and to identify core *N. nucifera* accessions.

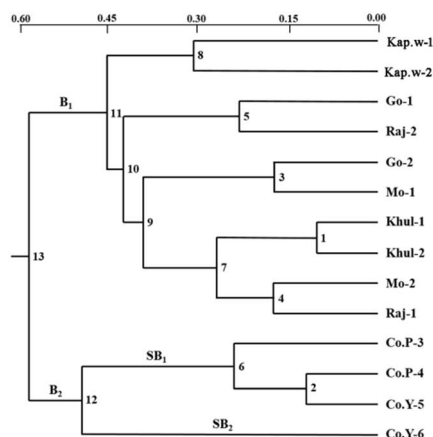


Fig. 5. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among 14 collected lotus variants by RAPD analysis. The variant Co.Y-6 was mostly different from others.

In conclusion, the results from this study showed a moderate level of variation present among the collected lotus germplasm. Based on the present findings, Kap-1, Kap-2 and Co.Y-6 considered superior and could be considered as the most valuable accessions. The current study indicated that although many accessions collected from different locations, they congregated into the same group. This study provides the knowledge for future basic and applied research related to *Nelumbo nucifera*. The results open a door for new opportunities in construction of core collection in lotus and breeding program.

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