



Genetic Relationship Assessment of Micropropagated *Nymphaea colorata* Peter Seedlings using HAT-RAPD

Itsaraphong Khaenthong^{1,2}, Theerachai Thanananta², and Narumol Thanananta^{3*}

¹ Division of Central Administration, Office of the President, Rajamangala University of Technology Thanyaburi, Pathum Thani, 12110, Thailand; <https://orcid.org/0000-0003-2307-577X>

² Faculty of Science and Technology, Thammasat University, Pathum Thani, 12120, Thailand

³ Faculty of Science and Technology, Valaya Alongkorn Rajabhat University Under The Royal Patronage, Pathum Thani, 13180, Thailand

* Correspondence: narumol@vru.ac.th

Citation:

Khaenthong, I.; Thanananta, T.; Thanananta, N. Genetic relationship assessment of micropropagated *Nymphaea colorata* Peter seedlings using HAT-RAPD. *ASEAN J. Sci. Tech. Report.* **2026**, 29(7), e262883. <https://doi.org/10.55164/ajstr.v29i7.262883>

Article history:

Received: December 19, 2025

Revised: May 10, 2026

Accepted: May 16, 2026

Available online: June 14, 2026

Publisher's Note:

This article is published and distributed under the terms of the Thaksin University.

Abstract: *Nymphaea colorata* Peter and its natural hybrids are typical African waterlily cultivars with beautiful, blue-flowered shading, which were precisely selected to be subjects in this study. We have proposed to assess the genetic relationships and variability of *in vitro* seedlings derived from cross pollination of three *N. colorata* cultivars. The surface-sterilized mature seeds were cultured on half Murashige and Skoog's (1/2MS) semi-solid medium supplemented with 1.5% (w/v) sucrose and 1.0 mg/L 6- (N⁶)-benzylaminopurine (6-BAP). After 4 weeks of culture, leafy-shooted seedlings (microgreens) were observed in all cultivars. For *ex situ* conservation and certain germplasm utilization, it is necessary to confirm that true-to-type waterlily species, wildtype and hybrid cultivars, are identified by their genetic backgrounds and genotyping. Thus, high-temperature random amplified polymorphic DNA (HAT-RAPD) markers were applied to examine genetic relationships among *in vitro* cultured seedlings compared with their field grown seed donor plants. Fourteen random primers were screened and selected from seventy-two universal primers for polymerase chain reaction (PCR) amplification and DNA fingerprinting. Genetic distances were represented as similarity coefficients ranging from 0.64 to 0.94 and then classified into two main clusters : the parental plant populations and their next generation populations. Therefore, HAT-RAPD might be effective for analyzing genetic relationships and variability in these waterlily cultivars. These scientific findings will support further conservation, breeding, genetic engineering and production of waterlily germplasm.

Keywords: *Nymphaea*; micropropagation; genetic relationship; HAT-RAPD

1. Introduction

Waterlilies are commonly known and called as 'Bua Sai' (in Thai), they are frequently represented to be the iconic queen of aquatic plants and aquarium landscapes, which includes an approximately 40 to 50 waterlily species and are the largest group in genus *Nymphaea* L., family Nymphaeaceae, followed by about discovered 800 waterlily species belonging to the order Nymphaeales [1, 2, 4, 7, 8, 9, 14, 15, 16, 17, 24, 25, 29, 31, 35]. Subgenus *Bachyceras* is recognized to various tropical day-blooming waterlilies, which are described as blooming time starting from 6.00 a.m. – 2.00 p.m. in each circadian rhythm. They are normally used for living plant collection, water gardening, traditional propagation, and

breeding [7, 8, 22, 25, 28, 29, 30, 35]. Members of this subgenus are considered diverse in both phenotypic and genotypic traits [3, 30]. Due to their pan-, core-, and niche-wide distribution, they are widely distributed and have adapted to propagate in several world regions, except Antarctica, under both hot and cold extreme conditions [20, 28, 29, 30, 32, 34]. However, different ecosystems, habitat fragmentation, and urbanization also affect their botanical characteristics and genetic backgrounds, both directly and indirectly. Likewise, *Nymphaea colorata* Peter (Subgenus *Bachyceras*, Genus *Nymphaea* L., Family Nymphaeaceae), a tropical day-blooming waterlily with floral uniqueness characteristics and notably blue flowered bloomer, is widely distributed from East Africa to the other tropical zones, which is usually used as a plant model in waterlily research [1, 5, 6, 10, 11, 18, 29, 30, 32, 33]. The niche conservation of native, exotic, and alien waterlily species has been investigated in numerous experiments and studies, thereby fostering species diversification in this group. In addition to *in situ* and *ex situ* conservation, *N. colorata* and its hybrids, including natural and artificial hybrid lines/cultivars, are chosen as plant models and plant materials for developing precision biotechnologies, especially mass-propagation and ornamental crop production systems, germplasm preservation, and breeding programs. For instance, *Nymphaea* ‘Chalong Khwan’ or *Nymphaea* ‘King of Siam’, an artificial waterlily hybrid with its commercially valuable traits of amaranth-flowered flowers, which was created by outcrossing between *Nymphaea* ‘Larpprasert’ and *Nymphaea colorata*. This waterlily hybrid cultivar was officially registered to the International Waterlily and Water Gardening Society (IWGS) by Thai breeder Chaiyaphon Thamasuwan [1, 7, 13, 18]. Moreover, the natural hybrids of *N. colorata* are also required for hybridization, varietal improvement, and genetic manipulation. Though the conservation and utilization of the waterlily genetic resource are necessary for understanding, they are essential for reliable, sustainable biological resource management within the ecosystem. In the context of novel agricultural biotechnologies, precision procedures are important for adaptive responses and innovation in the face of climate change. Thus, we aimed to develop suitable molecular markers for germplasm detection and genetic identification of *N. colorata* and its natural hybrids, which were tested in both parental plants and *in vitro* seedlings, and to assess the genetic relationships of these vulnerable waterlilies using HAT-RAPD markers.

2. Materials and Methods

2.1 Plant material preparation and *in vitro* seed culture

Mature seeds of three *N. colorata* cultivars obtained from outcrosses of the first day-bloomers as maternal (seed) plants with the second and third day-blooming flowers, which were from the same cultivar as paternal (pollen) plants, which caused from the protogynous phenomena (data were shown in figure 1A-F). After waterlily germplasm sampling, seed samples were used as explants and then disinfected with suitable sterilization protocols. Surface-sterilized seeds were cultured on half Murashige and Skoog’s (1/2MS) semi-solid medium supplemented with 1.5% (w/v) sucrose and 1.0 mg/L 6- (N⁶)-benzylaminopurine (6-BAP) for primary *in vitro* seed culture establishment. The results showed that germination and survival percentages of *in vitro* seedlings ranged approximately 30–45% after 4 weeks of culture. 1-2 cm-long, leafy-shooted seedlings representing microgreens were selected to test the genetic background and identify them, compared with their field grown seed donor plants. *In vitro* propagated seedlings were continuously maintained in standard tissue culture conditions until which were finally transplanted to the nursery [26, 27].

Table 1. List of twelve *N. colorata* accessions used in this study.

Sample No.	Sample Name: the concise details [†]	Sample Code
1	<i>Nymphaea colorata</i> Peter (a blue-petal cultivar; wildtype): Field-grown seed donor plant/wildtype #1	NCB1_WT
2	<i>Nymphaea colorata</i> Peter (a blue-petal cultivar): Field-grown seed donor plant/wildtype #2	NCB2
3	<i>Nymphaea colorata</i> Peter (a light purple-petal cultivar): Field-grown seed donor plant/natural waterlily hybrid line No. 1 #1	NCL1
4	<i>Nymphaea colorata</i> Peter (a light purple-petal cultivar): Field-grown seed donor plant/natural waterlily hybrid line No. 1 #2	NCL2

Table 1. List of twelve *N. colorata* accessions used in this study (continued).

Sample No.	Sample Name: the concise details [†]	Sample Code
5	<i>Nymphaea colorata</i> Peter (a purple-petal cultivar): Field-grown seed donor plant/natural waterlily hybrid line No. 2 #1	NCP1
6	<i>Nymphaea colorata</i> Peter (a purple-petal cultivar): Field-grown seed donor plant/natural waterlily hybrid line No. 2 #2	NCP2
7	<i>Nymphaea colorata</i> Peter (a blue-petal cultivar): <i>In vitro</i> grown seedling/wildtype #1	NCB_IS1
8	<i>Nymphaea colorata</i> Peter (a blue-petal cultivar): <i>In vitro</i> grown seedling/wildtype #2	NCB_IS2
9	<i>Nymphaea colorata</i> Peter (a light purple-petal cultivar): <i>In vitro</i> grown seedling/natural waterlily hybrid line No. 1 #1	NCL_IS1
10	<i>Nymphaea colorata</i> Peter (a light purple-petal cultivar): <i>In vitro</i> grown seedling/natural waterlily hybrid line No. 1 #2	NCL_IS2
11	<i>Nymphaea colorata</i> Peter (a purple-petal cultivar): <i>In vitro</i> grown seedling/natural waterlily hybrid line No. 2 #1	NCP_IS1
12	<i>Nymphaea colorata</i> Peter (a purple-petal cultivar): <i>In vitro</i> grown seedling/natural waterlily hybrid line No. 2 #2	NCP_IS2

[†] 12 accessions tested: the individually randomized plant samples were collected and selected from field grown seed donor plants and *in vitro* grown seedlings, which covered all *N. colorata* and its natural hybrids.

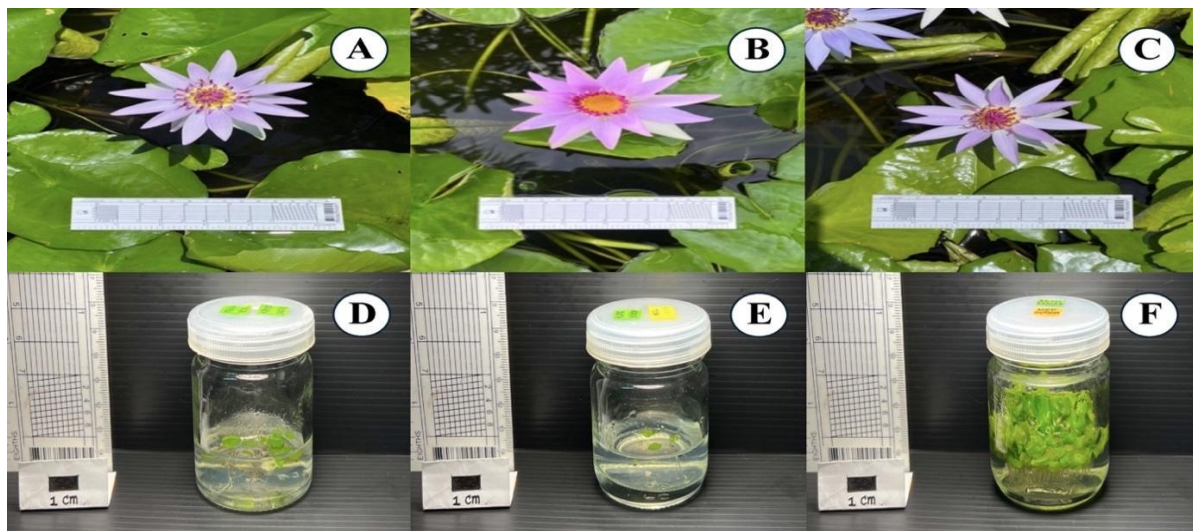


Figure 1. This is a picture of the (A) field grown seed donor plant of *N. colorata* (a blue-petal cultivar; wildtype), (B) field grown seed donor plant of *N. colorata* (a light purple-petal cultivar; natural waterlily hybrid line no. 1), (C) field grown seed donor plant of *N. colorata* (a purple-petal cultivar; natural waterlily hybrid line no. 2), (D) *in vitro* cultured seedlings of *N. colorata* (a blue-petal cultivar; wildtype), (E) *in vitro* cultured seedlings of *N. colorata* (a light purple-petal cultivar; natural waterlily hybrid line no. 1), and (F) *in vitro* cultured seedlings of *N. colorata* (a purple-petal cultivar; natural waterlily hybrid line no. 2). White ruler bar = 14 cm., Black bar = 1 cm.

2.2 DNA extraction and quantification

Fresh leaves were collected from vigorous seed donor plants that were planted and grown in the fields of Lotus and Waterlily Museum, Division of Central Administration, Office of the President, Rajamangala University of Technology Thanyaburi (RMUTT) (data were shown in figure 1A-C and table 1). Three cultivars of *N. colorata* were chosen to examine in this experiment, especially wildtype (a blue-petal cultivar), natural hybrid line no. 1 (a light purple-petal cultivar), and natural hybrid line no. 2 (a purple-petal cultivar). Healthy leaves were picked from the fields before pre-sterilization with mild detergent, then washed three times with running tap water. Fresh leaf samples were stored at -20 °C in a refrigerator before use. The Cetyltrimethyl ammonium bromide (CTAB) extraction used in this experiment was modified and then followed by the traditional CTAB extraction procedure that reported by Doyle and Doyle in 1987 [12, 16, 24, 25]. Genomic DNA (gDNA) pellets were dissolved in 500 µl TE buffer and stored at -20 °C. For *in vitro* cultured explant samples, about 100 mg of six-month-old *in vitro* *N. colorata* seedlings and its natural hybrids were also gently prepared for gDNA extraction using the modified CTAB extraction method as above (data showed in figure 1D-F and table 1) [12, 16, 24, 25]. The quantity of all stock gDNA samples was examined by spectrophotometer at the wavelength of optical density (OD) at 260 and 280 nanometer (nm), which were used to evaluate the amount of extracted gDNA samples, DNA concentration, and purification, respectively. At the same time, gDNA samples were also used for quality control by 0.8% (w/v) smart agarose gel electrophoresis and then visualized under UV trans-illumination.

2.3 Genetic analysis by high annealing temperature-random amplified polymorphic DNA (HAT-RAPD)

Polymerase chain reaction (PCR-based) technique was studied by using HAT-RAPD, which has been applied to investigate genetic relationship and identification in various *Nymphaea* species and their hybrids, and then reported by Suksakul et al., 2018 [16]. Seventy-two primers containing 12 nucleotides were also tested and produced DNA bands on the agarose gel. PCR reactions of the HAT-RAPD technique were applied and conducted in a BIO-RAD T100™ Thermal Cycler. A total reaction mixture was prepared for each PCR reaction, adjusted to 30 µl, containing about 100 ng/µl gDNA template. PCR amplification conditions were performed by involving in initial denaturation of 1 cycle of 94 °C for 3 minutes (the main step no. 1), following by the main step no. 2 contained 40 cycles of 94 °C for 45 seconds (Denaturation), 46 °C for 45 seconds (Annealing), 72 °C for 1 minute (Extension) and then a final cycle in the main step no. 3: 1 cycle of 72 °C for 5 minutes (Final Extension) [16]. After the thermal cycling procedure was finished, the amplified PCR products were stored at 4 °C overnight before the gel electrophoresis. PCR amplified products were electrophoresed on 1.5% (w/v) smart agarose gel that was incubated in 1X TAE (Tris-Acetate-EDTA) Buffer Solution at 100 V (Voltage), with running time (~30 minutes), depending on PCR product size, and stained with 6X ready-to-use (RTU) loading dye solution. The different stained DNA sizes of PCR amplicons were verified against a volume of 10 µl 0.1% (µg/µl) VC 100 bp Plus ready-to-use (RTU) DNA ladder molecular size standard (Vivantis Technologies Sdn Bhd, Malaysia), which was used as a DNA marker, compared to the negative control lane (deionized water, DI). The electrophoresed gels were stained with 0.1% (w/v) ethidium bromide (EtBr) solution for 10 minutes before gel documentation. The reproducibly obtained DNA fragments were repeated at least three times. Clearly, the resultant bands were counted and analyzed using a binary coding system (1/0). For all genotypes, amplified banding results were scored using a 1/0 coding method, where '1' indicated the presence of a DNA band and '0' its absence at the loci used for each HAT-RAPD primer. Numeric data generated from each primer were recorded and then compared between samples using the Numerical Taxonomy System Package (NTSYS-pc, version 2.01e) (Applied Biostatistics Inc., New York, USA) [16, 24, 25]. Leading to a genetic similarity index and phylogenetic dendrogram, the SimQual and SAHN modules were also used to compute the similarity coefficient, as the resultant similarity matrix was utilized for constructing an accurate phylogenetic dendrogram throughout an unweighted pair group method with arithmetic mean (UPGMA)-based technique (Applied Biostatistics Inc., New York, USA) [16, 24, 25].

3. Results and Discussion

Regarding to the micropropagated seedlings grown *in vitro*, no abnormal morphological characteristics or malformed leafy seedlings were observed; however, germination and growth performance showed significant differences among *N. colorata* cultivars and treatments. Fourteen primers containing twelve nucleotides were also screened and then selected that these HAT-RAPD markers for PCR product amplification and DNA fingerprinting on the 1.5% (w/v) smart agarose gel, especially primer A32, B22, B23, B27, B32, C21, C24, C28, C29, D23, E24, E32, F23, and F31 (data were shown in figure 2 and table 2). Depending on the HAT-RAPD technique, twenty-fifth waterlily accessions contained in both tropical and hardy waterlily hybrids have also been demonstrated by precisely selected seventeen primers out of seventy-two primers (i.e. A22, A24, A27, A29, A30, B21, C28, C29, C31, D23, D31, E22, E24, E32, F23, F25, and F31), which produced the total clearly scored of 226 bands (sized in ~ 200-3,000 bp). It gave a total polymorphic loci percentage of 98.67%. While the genetic similarities among all accessions ranged from 0.47 to 0.88, they were used to infer their genetic relationships with three major groups [16]. Therefore, genetic variation assessment and analysis could be investigated to understand the genetic basis of various biological phenomena in plants [21]. In particular, true-to-type plantlets grown *in vitro* have also been shown to exhibit clonal fidelity in tissue-cultured plants using DNA-based markers, such as ISSR, RAPD, and HAT-RAPD, which were investigated in *Aloe vera* and *Hedychium longicornutum* Griff. ex Baker [21, 26]. Interestingly, some HAT-RAPD markers, including primer E32, could be used to characterize *in vitro* regenerated banana plantlets (*Musa* spp., AAA genome) induced to mutate using gamma ray radiation [25]. From our molecular marker detection, the DNA amplicons showed clear bands, which were counted and scored in numeric results (data showed in Table 2). Numeric data from each effective primer yielded a total of 240 distinct DNA bands, with an average frequency of 17.14 bands per primer, spanning approximately 150-3000 base pairs (bps) (data showed in Table 2). The genetic distance matrix obtained for individual samples of three cultivars/populations according to the field grown seed donor plants and their seedlings grown *in vitro* showed that the genetic distance among twelve genotypes ranged from moderate to high values of 0.64 to 0.94, with an average of 0.82 (data were shown in figures 3-4). Hence, the polymorphic loci obtained from HAT-RAPD genotyping of appropriate markers were analyzed for total polymorphic loci percentage (73.33%) and then compared for genetic similarities between the samples using the program NTSYS-pc (data showed in figures 2-4 and table 2). From the accurately generated dendrogram, each sample was constructed and classified into two main clusters, especially cluster no. 1: field-grown seed donor plants and cluster no. 2: *in vitro* grown seedlings, respectively. Each clustering group revealed genetic relationships within and between *N. colorata* cultivars (data were shown in Figures 3 and 4). Whilst the genetic similarities within the same cultivars according to blue-petal cultivars versus (vs.) blue-petal cultivars, light-purple petal cultivars vs. light-purple petal cultivars, and purple-petal cultivars vs. purple-petal cultivars showed closer genetic relationship than another cultivar, except in some individuals of cluster no. 2 that showed the genetic variation during the outcrossing. Leading to some blue-petal *N. colorata* individual samples being particularly separated as another genotypic trait with different genetic inheritances, caused by occurrences and possibilities in both natural and artificial hybridization, apart from somaclonal variation in *in vitro* grown seedlings and plantlets. However, the specifically designed DNA-based markers and advanced high-throughput data derived from the multiple omics technologies are still more importantly applied to characterize and double-check in the genetic relationship assessment and variability of the living plants and certain germplasms of *N. colorata* than the one-trial or one-dimensional laboratory practice [1, 21, 22, 23, 24]. Using primer C24 of HAT-RAPD yielded a unique monomorphic set of PCR products and the lowest percentage of polymorphic loci (60%) among the others (data showed in Figure 2A-D and Table 2). By contrast, primer C21 not only yielded a monomorphic set of amplified PCR products, but also generated different alleles within the same locus, as shown in the alliant alleles, and this occurred in some individual plant samples. They are caused by genetic manipulation, for instance, gene introgression, nucleotide insertion, nucleotide deletion, nucleotide duplication, and so on (data were shown in Figure 2A-D and Table 2). Previous studies have confirmed the genetic uniformity and clonal fidelity of micropropagated *Aloe* plants derived from shoot-tip explants [21]. On the other hand, RAPD markers also showed consistent genetic uniformity and maintained genetic fidelity in the field-grown mother plants and *in vitro*-propagated plantlets of *H. longicornutum* [21].

Application of some efficient molecular markers in the tropical and hardy waterlilies, the selected DNA-based markers of ISSR, RAPD, PCR-RFLPs, HAT-RAPD and SCoT techniques and additive nuclear internal transcribed spacer (nrITS sized in approximately 900 bps) characterized sequences containing the partial regions of *18S*, *ITS1*, *5.8S*, *ITS2*, and *26S* rRNA genes were also used to identify and classified the interspecific, intersubgeneric (ISG) waterlily hybrid cultivars and several waterlily species [3, 4, 6, 7, 8, 15, 16, 17, 19, 21, 22, 23, 24, 25, 28, 29, 30]. Thus, we have aimed to assess and identify the genetic relationship and variation among the field grown seed donor or mother plants and *in vitro* grown seedlings by using reasonable molecular markers that will not be an arduous procedure for confirming the genetic relationship and authentication of *N. colorata* and its natural hybrids, but also used to characterize its artificial hybrids and mutant lines/cultivars in further studies.

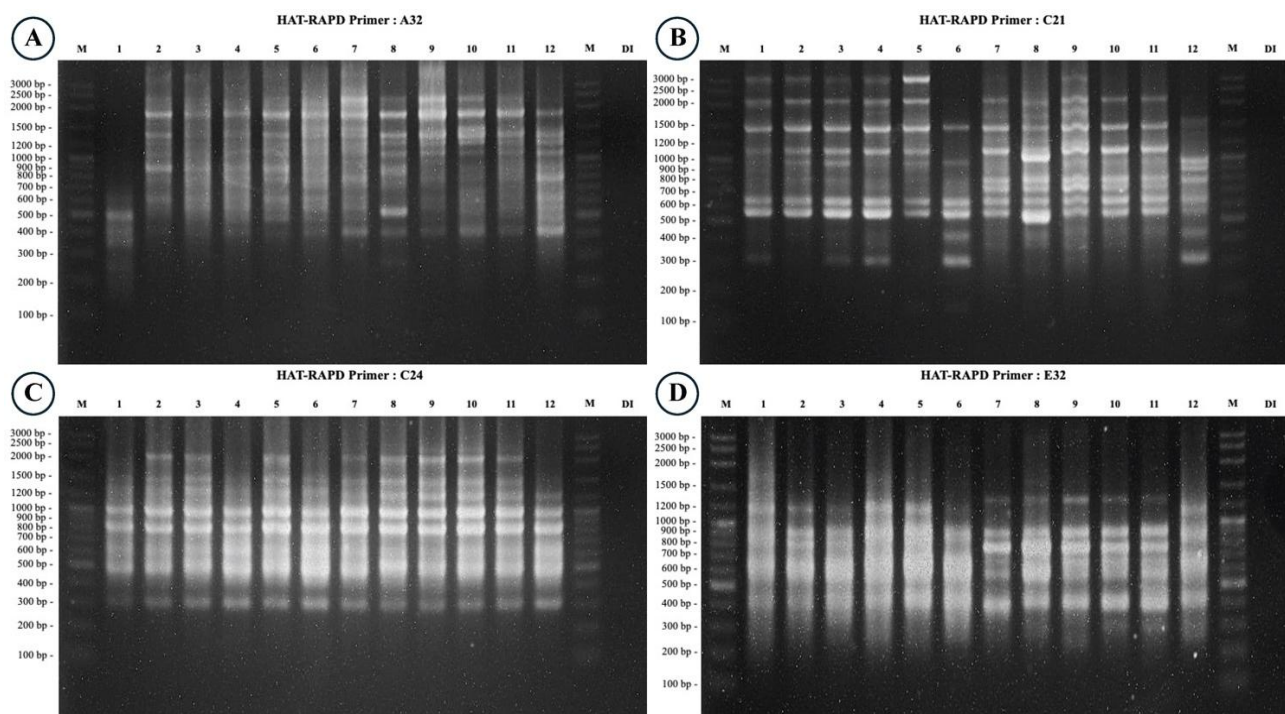


Figure 2. Representing pictures of polymerase chain reaction (PCR) amplified with the screened and selected high annealing temperature (HAT-RAPD) primers from *in vitro* cultured seedlings and their field grown seed donor plants of *N. colorata* and its natural hybrids. Primers used: (A) HAT-RAPD Primer-A32, (B) HAT-RAPD Primer-C21, (C) HAT-RAPD Primer-C24, and (D) HAT-RAPD Primer-E32. Lane M represents 100 bp Plus DNA ladder RTU, Lane DI represents deionized water (negative control), Lanes 1-6 represent field-grown seed donor plants of *N. colorata* and its natural hybrids, and Lanes 7-12 represent *in vitro* cultured seedlings of *N. colorata* and its natural hybrids. The sample loading lanes (lanes 1-12) were assigned to the sample codes in Table 1.

Table 2. List of high annealing temperature – random amplified polymorphic DNA (HAT-RAPD) primers used for genetic relationship and fidelity assessment of *in vitro* cultured *N. colorata* seedlings, compared to their field grown seed donor plants. The nucleotide sequences of selected primers, PCR product sizes, the number of total bands, and the percentage of polymorphic loci are shown in the column.

Primer name	Nucleotide sequence (5' → 3')	Approximately amplified DNA range/ PCR products sized (bp)	Total scorable bands (Sb/p [†])	Polymorphic loci examined with scorable bands	Polymorphic loci percentage (%)
A32	TTGCCGGGACCA	200 – 2250	20	14	70.00
B22	GGTGACTGGTGG	300 – 2250	16	13	81.25
B23	GGTGCCGGAGCA	300 – 3000	19	14	73.68
B27	GGCGGTTATGAA	150 – 2250	20	14	70.00
B32	ATCGCGGCTTAT	250 – 2250	15	12	80.00
C21	GGAGAGCGGACG	150 – 3000	23	18	78.26
C24	CCTTGGCATCGG	300 – 2250	15	9	60.00
C28	GTCGACGCATCA	300 – 3000	13	9	69.23
C29	GTCGCCTTACCA	150 – 3000	17	11	64.71
D23	ACCATCAAACGG	300 – 1800	12	10	83.33
E24	CCGGAGTGGATG	200 – 2500	18	14	77.78
E32	CAGGAACAGCAA	250 – 1300	12	8	66.67
F23	CCATCCGCACGA	200 – 3000	20	15	75.00
F31	ATCGTGACGCCG	200 – 2750	20	15	75.00
Total numeric result (s)		~ 150 – 3000	240	176	73.33

† Sb/p = total scorable amplified DNA bands per primer.

NCB1 WT	1.00											
NCB2	0.91	1.00										
NCL1	0.87	0.92	1.00									
NCL2	0.82	0.86	0.94	1.00								
NCP1	0.82	0.84	0.87	0.88	1.00							
NCP2	0.76	0.80	0.82	0.84	0.86	1.00						
NCB IS1	0.68	0.71	0.74	0.74	0.73	0.76	1.00					
NCB IS2	0.64	0.66	0.66	0.66	0.70	0.67	0.81	1.00				
NCL IS1	0.68	0.72	0.73	0.71	0.72	0.72	0.91	0.81	1.00			
NCL IS2	0.67	0.72	0.72	0.71	0.72	0.74	0.84	0.74	0.89	1.00		
NCP IS1	0.67	0.69	0.71	0.70	0.68	0.70	0.81	0.73	0.83	0.84	1.00	
NCP IS2	0.68	0.69	0.71	0.71	0.71	0.73	0.75	0.67	0.74	0.79	0.86	1.00
Sample	NCB1 WT	NCB2	NCL1	NCL2	NCP1	NCP2	NCB IS1	NCB IS2	NCL IS1	NCL IS2	NCP IS1	NCP IS2

Figure 3. The similarity coefficient matrix of HAT-RAPD data of the 12 *N. colorata* accessions with 14 primers.

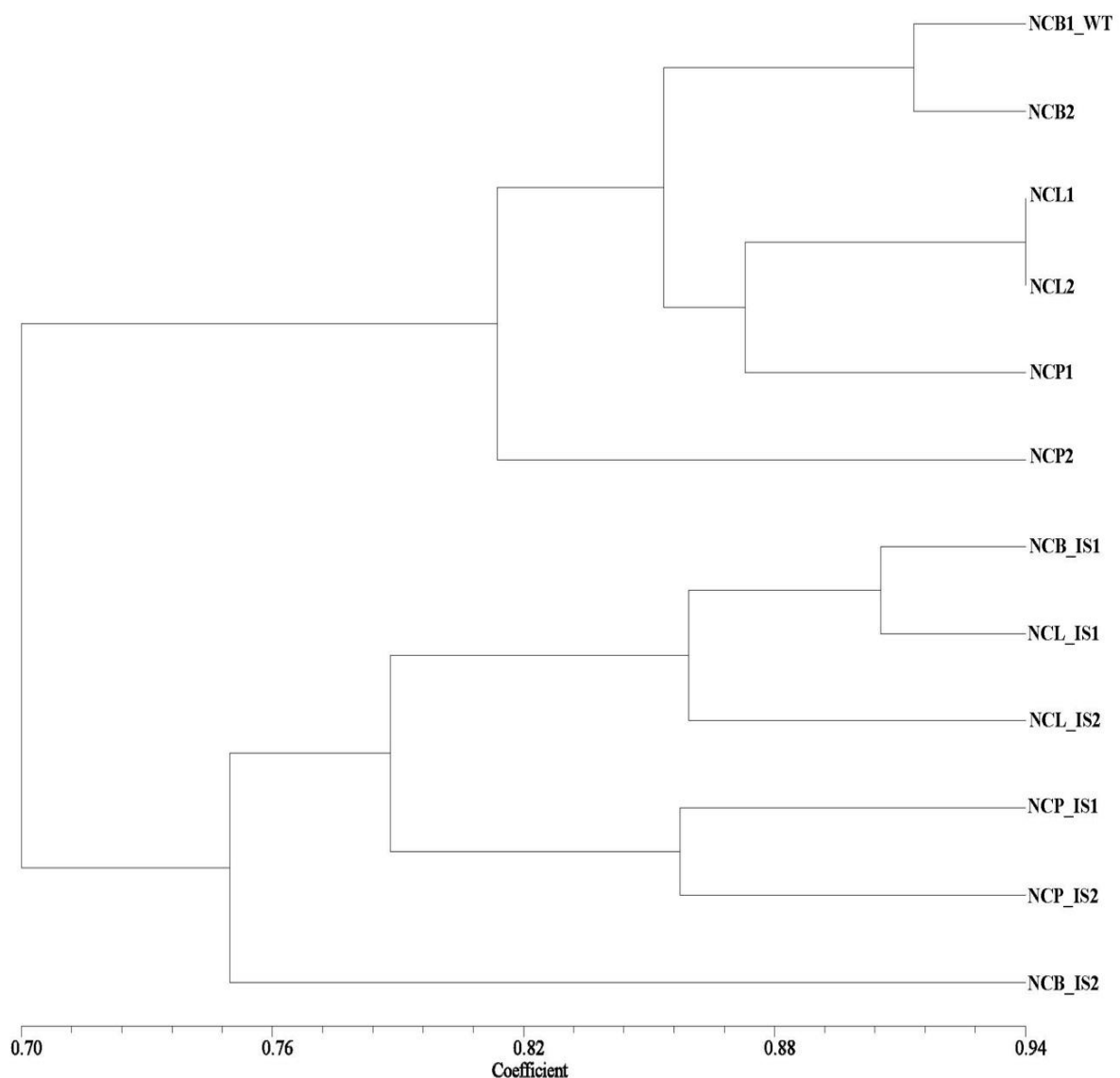


Figure 4. Phylogenetic dendrogram constructed by cluster analysis (UPGMA) of HAT-RAPD data of twelve *N. colorata* accessions with 14 primers.

4. Conclusions

This is the HAT-RAPD application specifically reported for *N. colorata* accessions and their *in vitro* progenies, which distinguishes it from previous studies. It has the potential to serve as molecular markers for assessing genetic relationships, species-specific identification, authentication, variability, and heterogeneity that expected due to reciprocal outcrosses and cross pollination among the field grown seed donor plants. However, *in vitro* seed culture and storage of these *N. colorata* cultivars will be demonstrated in further studies. Our research results not only served as a precise database for micropropagation and genetic characterization of *N. colorata* and its hybrids, but also developed effective molecular markers for seed-derived plants, true-to-type species, and pedigree analysis. Hence, a combination of plant biotechnological approaches would be useful for establishing *in situ* and *ex situ* conservation of all *N. colorata* hybrid lines/cultivars. There are also opportunities to apply in commercial ornamental crop production, breeding technology, genetic engineering, and the utilization of certain waterlily germplasm resources.

5. Acknowledgments

This research was financially supported by the Internationalization, Education and Research Promotion Scholarship from Thammasat University (TU) (Contract No. 4/2563). At the same time, Itsaraphong Khaenthong would like to thank all academic and supporting staffs driven the Doctor of Philosophy Program in Biotechnology and Agriculture, Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Campus, and the executives and the up-skilled/re-skilled university staff committees for supporting the personnel development funds for education and research that approved and provided by Rajamangala University of Technology Thanyaburi (RMUTT) due to related authors during year 2020-2026, which were referred to the Contract No. 0022/2563, Contract No. 0001/2567, Contract No. 0002/2567, Contract No. 0005/2567, Contract No. 0006/2567, Contract No. 0004/2568, Contract No. 012/2569 and the related RMUTT official working contracts and commitments.

The authors also acknowledge the full supports, which are perfectly provided by Lotus and Waterlily Museum, Division of Central Administration, Office of the President, Rajamangala University of Technology Thanyaburi (RMUTT), joined with Plant Genetic Conservation Project Under The Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) and Department of Biotechnology, Faculty of Science and Technology, Thammasat University (TU), Rangsit Campus, Pathum Thani, Thailand for providing all laboratory equipment and facilities, according to waterlily genetic resources and germplasms, living plant materials grown in the field study sites and sampling.

Author Contributions: Conceptualization, I.K., T.T., and N.T.; methodology, I.K., T.T., and N.T.; software, I.K.; validation, I.K.; formal analysis, I.K., T.T., and N.T.; investigation, I.K., T.T., and N.T.; resources, I.K., T.T., and N.T.; data curation, I.K.; writing—original draft preparation, I.K., T.T., and N.T.; writing—review and editing, I.K., T.T., and N.T.; visualization, T.T. and N.T.; supervision and project administration. All authors have read and agreed to the published version of the manuscript.

Funding: During year 2020-2026, this research was funded by Internationalization, Education and Research Promotion Scholarship from Thammasat University (TU) (Contract No. 4/2563) and the personnel development funds for education and research from Rajamangala University of Technology Thanyaburi (RMUTT), which were referred to the Contract No. 0022/2563, Contract No. 0001/2567, Contract No. 0002/2567, Contract No. 0005/2567, Contract No. 0006/2567, Contract No. 0004/2568, Contract No. 012/2569 and the related RMUTT official working contracts and commitments.

Conflicts of Interest: The authors declare no conflict of interest.

References

- [1] Yu, C.; Qiao, G.; Qiu, W.; Yu, D.; Zhou, S.; Shen, Y.; Yu, G.; Jiang, J.; Han, X.; Liu, M.; Zhang, L.; Chen, F.; Chen, Y.; Zhuo, R. Molecular breeding of water lily: Engineering cold stress tolerance into tropical water lily. *Hortic. Res.* **2018**, *5*, 73. <https://doi.org/10.1038/s41438-018-0086-2>
- [2] Borsch, T.; Hilu, K. W.; Wiersema, J. H.; Löhne, C.; Barthlott, W.; Wildes, V. Phylogeny of *Nymphaea* (Nymphaeaceae): Evidence from substitutions and microstructural changes in the chloroplast *trnT-trnF* region. *Int. J. Plant Sci.* **2007**, *168*(5), 639–671. <https://doi.org/10.1086/513476>
- [3] Nzei, J. M.; Médez, N. M.; Mwanzia, V. M.; Kurauka, J. K.; Wang, Q. F.; Li, Z. Z.; Chen, J. M. Climatic niche evolution and niche conservation of *Nymphaea* species in Africa, South America, and Australia. *BMC Plant Biol.* **2024**, *24*, 476. <https://doi.org/10.1186/s12870-024-05141-1>
- [4] Borsch, T.; Wiersema, J. H.; Hellquist, C. B.; Löhne, C.; Govers, K. Speciation in North American water lilies: Evidence for the hybrid origin of the newly discovered Canadian endemic *Nymphaea lorianae* sp. nov. (Nymphaeaceae) in a past contact zone. *Botany* **2014**, *92*, 867–882. <https://doi.org/10.1139/cjb-2014-0060>
- [5] Löhne, C.; Borsch, T.; Jacobs, S. W. L.; Hellquist, C. B.; Wiersema, J. H. Nuclear and plastid DNA sequences reveal complex reticulate patterns in Australian waterlilies (*Nymphaea* subgenus *Anecphyia*, Nymphaeaceae). *Aust. Syst. Bot.* **2008**, *21*, 229–250. <https://doi.org/10.1071/SB07010>

- [6] Borsch, T.; Löhne, C.; Wiersema, J. Phylogeny and evolutionary patterns in Nymphaeales: Integrating genes, genomes and morphology. *Taxon* **2008**, 57(4), 1052–1081. <https://doi.org/10.1002/tax.574004>
- [7] Sangsri, N.; Kuleung, C.; Hongtrakul, V. Analysis of nrITS sequence in Thai waterlilies, *Nymphaea* spp. *Thai J. Sci. Technol.* **2018**, 8(3), 258–270.
- [8] Songpanich, P.; Hongtrakul, V. Intersubgeneric cross in *Nymphaea* spp. L. to develop a blue hardy waterlily. *Sci. Hortic.* **2010**, 124, 475–481. <https://doi.org/10.1016/j.scienta.2010.01.024>
- [9] Parveen, S.; Singh, N.; Adit, A.; Kumaria, S.; Tandon, R.; Agarwal, M.; Jagannath, A.; Goel, S. Contrasting reproductive strategies of two *Nymphaea* species affect existing natural genetic diversity as assessed by microsatellite markers: Implications for conservation and wetlands restoration. *Front. Plant Sci.* **2022**, 13, 773572. <https://doi.org/10.3389/fpls.2022.773572>
- [10] Murashige, T.; Skoog, F. A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiol. Plant.* **1962**, 15(3), 473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- [11] Khan, S.; Al-Qurainy, F.; Nadeem, M. Biotechnological approaches for conservation and improvement of rare and endangered plants of Saudi Arabia. *Saudi J. Biol. Sci.* **2012**, 19(1), 1–11. <https://doi.org/10.1016/j.sjbs.2011.11.001>
- [12] Doyle, J. J.; Doyle, J. L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, 19(1), 11–15.
- [13] Noimai, Y.; Charoenwattana, P. Effects of BA and IAA on *in vitro* shoot induction of *Nymphaea* sp. 'King of Siam'. *Agric. Sci. J.* **2012**, 43(2, Suppl.), 249–252.
- [14] Khaenthong, I.; Chuenboonngarm, N.; Muengkrut, A. *In vitro* self and cross pollinated seed culture of *Nymphaea rubra* 'Maeploi', a night-blooming tropical waterlily hybrid (Nymphaeaceae) from Thailand. *Res. J. Rajamangala Univ. Technol. Thanyaburi* **2021**, 20(1), 123–133. <https://doi.org/10.14456/rj-rmutt.2021.12>
- [15] Pereva, P. I. P.; Attanayaka, D. P. S. T. G.; Yakandawala, K.; Yakandawala, D. M. D.; Fernando, C. N.; Arachchi, U. P. E.; Gunawardhana, W. P. D. S.; Weerashighe, W. D. C. D. *In vitro* culture of *Nymphaea nouchali* seeds: A conservation approach for a vulnerable species. *J. Natl. Sci. Found. Sri Lanka* **2021**, 49(3), 393–402. <https://doi.org/10.4038/jnsfsr.v49i3.10305>
- [16] Suksakul, S.; Thanananta, T.; Thanananta, N. Genetic relationship assessment and identification of *Nymphaea* sp. and their hybrids using HAT-RAPD. *Thai J. Sci. Technol.* **2018**, 7(4, Suppl.), 418–426. <https://doi.org/10.14456/tjst.2018.39>
- [17] Poczai, P.; Mátyás, K. K.; Szabó, I.; Vanga, I.; Hyvönen, J.; Cernák, I.; Gorji, A. M.; Decsi, K.; Taller, J. Genetic variability of thermal *Nymphaea* (Nymphaeaceae) populations based on ISSR markers: Implications on relationships, hybridization, and conservation. *Plant Mol. Biol. Rep.* **2011**, 29, 906–918. <https://doi.org/10.1007/s11105-011-0302-9>
- [18] Rodboot, N.; Yenchon, S.; Te-chato, S. Optimization of explant sterilization and plant growth regulators for enhancing the *in vitro* propagation of *Nymphaea colorata* Peter. *Plant Cell Tissue Organ Cult.* **2024**, 159, 59. <https://doi.org/10.1007/s11240-024-02911-5>
- [19] Williams, J. G. K.; Kubelik, A. R.; Livak, K. J.; Rafalski, J. A.; Tingey, S. V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **1990**, 18(22), 6531–6535. <https://doi.org/10.1093/nar/18.22.6531>
- [20] Lin, F.; Kang, Y.; Huqail, A. A. A.; Alshehri, D.; Li, Y.; Guo, Y.; Yang, G.; Tang, F.; Yin, J.; Ding, Z.; Eissa, M. A. Novel cultivation techniques for water lily (*Nymphaea micrantha* Guill. & Perr.) production based on *in vitro* technology. *Plant Methods* **2025**, 21, 56. <https://doi.org/10.1186/s13007-025-01377-6>
- [21] Khatun, M. M.; Tanny, T.; Yesmin, S.; Salimullah, M.; Alam, I. Evaluation of genetic fidelity of *in vitro*-propagated *Aloe vera* plants using DNA-based markers. *ScienceAsia* **2018**, 44, 87–91. <https://doi.org/10.2306/scienceasia1513-1874.2018.44.087>
- [22] Chen, F.; Liu, X.; Yu, C.; Chen, Y.; Tang, H.; Zhang, L. Water lilies as emerging models for Darwin's abominable mystery. *Hortic. Res.* **2017**, 4, 17051. <https://doi.org/10.1038/hortres.2017.51>
- [23] Dong, S.; Zhao, C.; Chen, F.; Liu, Y.; Zhang, S.; Wu, H.; Zhang, L.; Liu, Y. The complete mitochondrial genome of the early flowering plant *Nymphaea colorata* is highly repetitive with low recombination. *BMC Genomics* **2018**, 19, 614. <https://doi.org/10.1186/s12864-018-4991-4>

- [24] Suksakul, S.; Thanananta, T.; Thanananta, N. Genetic relationship assessment and identification of *Nymphaea* sp. and their hybrids using SCoT markers. *Thai J. Sci. Technol.* **2018**, 7(6), 570–579.
- [25] Kashima, S.; Thanananta, T.; Kachonpadungkitti, Y.; Bodhipadma, K. Induced mutation in *Musa* spp. genomes AAA using acute gamma ray and determination with HAT-RAPD markers. *Thai J. Sci. Technol.* **2020**, 9(5), 668–679.
- [26] Chuengpanya, R.; Muangkroot, A.; Jenjittikul, T.; Thammasiri, K.; Umpunjun, P.; Viboonjun, U.; Chuenboonngarm, N. *In vitro* propagation and genetic fidelity assessment of *Hedychium longicornutum* Griff. ex Baker, a vulnerable zingiberaceous plant of Thailand. *Curr. Appl. Sci. Technol.* **2022**, 22 (6), 1–21. <https://doi.org/10.55003/cast.2022.06.22.012>
- [27] Rittirat, S.; Thammasiri, K.; Klaocheed, S. *In vitro* rapid multiplication of a highly valuable ornamental aquatic plant *Anubias heterophylla*. *Trends Sci.* **2021**, 18 (19), 3. <https://doi.org/10.48048/tis.2021.3>
- [28] Zhang, L.; Chen, F.; Zhang, X.; Li, Z.; Zhao, Y.; Lohaus, R.; Chang, X.; Dong, W.; Ho, S. Y. W.; Liu, X.; Song, A.; Chen, J.; Guo, W.; Wang, Z.; Zhuang, Y.; Wang, H.; Chen, X.; Hu, J.; Liu, Y.; Tang, H. The water lily genome and the early evolution of flowering plants. *Nature* **2020**, 577(7788), 79–84. <https://doi.org/10.1038/s41586-019-1852-5>
- [29] Tang, H.; Zhang, L.; Chen, F.; Zhang, X.; Chen, F.; Ma, H.; Van de Peer, Y. *Nymphaea colorata* (Blue-Petal Water Lily). *Trends Genet.* **2020**, 36 (9), 718–719. <https://doi.org/10.1016/j.tig.2020.06.004>
- [30] Xiong, X.; Zhang, J.; Yang, Y.; Chen, Y.; Su, Q.; Zhao, Y.; Wang, J.; Xia, Z.; Wang, L.; Zhang, L.; Chen, F. Water lily research: Past, present, and future. *Trop. Plants* **2023**, 2(1), 1–8. <https://doi.org/10.48130/TP-2023-0001>
- [31] Khaenthong, I.; Phairin, U.; Mattisinlapin, P.; Subudom, S.; Sripan, S.; Boonthongto, A.; Malaithong, S. The effects of seed coat scarification, priming, and cold stratification on seed germination of a tropical night-blooming waterlily hybrid *Nymphaea rubra* 'Maeploi'. *Acta Hort.* **2020**, 1298, 581–586. <https://doi.org/10.17660/ActaHortic.2020.1298.80>
- [32] Orban, I.; Bouharmont, J. Megagametophyte development of *Nymphaea nouchali* Burm. f. (Nymphaeaceae). *Bot. J. Linn. Soc.* **1998**, 126, 339–348. <https://doi.org/10.1111/j.1095-8339.1998.tb01386.x>
- [33] Rodboot, N.; Te-chato, S.; Yenchon, S. Influence of MS medium strengths and types on *in vitro* shoot multiplication and development of *Nymphaea colorata*. *ASEAN J. Sci. Technol. Rep.* **2025**, 28(4), e258176. <https://doi.org/10.55164/ajstr.v28i4.258176>
- [34] Khaenthong, I.; Akkarakultron, P. Seed coat scarification of some Australian *Nymphaea* species by vinegar. *Agric. Sci. J.* **2016**, 47(2, Suppl.), 537–540.
- [35] Song, W.; Shi, W.; Wang, H.; Zhang, Z.; Tao, R.; Liu, J.; Wang, S.; Engel, M. S.; Shi, C. Comparative analysis of 12 water lily plastid genomes reveals genomic divergence and evolutionary relationships in early flowering plants. *Mar. Life Sci. Technol.* **2024**, 6, 425–441. <https://doi.org/10.1007/s42995-024-00242-0>