Adv. Hort. Sci., 2022 36(3): 247-251 DOI: 10.36253/ahsc-12347

Short note

AHS
Advances in Horticultural Science

Evaluation of *Hemerocallis* germplasm using single nucleotide polymorphisms of nrITS and chloroplast interspacer region

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OPEN ACCESS

Key words: Daylily, haplotype, nocturnal flowering, polymerase chain reaction, sequence analysis.

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Citation: PARK S.Y., JOUNG Y.H., SUH J.K., ROH M.S., 2022 - *Evaluation of Hemerocallis germplasm using single nucleotide polymorphisms of nrITS and chloroplast interspacer region.* - Adv. Hort. Sci., 36(3): 247-251

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 24 November 2021
Accepted for publication 31 August 2022

Abstract: This study was initiated to distinguish nocturnal (night) flowering *Hemerocallis* species from day flowering species based on the single nucleotide polymorphisms (SNPs) of nuclear internal transcribed spacers 1, 2 in a ribosomal RNA gene (nrITS) and a chloroplast interspacer region (cpIS). Four nocturnal flowering species, *H. citrina*, *H. thunbergii*, *H. minor*, and *H. lilioasphodelus* were collected including Korea, and compared with day flowering species that included *H. vespertina* and *H. hongdoensis.* Based on the haplotypes of nrITS and cpIS, nocturnal species cannot be distinguished from day flowering species. Discrepancies in flowering time and haplotypes among *H. minor* accessions suggest that more germplasm with diverse geographic origins should be evaluated and identification of other genes is required to effectively distinguish nocturnal species from day flowering species.

1. Introduction

There are about 15-26 species/varieties in the genus *Hemerocallis* (USDA, ARS, National Genetic Resources Program, 2015). Using amplified fragment length polymorphisms (AFLP) markers, *H. fulva* L. were grouped separately from the nocturnal species *H. thunbergii* Baker and *H. lilioasphodelus* L., while nocturnal *H. minor* Mill. and *H. citrina* Baroni, were grouped together in a different sub-cluster (Tomkins *et al*., 2001). The flowers of nocturnal *Hemerocallis* open late in the afternoon and wither the next morning (Fig. 1) (Chen and Noguchi, 2000). However, Gulia *et al*. (2009) classified *H. minor* as a diurnal species rather than a nocturnal species, confirming the study by Krestova and Nesterova (2003) that *H. minor* flowered in the morning under sunny weather at >16°C and with-

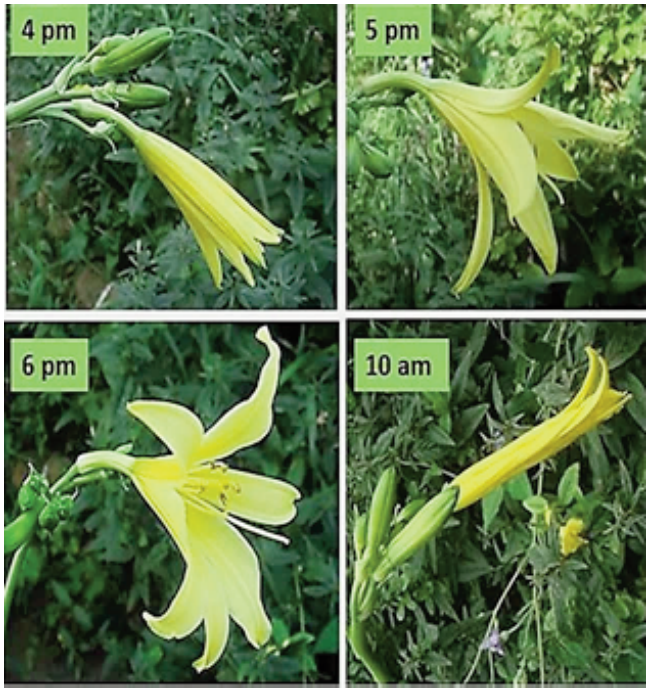

Fig. 1 - Nocturnal *H. thunbergii* accession 7 collected from Korea (K7) showing flower opening at 4, 5, and 6 pm and closed by 10 AM following day.

ered in the afternoon.

Molecular markers have not previously been tested to determine timing of flowering among *Hemerocallis* species. Therefore, this study was conducted to investigate the use of SNPs of nuclear internal transcribed spacers 1, 2 in a ribosomal RNA gene (nrITS) and a chloroplast interspacer region (cpIS) to evaluate the genetic relationships between nocturnal and day flowering species of *Hemerocallis*.

2. Materials and Methods

The nocturnal flowering species *Hemerocallis thunbergii, H. minor, H. lilioasphodelus,* and *H. citrina,* and the day flowering species *H. hongdoensis* M.G. Chung & S.S. Kang*, H. vespertina* H. Hara*, H. dumortierii* C. Morren and hybrid 'Stella de Oro' were collected from Korea (K), China (C), United Kingdom (UK), United States of America (UA) and Germany (GE) (Table 1). Samples were designated as, for example, K1 (mother plant)/1-3 (seedling 1, 2, 3 from mother plant K1).

Genomic DNA was isolated from young leaves using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Polymerase chain reaction (PCR) for nrITS was performed with a 18S rRNA gene specific forward primer ITS1 (5'-TAG AGG AAG GAG AAG TCG TAA CAA GG-3') and primer ITS2 (5'- GATTTTCAGTCCTCTGCTC-TAC-3'). The reaction mix consisted of 12.5 µl of 2X F-star *Taq* Smartmix (SolGent Co., Daejeon, Korea), 2 µl of each primer (0.4 µM final concentration), and 2 µl of genomic DNA. For cpIS, forward primer (5' -TCGT-GAGGGTTCAAGTCCCTCT-3') and reverse primer (5'-GATTTTCAGTCCTCTGCTCTAC-3') were used.

The reaction mix consisted of 12.5 µl of 2X F-star *Taq* Smartmix (SolGent Co., Korea), 2 µl of each primer (0.4 µM final concentration), and 2 µl of genomic DNA (50 ng) and 5 µl of 5× Band Doctor buffer (SolGent), and made up to the 25 µl final volume with PCR ultrapure water. The PCR conditions were: 2 min at 95°C, followed by 35 cycles of 20 sec at 95°C, 40 sec at 65°C, and 1 min at 72°C, followed by 5 min of 72°C using an ABI Veriti Dx Thermal Cycler (Life Technologies, Grand Island, NY, USA). PCR products were direct sequenced as described by Park *et al*. (2014). Sequences were registered in the National Center for Biotechnology Information GenBank (NCBI, http://www.ncbi.nlm.nih.gov/).

3. Results and Discussion

Based on the sequences of nrITS, three haplotypes were identified: T I-1, T I-2, and T II (Table 2) and based on the sequences of cpIS, 7 haplotypes were identified: T I, TI-1, TII, TIII, TIV, TV, and TVI (Table 3). Sequences of cpIS were more informative to separate accessions than those of nrITS, suggesting that the ITS region in *Hemerocallis* may not be useful as a potential source for species identification, although accessions of cultivated origin *Kolkwitzia amabilis* (Graebn.) Christenh were derived from the accession of known wild origin (AA816-84A) (Park *et al*., 2014).

Nocturnal flowering *H. citrina*, *H. thunbergii* and *H. lilioasphodelus* flower in the afternoon and wither the following morning (Table 1, Fig. 1). The difference between two types of floral morphology of *H. thunbergii,* based on the presence or absence of bracts subtending the flower buds; K7 lacked bracts while K8-9 had bracts was detected in the haplotype of cpIS, with K7 belonging to type I-1 and K8-9 belonging to type I that may result from that the sequence of the ITS 2 region is more variable than that of the ITS 1 region (Ma *et al*., 2014). Nocturnal flowering *H. thunbergii* accessions collected from the same location in Korea (K8-9, and K 8-13) should be evaluated

further since these accessions were grouped together with other *H. thunbergii* accessions (K1-3).

There is no correlation between these nrITS haplotypes and timing of flowering observed in *H. citrina*; collected from China (C2) and the United Kingdom (UK5), both belonging to type I, from Germany (GE4), belonging to type II, and from the United Kingdom (UK1), belonging to type III. This requires further examination collecting more accessions from different collection sites. Further, in cpIS, day flowering *H. hongdoensis* and *H. vespertina* belonged to type I and II, respectively.

When T was assigned for the ambiguous code C or T at the positions 78, 86, 99, 113, and 120 of nrITS sequence for type I-1, and A was assigned for A or G at 231, types I-1 and I-2 can be combined and all accessions can be assigned as type I, separated from *H. minor* (UK6 and GE3) as type II (Tables 1 and 2). They may be derived from different geographic origins of K14 or UA5 which were collected from Korea, belonging to type I-2, and exhibit some degree of genetic variation revealed in this study using universal primers for nrITS. However, different strains or populations of *H. minor* may exist since *H. minor*

Table 1 - Accession information on *Hemerocallis taxa* (mother plants and their seedlings) with flowering characteristics. Haplotype based on the sequence analysis of nrITS and cpIS are indicated

Scientific name	Mother plant (leaf)	Seedlings [a]	Flowering time [b]	Source, Country	Haplotype (T) [c]	
					nrITS	cpIS
H. thunbergii	K1 [d]	K1/1-3	N		I-2	I
H. thunbergii	K2	K2/1-3	N	Jungseon-gun, Korea	I-2	I
H. thunbergii	K3	K3/1-3	N		I-2	I
H. thunbergii	K7		N	J.W. Chang, Gomyeong-dong, Jecheon-si, Chungcheongbuk-do, Korea	I-2	I-1
H. thunbergii	K8,9 [e]		N	E.J. Kim, Gomsi-gil, Ungdam-ri, Paju-gun, Kyunggi-do, Korea	I-2	I
	Ka8-13 [d]		N	E.J. Kim, M.S. Roh, Gomsi-gil, Ungdam-ri, Paju-gun, Kyunggi-do, Korea	I-2	IV
H. thunbergii	K11		N	Hantaek Botanical Garden, Korea	I-2	I
H. thunbergii	UK3, 4		N	Royal Botanical Garden Edinburgh, UK (19300128A [f])	I-2	I
H. thunbergii	UA7	-	N	United States National Arboretum, Washington, DC, USA (USNA; NA54757.3 collected from Korea)	I-2	I
H. minor	-	K14/1-3	D	Hantaek Botanical Garden, Korea	I-2	I-1
H. minor	UK6	UK6/1-3	N?	Royal Botanical Garden Edinburgh, UK	II	V
H. minor		GE3/1-3	N?	Botanischer Garten Leipzig (XX-O-LZ-AD439/2006)	II	V
H. minor	UA5	-	D	USNA; NA31800.1	I-2	I
H. lilioasphodelus	-	C1/1	N	X.W. Wu, China	I-2	I
		C1/2-3			I-1	I
H. liliasphodelus	UA6	-	N	USNA; NA54879.3	I-2	I
H. citrina	-	C2/1-3	N	X.W. Wu, China	I-2	I
H. citrina	UK1		N	Royal Botanical Garden Edinburgh, UK (19685548A [u])	I-2	III
H. citrina	UK5		N	Royal Botanical Garden Edinburgh, UK	I-2	I
H. citrina		GE4/1-3	N	Botanischer Garten Leipzig (XX-O-LZ-AW78/1998, 2000)	I-2	II
H. citrina 'April Flower'	-	C3/1-3		X.W. Wu, China	I-2	I
H. vespertina	K12	K12/1-3	D	Hantaek Botanical Garden, Korea	I-2	II
H. dumortierii C. Morren	K13		D	Hantaek Botanical Garden, Korea	I-2	VI
H. hongdoensis	-	K15/1-3	D	Hantaek Botanical Garden, Korea	I-2	I
'Stella de Oro'	UA2	UA2/1-3	D	M.S. Roh, Ann Arbor, MI., USA	I-1	I

[a] Seedlings 1, 2, 3 from mother plant K1. Designations of accession of mother plant and three seedlings are indicated as K1 and K1/1-3, respectively.

[b] Flower opens in the morning and withers in the late afternoon (day, D) or opens in the late afternoon and withers early on the next morning (night, N). Flowering characteristics were not evaluated in this study for *H. minor* collected from Royal Botanical Garden Edinburgh, UK and Botanischer Garten Leipzig (XX-O-LZ-AD439/2006).

[c] Refer to Table 2 for nrITS and Table 3 for cpIS haplotypes and single nucleotide polymorphisms.

[d] Collected or received from Korea (K, Ka), United States of America (UA), United Kingdom (UK), Germany (GE), and China (C).

[e] Samples of K8 and 9 and of Ka 8-13 were collected from the same location in 2011 and 2014, respectively.

[f] Samples were of garden origin from Dendrologische Gartenerei in Pruhonice, Czech Republic via Peter Brownless.

flowers in the morning under sunny weather at >16°C and withers in the afternoon, as reported at the Botanical Garden Institute, Far East Branch, Russian Academy of Sciences (Krestova and Nesterova, 2003). The *H. minor*, received from the US National Arboretum (USNA; NA31800.1) originally collected from Korea, flowers in the morning in Ann Arbor, MI, USA. Therefore, further investigation of *Hemerocallis minor* is needed to determine whether accessions collected from China, Korea and the far eastern part of Russia are of two different flowering types.

Hemerocallis hybrid 'Stella de Oro' (UA2), day flowering landscape plant of unknown parentage, was grouped with *H. minor* collected from Korea (K14), but not with *H. minor* (UK6 and GE3) accessions and their seedlings (K14/1-3) and UK6/1-3, based on the haplotypes of SNPs with a nrITS region (Table 2) and with cpIS (Table 3). Grouping of *H. dumortieri* (K13) with *H. minor* (UK6 and GE3) with nrITS region was also different from that with cpIS (Table 3). McGarty (2006) used AFLP markers from *Hemerocallis* species, and placed *H. lilioasphodelus* with *H. thunbergii* in one sub-group and *H. citrina*, *H. minor*, and *H. dumortieri* in another. This suggests that day and night flowering species cannot be separated either by AFLP markers, or by SNPs from a nrITS region or cpIS, as attempted in this study. Difficulties with identifying species of *Hemerocallis* native to Korea may not be easy to resolve and flowering time in F_1 hybrids between *H. fulva* (day flowering) and *H. citrina* (nocturnal flowering) showed discontinuous bimodal distribution (Hasegawa *et al*., 2006).

Beyond the identification issue for the accessions *H. thunbergii* Ka8-13, grouping of species investigated in this study differs significantly between nrITS and cpIS (Tables 2 and 3). Difficulties with identifying species of *Hemerocallis* native to Korea may not be easy to resolve and flowering time in F1 hybrids between *H. fulva* (day flowering) and *H. citrina* (night flowering) showed discontinuous bimodal distribution (Hasegawa *et al*., 2006).

Distinguishing nocturnal flowering forms of *H. minor* from day flowering forms is not possible due to the existence of two different genotypes collected from China, Korea and far eastern Russia and by testing the universal primers by SNPs of nrITS region and cpIS. However, these primers were used successfully to identify mother plants and seedlings of *Ligustrum quihoui* Carrière (Ma *et al*., 2014).

Table 2 - Haplotype based on single nucleotide polymorphisms (SNPs) and insertion and deletion (IN/Del) of a nuclear internal transcribed spacer 1, 2 in a ribosomal RNA gene of Hemerocallis accessions

Haplotype	NCBI Registration	Positions and codes of nucleotide												
		Single nucleotide polymorphisms												In/Del
		78	86	99	113	120	170	206	210	231	254	432	568	597	435-441
I-1	KT189161	C/T	C/T	C	C/T	C/T	A	G	A	A/G	A	A	G	G	C7
I-2	KT189162	C	C	C	C	C	A	G	A	A	A	A	G	G	C6-7
II	KT189163	C	C	T	C	C	G	C	C	C	C	G	A	A	C7

Table 3 - Haplotypes based on single nucleotide polymorphisms (SNPs) and insertion and deletion (In/Del) of a chloroplast interspacer region of *Hemerocallis* accessions

Haplotype	NCBI registration	Positions and codes of nucleotide							
		Single nucleotide polymorphisms						In/Del	
		26	216	243	246	291	315	37	302
I	KT189164	T	C	C	C	A	A	T9	T7
I-1	KT189165	T	C	C	C	A	A	T9	T8
II	KT189166	T	C	C	C	C	A	T9	T8
III	KT189167	T	C	C	A	A	A	T9	T8
IV	KT189168	C	C	A	A	A	A	T8	T8
V	KT189169	C	C	C	A	A	A	T8	T8
VI	KT189170	C	A	C	A	A	A	T10	T8

Seedlings grouped in the haplotype with their mother plants, except the mother plant (C1) and seedlings 2-3 of *H. liloasphodelus* (C1/2-3), which belonged to type I, type I-2 and I-1, respectively, in nr-ITS region (Tables 1 and 2). The current primers for nrITS region and cpIS cannot be used to differentiate nocturnal flowering species from day flowering species. Lee and Maki (2015) reported that cpDNA in the majority of cultivars were inherited from *H. albomarginata*, although the leaf morphology was similar to *H. sieboldiana*, indicating that nrITS should further be investigated.

4. Conclusions

The sequence variations of nrITS region and cpIS cannot be used to distinguish nocturnal flowering species from day flowering *Hemerocallis* species. Markers other than those evaluated in this study should be evaluated. Genetic variations among seedlings or between mother plant and their seedlings were observed in *H. lilioasphodelus* (C1/1 vs. C1/2-3), requiring seedlings of *H. lilioasphodelus* to investigate to confirm the results of this study. Discrepancies in flowering time among *H. minor* accessions also suggest that more germplasm with diverse geographic origins should be evaluated.

Acknowledgements

Critical review and English editing of the manuscript by Dr. C.J. Catanzaro, Virginia State University, VA, USA, is greatly appreciated.

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