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Genetic variation of wild *Bulbophyllum reclusum* Seidenf. in Northeast Thailand based on chloroplast *mat*K sequence analysis

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Abstract

Bulbophyllum reclusum is one of the endangered wild orchid species in Thailand, and its genetic variation has never been elucidated using molecular techniques. The present study is the first to provide genetic variation information of wild *B. reclusum* collected from Northeast Thailand using the chloroplast maturase K (*mat*K) gene as a marker. Based on the *mat*K sequence analysis of 43 orchid samples from Sakon Nakhon and Ubon Ratchathani Provinces, 25 variable nucleotide sites were detected. There was no genetic variation in the samples collected from Sakon Nakhon Province. In contrast, high levels of intra- and inter-population genetic variation were observed in the samples collected from Ubon Ratchathani Province. However, the genetic differences between the populations were not significant and in a phylogenetic analysis, they clustered as a monophyletic group.

Keywords: *Bulbophyllum reclusum*, Genetic variation, *Mat*K gene, Orchid

1. Introduction

Bulbophyllum is one of the largest genera of Orchidaceae and is mainly distributed in Asia. It is also one of the largest genera of vascular plants, comprising more than 2,000 species [1]. Due to the significant number of species and their variety in nature, systematic classification is complicated. However, based on classical taxonomic identification, approximately 17 sections with more than 100 subgenera have been reported [1].

The orchids in *Bulbophyllum* are primarily epiphytic and can be found in different habitats ranging from subtropical dry forests to wet montane cloud forests. Most of them are pollinated by flies [2,3]. Although *Bulbophyllum* orchids are diverse in Thailand, some are rarely collected, and their genetic variations are limited. More recently, *B. reclusum* Seidenf. has been reported to be found only in Thailand and Myanmar [4]. It occurs mainly in the northeastern part of Thailand, especially in deciduous dipterocarp forests in Sakon Nakhon and Ubon Ratchathani Provinces. It has been considered as one of the 207 endangered species listed in Thailand based on IUCN Red List [5]. Due to climate change and the destruction and fragmentation of its habitat, the natural population of *B. reclusum* has dramatically declined. Thus, it is urgently necessary to explore basic information about the species, including its genetic variation. No information regarding the genetic variation of this orchid species has been reported in the literature.

Plant genetic diversity is evaluated for many purposes, e.g., updating taxonomic status, strain improvement, as well as genetic conservation in endanged species. To explore genetic diversity comprehensively, appropriate molecular techniques and/or genetic markers should be selected. Several molecular techniques have been employed to investigate the genetic variations of Orchidaceae. Among the Deoxyribonucleic acid (DNA) markers, chloroplast and nuclear DNA sequences are widely used [6-11]. The chloroplast DNA maturase K gene (*mat*K)

is one of the most commonly used regions as a core barcode for assessing the genetic variation and phylogenetic relationships in Orchidaceae [10-15]. In addition, the universal *mat*K primers have been widely used for DNA barcoding of angiosperms and other plants [16]. This research examines the genetic variation of *B. reclusum* collected in Northeast Thailand using the chloroplast *mat*K gene as a molecular marker.

2. Materials and methods

2.1 Sample collection

Briefly, a young leaf of the orchid was collected, dried in silica gel, and then transferred to the laboratory for molecular analysis. Forty-three samples from four natural populations of *B. reclusum* (Figure 1) were collected from their natural habitats in Sakon Nakhon and Ubon Ratchathani Provinces, Northeast Thailand, from April 2018 to March 2019 (Figure 2). A map of sampling sites (Figure 2) was generated using ArcGIS Desktop version 10.8 (Esri Inc, USA). The sample size and habitats of the sampled populations and their approximate locations are shown in Table 1.

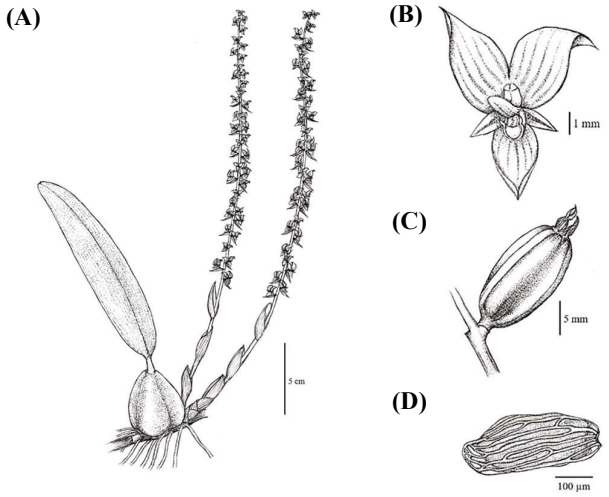

Figure 1 Drawing of *Bulbophyllum reclusum*; (A) inflorescences and pseudobulb with a single leaf, (B) flower, (C) fruit capsule, and (D) seed.

2.2 DNA extraction, polymerase chain reaction (PCR) amplification, and DNA sequencing

Total genomic DNA was extracted from individual leaf tissues of orchids by grinding to a powder in liquid nitrogen using a sterile mortar and pestle. The resulting powder was subjected to a Plant Genomic DNA Extraction Kit (RBC Bioscience, Korea) using the protocol described in the manufacturer's instructions. The chloroplast *mat*K region was then PCR-amplified using purified DNA as a template and specific forward primer (matK-bulboF: 5′-TCG GCA ACA AAA CTT CCT AT-3′) and reverse primer (matK-bulboR: 5′-TAC GTT CTC TAT GTA ATC CGT G-3′), which are currently designed based on the *mat*K gene in the GenBank database. DNA amplification was performed in a PCR thermocycler (BioRad, USA) using 25 mL of reaction mixtures consisting of 20-50 ng of template DNA, 1× Ex buffer (Takara, Japan) with 0.2 mM dNTPs, 0.2 µM each of forward and reverse primers, and 0.625 U of Ex *Taq* DNA polymerase (Takara, Japan). The PCR conditions comprised initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 8 min. The amplicon products were electrophoresed on 1.5% agarose gels buffered with 0.5× TBE and detected using GelRedTM Nucleic Acid Gel Stain (Biotium, USA) staining. The specific band size of approximately 1.4 kb corresponding to the *mat*K gene was excised from a gel, purified using an E.Z.N.A.® Gel Extraction kit (Omega biotek, USA), and subjected to DNA sequencing using the Dideoxy chain termination method at ATGC Co., Ltd., Thailand.

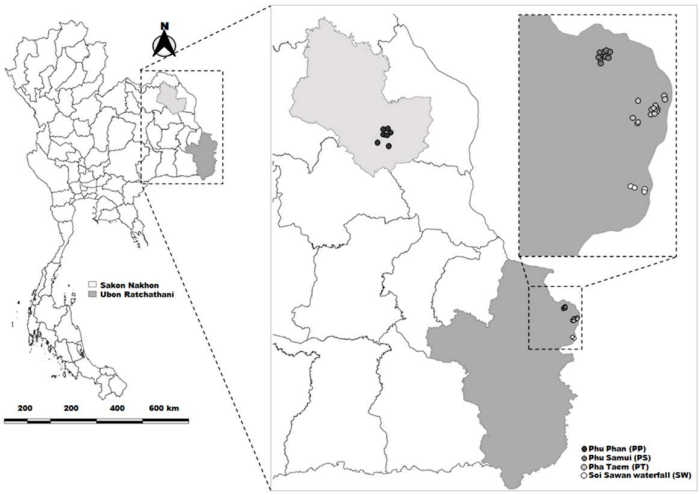

Figure 2 Map of the sampling sites of wild *Bulbophyllum reclusum* collection.

Table 1 Details of the sample collection of *B. reclusum* in Northeast Thailand.

Locality (Sample number)	Sample code	Haplotype	Habitat	Latitude	Longitude	Altitude (m)
PP (7)	PP1-1	H1	*Hopea ferrea* Laness	391142.0	1886497.0	341
	PP2-1	H1	*Sindora siamensis* Teijsm. ex Miq.	395975.0	1893192.0	316
	PP4-1	H1	*Hopea ferrea* Laness	395892.0	1893185.0	323
	PP5-1	H1	*Ficus* sp	395816.0	1893190.0	332
	PP5-2	H1	*Ficus* sp	395816.0	1893190.0	332
	PP32-1	H1	*Hopea ferrea* Laness	395937.0	1893187.0	317
	PP33-1	H1	Rock	401427.4	1883472.6	395
PS (14)	PS7-1	H1	*Madhuca kerrii* Fletch	555590.0	1737156.0	356
	PS7-2	H8	Rock	555210.0	1737314.0	344
	PS8-1	H1	*Hopea ferrea* Laness	555598.0	1737133.0	357
	PS9-1	H1	Rock	555075.0	1736541.0	385
	PS9-2	H9	Rock	555474.0	1737333.0	339
	PS10-1	H1	*Shorea siamensis* Miq./Rock	555230.0	1737171.0	346
	PS11-1	H1	*Shorea siamensis* Miq.	555475.0	1737362.0	337
	PS12-1	H1	Rock	555602.0	1737153.0	361
	PS13-1	H1	*Shorea siamensis* Miq.	555035.0	1736929.0	378
	PS13-2	H10	*Memecylon pauciflorum Bl.*	555213.0	1737305.0	348
	PS14-1	H1	*Hopea odorata* Roxb.	555487.0	1737369.0	338
	PS14-2	H11	*Microcos tomentosa* Sm./rock	555502.0	1737367.0	338
	PS15-1	H1	*Shorea siamensis* Miq./rock	555574.0	1737199.0	353
	PS16-1	H3	*Hopea ferrea* Laness	555583.0	1737173.0	356
PT (18)	PT1-1	H1	*Madhuca kerrii* Fletch	553718.0	1702854.3	227.38
	PT1-2	H1	*Madhuca kerrii* Fletch/rock	563036.1	1726640.1	354.18
	PT4-1	H2	*Madhuca kerrii* Fletch	553702.4	1702875.6	175.8
	PT6-1	H1	*Shorea siamensis* Miq.	561735.8	1724881.7	266
	PT6-2	H7	Rock	562255.0	1724474.0	255
	PT8-1	H3	*Calophyllum calaba* L.	565250.0	1725951.0	448
	PT10-1	H5	*Shorea siamensis* Miq.	537952.9	1699344.2	390
	PT13-1	H1	*Shorea siamensis* Miq.	564584.0	1726139.0	445
	PT19-1	H1	*Shorea siamensis* Miq.	555092.0	1702756.8	225.58
	PT20-1	H1	*Madhuca kerrii* Fletch	555084.6	1702784.0	225.74
	PT20-2	H12	*Irvingia malayana* Oliv. ex A. Benn.	566041.0	1726930.0	418
	PT21-1	H1	*Schima wallichii* (DC.) Korth.	565341.0	1726252.0	458
	PT22-1	H1	*Calophyllum calaba* L.	566053.0	1727207.0	402
	PT23-1	H1	*Parinari anamense* Hance	565095.0	1725687.0	451
	PT23-2	H13	*Parinari anamense* Hance	565095.0	1725687.0	451
	PT24-1	H1	*Calophyllum calaba* L.	565000.0	1725696.0	459
	PT25-1	H6	*Cratoxylum* sp.	565000.0	1725696.0	453
	PT30-1	H1	*Dipterocarpus obtusifolius* Teijsm. ex Miq.	564749.0	1725982.0	453
SW (4)	SW1-1	H4	Shorea roxburghii G. Don	565226.0	1726012.0	255
	SW2-1	H1	*Madhuca kerrii* Fletch	565196.0	1726005.0	266
	SW3-1	H4	*Irvingia malayana* Oliv. ex A. Benn.	565067.0	1726291.0	243
	SW4-1	H1	*Shorea siamensis* Miq.	562311.0	1708979.0	239

Note: PT; Pha Taem, PS; Phu Samui, SW; Soi Sawan waterfall.

2.3 Nucleotide diversity and phylogenetic analysis

The *mat*K sequences of *B. reclusum* determined in this study were deposited in GenBank under the accession numbers ON814159 – ON814171. For species confirmation, the *mat*K sequences were subjected to BLAST searches [17] in the National Center for Biotechnology Information (NCBI) GenBank. The *mat*K sequences were assembled and manually edited using BioEdit ver. 7.2.6 [18]. Genetic differentiation (Φ_{ST}) between the populations of *B. reclusum* was calculated using Arlequin ver. 3.5.2.2 [19]. Diversity indices, including the number of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity (л), were calculated using DnaSP ver. 5.10.01 [20]. A phylogenetic tree was reconstructed based on the neighbor-joining methods using the MEGA X program [21].

3. Results and discussion

The 1,417 bp of the *mat*K gene of 43 *B. reclusum* samples were examined, and 25 variable nucleotide sites were detected. Of these nucleotide sites, 11 nucleotide variable sites were singletons, while the other 14 were parsimony informative sites (Table 2). The 13 haplotypes (H1-H13) were classified based on these nucleotide variable sites. Of these, 11 haplotypes were uniquely found in a particular locality, while only two haplotypes, i.e., H1 and H3, were shared between populations. Haplotype H1 was the most commonly shared between all populations examined herein, while H3 was shared between the PT and PS populations. The population from Sakon Nakhon Province showed no variation among individuals. In contrast, the three populations, i.e., PT, PS, and SW, from Ubon Ratchathani Province, were revealed to have high genetic variation at both the intra- and inter-population levels, with haplotype diversity and nucleotide diversity ranging between 0.604±0.150 to 0.667±0.204 and 0.0014±0.0006 to 0.0174±0.0008, respectively (Table 3). Genetic differences between populations ranged from 0.0323 to 0.4815, with no significant difference ($p > 0.05$) observed (Table 4).

Genetic analysis using the *mat*K gene marker revealed that samples collected from Sakon Nakhon Province exhibited no genetic variation among the individuals, while high genetic variation at the intra- and inter-population levels was found in the samples collected from Ubon Ratchathani. Richards [22] and Tremblay et al. [23] pointed out that low intra-population genetic variation in orchids can result from founding events, leading to a small adequate population size that favors genetic drift. The occurrence of inbreeding within orchid populations is another possible explanation since self-fertilization decreases the proportion of heterozygous loci in individuals, resulting in the fixation of homozygous loci [24,25]. In addition, the potential of vegetative reproduction via pseudobulbs can also lead to the maintenance of low genetic variation within populations [25-27]. However, this is not the case for the inbreeding weed *Capsella bursa-pastoris* in which genetic differentiation was found between individuals and different populations [28]. Genetic drift has been proposed to cause high genetic differentiation in *C. bursa-pastoris* [28]. Recently, Ueno et al. [25] also pointed out that genetic drift could cause high genetic differentiation in the terrestrial orchid *Oeceoclades maculata*. Thus, the high genetic variation found in the *B. reclusum* samples collected from Ubon Ratchathani may also be related to genetic drift. However, more studies are required to test and verify this hypothesis.

Table 2 Comparison of the *mat*K nucleotide variable sites between the 13 haplotypes of *B. reclusum*.

Sample code* (Haplotype)	Variable positions																								
	1 5 2	2 0 7	2 0 8	2 3 0	3 1 4	3 6 0	3 7 8	4 0 5	4 1 6	6 3 3	6 4 0	6 4 2	6 4 5	6 4 8	6 6 9 3	7 2 8	7 3 9	7 7 0	8 3 2	8 5 8	8 6 1	9 9 0	1 0 5	1 1 6	1 3 0 8
PT1-1 (H1)	C	C	G	C	T	G	C	A	T	C	A	A	A	G	A	T	A	G	T	A	G	A	T	A	T
PT4-1 (H2)																G	C								
PT1-1 (H3)				A																					
PT4-1 (H4)		T			C						C														
PT1-1 (H5)		T	T																						
PT4-1 (H6)	A	T			C						C	C			G							C			
PT1-1 (H7)										T														C	
PT4-1 (H8)																		T							G
PT1-1 (H9)						T		C	C	T									G	G		C			
PT4-1 (H10)							A	C								G	C	T					G		
PT4-1 (H11)								C		T															
PT4-1 (H12)						T				T	C	C	C	T		G	C	T	G						
PT4-1 (H13)																			G	G	T				

Table 3 Diversity indices of the *mat*K sequences in the *B. reclusum* populations from four different sites.

Sample locality	n	s	h	Uh	Hd±SD	Nd±SD
PT	18	20	8	6	0.641±0.130	0.0021±0.0007
PP	7	0	1	0	0.000±0.000	0.0000±0.0000
PS	14	14	6	4	0.604±0.150	0.0174±0.0008
SW	4	3	2	1	0.667±0.204	0.0014±0.0006
Total	43	25	13	11	0.548±0.093	0.0016±0.0004

The 35 *mat*K sequences of the other 27 species of the genus *Bulbophyllum*, i.e., *B. hirundinis, B. yongtaiense, B. inconspicuum, B. amplifolium, B. umbellatum, B. bicolor, B. wendladnianum, B. rothschildiannum, B. tigridum, B. longiflorum, B. ayuthayense, B. nigrescens, B. candicum, B. yunnanense, B. affine, B. lobbii, B. cambodianum, B. meson, B. oblongum, B. leion, B. pictum, B. nasutum, B. disciflorum, B. roseum, B. cylindraceum, B. nitidum,* and *B. steyermarkii,* were retrieved from the GenBank database for phylogenetic analysis alongside our sequences. The outgroup was comprised of *mat*K sequences of the genus *Dendrobium*. Phylogenetic analysis revealed that the populations of *B. reclusum* examined in this study were clustered as a monophyletic group and separated from the other 27 species of the genus *Bulbophyllum* with high supporting value. However, *B. steyermarkii* from Brazil was clustered closely with the genus *Dendrobium* instead of the genus *Bulbophyllum* (Figure 3).

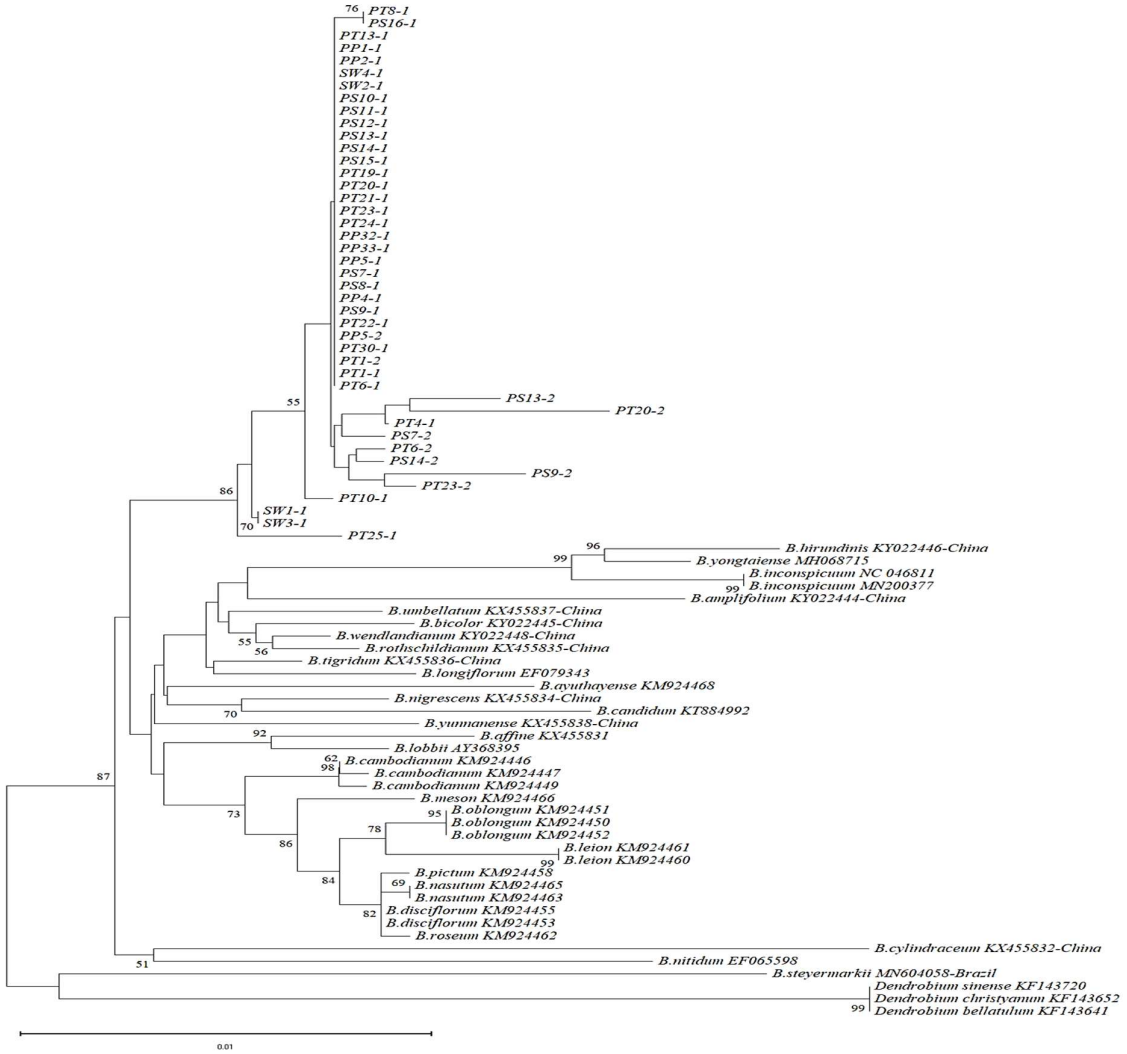

Figure 3 The neighbor-joining tree revealed a monophyletic group of *B. reclusum* reconstructed with the other 35 *mat*K sequences of 27 species of the genus *Bulbophyllum* retrieved from the GenBank database. Nodal supports are sequential values of bootstrap values generated by neighbor-joining method. The scale-bar indicates the expected number of substitutions per site. The *mat*K sequences of the genus *Dendrobium* were used as the out-group.

Due to the simplicity of amplification, sequencing, and a wide range of variability, chloroplast DNA sequences are one of the molecular markers widely utilized for plant genetic diversity analysis [29]. However, not all chloroplast DNA regions are used since some regions are difficult to align or generate low variability nucleotide sites. Due to a high sequence divergence compared to other coding regions, such as *rbc*L, the *mat*K gene is among the most variable plastid protein-coding regions used for the phylogenetic analysis of Orchidaceae [30]. However, the *mat*K gene provides few or no parsimony-informative sites between closely related species within orchid genera. It is also a pseudogene in some orchid taxa because it does not maintain a reading frame [25,31]. In such a case, another molecular marker, such as the hypothetical chloroplast open reading frame 1 (*ycf*1), may be more effective. Nearly all plant plastid genomes possess *ycf*1and it is essential for plant survival [32,33]. Its open reading frame (approximately 5,500 bp) covers a portion of the inverted repeat (IR) and the small-single copy (SSC), which generate more sequence variables than the *mat*K region. Furthermore, due to its conserved reading frame, the *ycf*1 region is relatively easy to amplify and align [25,34]. However, primer design for the *ycf*1 region is one of the most challenging due to many indels (insertions/deletions) and homopolymer stutter regions [35].

Table 4 Genetic differentiation (Φ_{ST}) of the *mat*K sequence of the populations from four different localities.

Code	PT	PP	PS	SW
PT	-			
PP	0.0769	-		
PS	0.0323	0.0723	-	
SW	0.0633	0.4815	0.0736	-

4. Conclusion

The endangered wild orchid *B. reclusum* is mainly found in Northeast Thailand, especially in Sakon Nakhon and Ubon Ratchathani Provinces. There was no genetic variation of the orchid population collected from Sakon Nakhon. This result may have been influenced by the low sample number from Sakon Nakhon Province. A high level of genetic variation was observed in the orchid populations from Ubon Ratchathani at both the intra- and inter-population levels based on the *mat*K sequences. We propose from this finding that a high genetic variation in the population from Ubon Ratchathani might be correlated with genetic drift, but this hypothesis has to be further tested and proven. In addition, more orchid populations from different sample sites in Sakon Nakhon Province should be collected and analyzed; and this is now in progress.

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