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Revealing genetic diversity of wild *Phalaenopsis* orchids in Thailand through Random Amplified Polymorphic DNA markers

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Abstract. *Kate-ngam S, Jantasuriyarat C, Lakote P, Promchot T, Chueakaew C. 2023. Revealing genetic diversity of wild* Phalaenopsis *orchids in Thailand through Random Amplified Polymorphic DNA markers. Biodiversitas 24: 5409-5417.* Wild *Phalaenopsis* orchids are widely distributed in Thailand, especially along the banks of the Mekong River. The tremendous decrease in the population of these orchids is a matter of concern for maintaining and preventing genetic loss in the natural ecosystem. This study aimed to determine the genetic diversity of 50 wild *Phalaenopsis* accessions collected from northeastern of Thailand using random amplified polymorphic DNA (RAPD) markers. The 204 polymorphic bands generated from 27 RAPD primers were employed for fingerprinting these orchid accessions. Dice's similarity coefficients ranged from 0.43 to 0.98, with an average of 0.65. In general, a dendrogram constructed based on the unweighted pair group method with arithmetic average (UPGMA) grouped these wild *Phalaenopsis* accessions into two clusters, namely *P. pulcherrima* and *P. ubonensis* clusters. The UPGMA clustering pattern and principal component analysis (PCA) corresponded well with their morphological classification and ploidy level. Our results suggest that this wild *Phalaenopsis* orchid exhibits a moderate level of relatedness. This might be a consequence of habitat destruction and human over-exploitation which limit gene flow and cause genetic drift among populations. The present finding supports the urgent need for a systematic conservation effort of this orchid species in Thailand.

Keywords: Conservation, genetic diversity, orchid, *Phalaenopsis*, RAPD markers

INTRODUCTION

Orchids, belonging to the family Orchidaceae are admired for their beautiful shapes, colors, and flowers (Chase et al. 2015; Givnish et al. 2015). However, their populations are rapidly declining worldwide due to habitat deterioration and unregulated commercial collection, drawing the attention of conservationists (Fay 2018; Pykälä 2019; Tikendra et al. 2019). The loss of genetic diversity within endangered populations can decrease adaptability to environmental changes and eliminate unique characteristics such as disease resistance and fruit set (Chung et al. 2023). Therefore, these sensational species are in concern for plant conservation.

The wild *Phalaenopsis* orchids (formerly known as *Doritis* orchids) are native to tropical Asia and widely distributed across Southeast Asia, spanning from Assam, the Chinese Himalayas, Myanmar, Thailand, Malaysia, Laos, Cambodia, Vietnam to Sumatra and Kalimantan (Christenson 2001; Averyanov 2009). In their natural habitat, these orchids thrive on sandy soils, sparse rocks, and rainforests, producing showy, medium-sized flowers on stiffly erect inflorescences (Averyanov 2009). The genus is initially classified under the genus *Doritis* by Sweet (1980), then Christenson (2001) treated *Doritis* as a synonym of *Phalaenopsis* and placed 3 species, including *D. pulcherrima*, *D. regnieriana*, and *D. buyssoniana* (presently known as *P. ubonensis*) in the section of *Esmeralda* under subgenus *Phalaenopsis*, within the genus *Phalaenopsis.* This generic treatment was supported by several molecular phylogeny studies, such as Goh et al. (2005), Padolina et al. (2005), Tsai et al. (2010), and Govaerts et al. (2021).

In Thailand, *P. pulcherrima* (Lindl.) J.J.Sm. and *P. regnieriana* Rchb.f. are prevalent in all regions of the country, while *P. ubonensis* (O.Gruss) J.M.H.Shaw (formerly known as *D. buyssoniana* (Rchb.f.) J.M.H.Shaw) is mainly distributed in the northeastern areas along the Mekong River, especially in Ubon Ratchathani province (Jantasuriyarat et al. 2012; Rungratchakanon et al. 2013). The local name of *P. pulcherrima* is 'Ma Wing' which means 'running horse' due to its flower shape resembling the head of a running horse, whereas *P. ubonensis* is named 'Daeng Ubon' (Red Ubon) because of the vivid pink to purple-violet color of its flowers and its region of origin Ubon Ratchathani in far eastern Thailand (Gruss 2014). To date, the number of these orchids in national parks in Thailand has significantly reduced due to suitable habitat loss, climatic change, and over-collecting from nature (Thipwong et al. 2022). Consequently, this situation could lead to further loss of genetic diversity and alterations in the population's genetic structure (Pykälä 2019). Therefore, an evaluation of genetic diversity is urgently needed for the implementation of this wild orchid conservation program.

Genetic diversity can be assessed using morphological markers and/or molecular markers. Morphological markers visually distinguish qualitative characters, such as flower color and growth habit. However, they have limitations due to the small number of traits considered and their susceptibility to environmental conditions and plant development stages. In contrast, molecular markers detect varietal differences based on DNA polymorphism and offer informative, abundant, and environment-independent information throughout the genome (Nadeem et al. 2018). Among DNA markers used in plant conservation research, random amplified polymorphic DNA (RAPD) has been widely employed to reveal genetic diversity and population structure in *Phalaenopsis* (Been et al. 2002; Goh et al. 2005; Mursyidin et al. 2022), jewel orchid (Tran et al. 2022), and several medicinal plants like *Cichorium spinosum* (Shidfar et al. 2018), edible honeysuckle (*Lonicera kamtschatica*) (Cehula et al. 2019), and *Curcuma comosa* (Boonsrangsom 2020). RAPD markers are amplification products of anonymous DNA sequences using a single, arbitrary deca-oligonucleotide primer, and thus do not require prior knowledge of DNA sequences. The technique's low expense, efficiency in developing a large number of DNA markers in a short time, and the requirement for less sophisticated equipment have made RAPD valuable in research (Nadeem et al. 2018; Babu et al. 2021).

Little information on wild *Phalaenopsis* diversity at molecular level has been reported. Therefore, this research aim to investigate genetic diversity in these wild orchids from northeastern Thailand using the RAPD technique. The findings will enhance our understanding of the genetic profile of these wild native orchids, aiding the development of conservation management strategies for these species.

MATERIALS AND METHODS

Plant materials

Fifty accessions of wild *Phalaenopsis* were collected from three provinces in northeastern Thailand including 2 accessions from Roi Et province, 27 accessions from Ubon Ratchatani province, and 21 accessions from Mukdahan Province (Figure 1; Table 1). Plants of each orchid accession were raised in the plant nursery at the Faculty of Agriculture, Ubon Ratchathani University, Thailand. Based on their morphological characteristics and floral structure, these orchid accessions were identified as *P. pulcherrima* (Lindl.) J.J.Sm. and *P. ubonensis* (O.Gruss) J.M.H.Shaw (Figure 2).

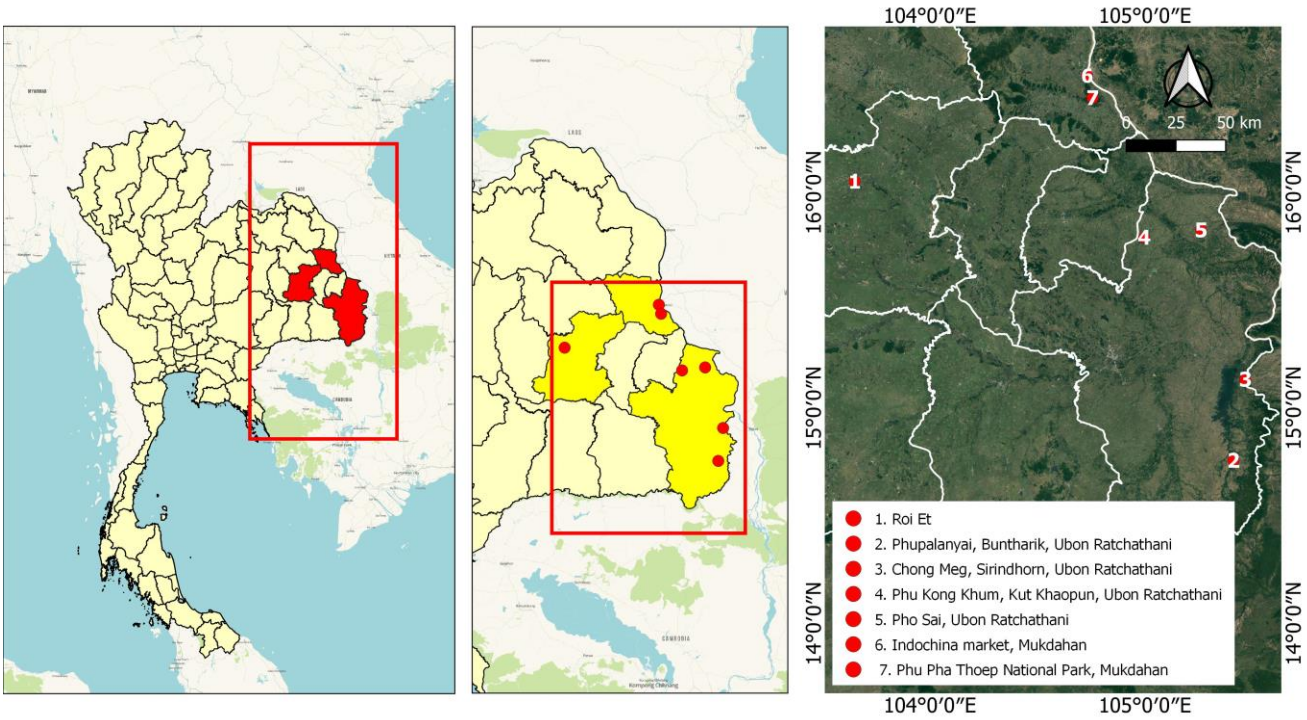

Figure 1. Map of Thailand showing regions from which the 50 wild *Phalaenopsis* accessions from the northeastern of Thailand were collected for genetic diversity assessment

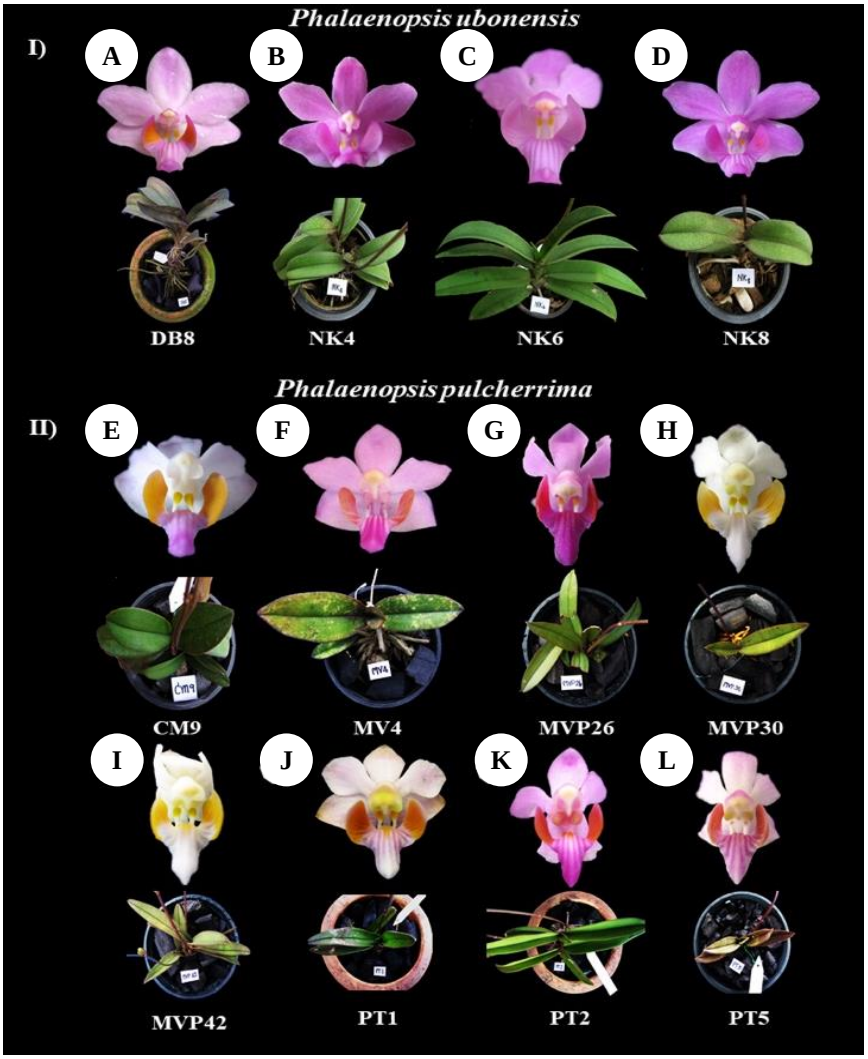

Figure 2. The morphological features of wild *Phalaenopsis* used in the study: *P. ubonensis* (IA-D) and *P. pulcherrima* (IIE-L). The accession codes with collection sites are presented in Table 1

Table 1. List of 50 wild *Phalaenopsis* accessions collected from the northeastern of Thailand used for genetic diversity assessment along with their taxon, habitat/collection sites, GPS coordinate, and altitude data

Accessions	Scientific name (1)	Habitat/collection sites (2)	GPS coordinate (3)	Altitude (m asl.)
RO4, RO10	*P. ubonensis*	Roi Et (No. 1)	16°03'6.60"N 103°39'2.39"E	150
NK4, NK6, NK7, NK8 , NK9	*P. ubonensis*	Phupalanyai, Buntharik district, Ubon Ratchathani (No. 2)	14°45′24″N 105°24′41″E	148
DB6, DB8, CM3, CM11, CM12, CM13, CM16, CM17	*P. ubonensis*	Chong Meg, Sirindhorn district, Ubon Ratchathani (No. 3)	15°7′59″N 105°28′1″E	178.77
CM8, CM9, MV4, MV8	*P. pulcherrima*	Chong Meg, Sirindhorn district, Ubon Ratchathani (No. 3)	15°7′59″N 105°28′1″E	178.77
PK4, PK8	*P. ubonensis*	Phu Kong Khum, Kut Khaopun district, Ubon Ratchathani (No. 4)	15°47′30″N 104°59′48″E	155
MVP2, MVP19, MVP24, MVP26, MVP27, MVP30, MVP39, MVP42	*P. pulcherrima*	Pho Sai district, Ubon Ratchathani (No. 5)	15°49′33″N 105°15′39″E	160
MDD1, MDD2, MDD3, MDD6, MDD10, MDD11, MDD15, MDD17, MDD22, MDD26	*P. ubonensis*	Indochina market, Mukdahan (No. 6)	16°32'32"N 104°43'51"E	151
MDD20, MDM7, MDM14, MDM15, MDM18, MDM21, MDM22	*P. pulcherrima*	Indochina market, Mukdahan (No. 6)	16°32'32"N 104°43'51"E	151
PT1, PT2 PT4, PT5	*P. pulcherrima*	Phu Pha Thoep National Park, Mukdahan (No. 7)	16°26′21″N 104°45′24″E	295

Note: [1)] scientific name of wild *Phalaenopsis* accessions was based on their morphological characteristics and floral structure, [2)] number in parentheses after collection sites correspond to the collection site number in the map of Figure 1, [3)] coordinates obtained from Google maps

DNA extraction

Total genomic DNA was extracted from 0.5 g of young leaf tissues using 2% CTAB with phenol-chloroform according to the method described by Porebski et al. (1997). DNA quality was assessed by 1.2% (w/v) agarose gel electrophoresis, diluted to 20 ng μL^{-1}, and stored at -20°C for genetic diversity assessment.

RAPD primer screening and RAPD fingerprinting

Optimized RAPD-PCR of these wild *Phalaenopsis* was carried out as described by Kate-ngam and Lakote (2008). The 110 RAPD primers from the Operon kit series (Operon Technologies, Germany) were initially screened with 4 randomly selected wild *Phalaenopsis* accessions, namely MDM No. 5, MDM No. 14, MDD No. 3, and MDD No. 13. The selection criteria for RAPD primers were based on their reproducibility, clear and countable DNA amplification and number of polymorphic RAPD markers. These selected RAPD primers were then used to fingerprint and evaluate the genetic diversity of these orchid accessions in this study.

RAPD fingerprints of 50 wild *Phalaenopsis* accessions were carried out in a final volume of 20 µL containing approximately 10 ng of DNA templates, 200 mM of each dNTPs (Promega), 0.6 µM of primer, 3.0 mM $MgCl_2$ 1.0 U of *Taq* DNA polymerase (Fermentas), and 1X PCR buffer (Promega). DNA amplification reactions were performed in a Thermal Cycler (Perkin Elmer 9700, USA) using a PCR amplification profile of initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, and extension at 72°C for 2 minutes. The PCR reaction was further extended for the final step at 72°C for 10 min.

The amplified PCR products were separated by electrophoresis in 1.2% (w/v) agarose gels in TBE buffer (Tris-boric acid-EDTA) for 2 hours at 80 volts and visualized by ethidium bromide staining under UV light. The size of RAPD markers was estimated by comparison with GeneRuler™ DNA Ladder Mix (Fermentas). Each reaction was repeated twice to ensure reproducible RAPD markers.

Data analysis

Only clear polymorphic RAPD markers were scored for data analysis as binary data, presence (1) or absence (0), across 50 wild *Phalaenopsis* accessions. The number of polymorphic bands (PB), percentage of polymorphic bands (PPB), the observed number of alleles per locus (A_o), effective number of alleles per locus (A_e), and Nei's genetic diversity (h^e) were calculated using POPGENE v.1.31 (Yeh et al. 1999). The NTSYSpc (Numerical Taxonomy System pc) software package (Exeter Software, New York, USA) (Rohlf 2004) was employed to perform cluster analysis of polymorphic RAPD markers. A pairwise similarity matrix was constructed using the Dice similarity coefficient; SD = $2N_{ab}/ (N_a+N_b)$, where N_{ab} is the number of shared RAPD alleles between a pair of accessions 'a' and 'b', N_a is the number of scored RAPD alleles in accession 'a' and N_b the number scored RAPD alleles in accession 'b'. The Dice similarity coefficient counts the percentage of shared alleles among two individuals and gives more weight to those alleles that are present in both. It considers that the absence of certain alleles has less biological significance, making this coefficient a meaningful measure of DNA similarity. Estimated similarity was analyzed by the unweighted pair group method with arithmetic average (UPGMA) and the resulting clusters were shown as dendrograms. The goodness of fit of cluster analysis was evaluated using the cophenetic correlation coefficient (r) from the MXCOMP program in NTSYS, which allows direct comparisons between the original similarity matrix that was clustered and the cophenetic value matrix. To determine the clustering patterns among wild *Phalaenopsis* accessions, principal coordinate analysis (PCA) was also operated using NTSYS-pc software.

RESULTS AND DISCUSSION

Primer screening and RAPD fingerprinting

The reproducibility of RAPD amplification is highly influenced by experimental factors including quantity and quality of DNA, and also concentration of PCR mixtures, such as $Mg^{(2+)}$, *Taq* DNA polymerase, and PCR cycling conditions, but reproducible fingerprinting results can be obtained by optimization of these parameters (Babu et al. 2021). In the present study, the optimized RAPD-PCR of these wild orchids (*Doritis*) reported by Kate-ngam and Lakote (2008) was used to detect DNA polymorphisms among wild *Phalaenopsis* accessions from the northeastern of Thailand. Out of 110 deca-oligonucleotide RAPD primers screened with four *Phalaenopsis* accessions, 27 RAPD primers showing distinctive DNA amplification patterns were chosen and employed to fingerprint the 50 wild orchid accessions. A total of 336 strongly stained bands were obtained, of which, 204 RAPD bands were found to be polymorphic ranging from 2 to 15 bands with an average of 7.5 polymorphic bands per primer (Table 2). The largest number of polymorphic RAPD markers was obtained from OPD20 (15 bands) and the lowest numbers of polymorphic bands were obtained from OPA03 and OPA08 (2 bands). The examples of RAPD fingerprints of 50 wild *Phalaenopsis* accessions generated by OPA1 and OPC19 are shown in Figure 3. The band sizes ranged from 400 to 3,000 bp. The OPE05 primers produced a maximum polymorphic rate (100%) of RAPD amplification, whereas OPA03 yielded only 20% of its amplification.

RAPD markers are dominant and DNA amplification occurs at primed sites is scored based on the presence or absence of DNA bands. In this study, approximately 24% of RAPD screened primers (27 primers) were able to produce 204 polymorphic bands with polymorphic rate of 60%. These scorable RAPD markers were obtained by standardizing concentration of $MgCl_2$, *Taq* DNA polymerase, and DNA template as described in Kate-ngam and Lakote (2008). Since RAPD technique is sensitive to the changes in PCR components especially the concentration of $MgCl_2$ and *Taq* DNA polymerase and PCR cycling conditions specifically annealing temperature, minor changes in DNA amplification efficiencies can affect the outcome of PCR

patterns and yield. The optimization of RAPD-PCR to achieve reproducible and interpretable results is thereby requisiteness (Kumari and Thakur 2014; Babu et al. 2021). Once RAPD-PCR reactions have been optimized, this relatively fast and cheap technique will offer a rapid molecular tool for a variety of applications related to the molecular genetics studies of wild *Phalaenopsis* accessions.

Table 2. List of 27 RAPD primers along with their sequences, GC content (%), total amplified bands, polymorphic bands, and band sizes of 50 wild *Phalaenopsis* accessions

Primer names	Sequence (5' to 3')	GC content (%)	Total amplified bands	Polymorphic bands	Percentage of polymorphic bands (%)	Band sizes (bp)
OPA01	CAGGCCCTTC	70	16	11	68.75	800-3000
OPA02	TGCCGAGCTG	70	14	6	42.86	600-1500
OPA03	AGTCAGCCAC	60	10	2	20.00	750-800
OPA08	GTGACGTAGG	60	8	2	25.00	550-600
OPA12	TCGGCGATAG	60	11	6	54.55	650-1300
OPA16	AGCCAGCGAA	60	12	10	83.33	800-1800
OPA20	GTTGCGATCC	60	12	6	50.00	600-1500
OPB01	GTTTCGCTCC	60	15	11	73.33	600-4000
OPB04	GGACTGGAGT	70	7	2	28.57	1200-1400
OPB15	GGAGGGTGTT	60	11	6	54.55	1000-1600
OPB20	GGACCCTTAC	60	10	8	80.00	600-1500
OPC02	GTGAGGCGTC	70	17	7	41.18	500-2000
OPC05	GATGACCGCC	70	15	9	60.00	700-2100
OPC15	GACGGATCAG	60	13	9	69.23	900-2500
OPC19	GTTGCCAGCC	70	11	8	72.73	900-1500
OPC20	ACTTCGCCAC	60	8	6	75.00	400-1000
OPD11	AGCGCCATTG	60	12	6	50.00	500-1200
OPD20	ACCCGGTCAC	70	20	15	75.00	600-2500
OPE05	TCAGGGAGGT	70	11	11	100.00	750-2500
OPF06	GGGAATTCCC	60	13	11	84.62	500-2500
OPF14	TGCTGCAGGT	70	15	11	73.33	500-1500
OPK07	AGCGAGCAAG	60	16	5	31.25	1400-2100
OPR04	CCCGTAGCAC	60	13	8	61.54	700-1300
OPR15	GGACAACGAG	60	9	6	66.67	550-1300
OPU02	CTGAGGTCTC	60	12	11	91.67	700-2000
OPU10	ACCTCGCCAC	70	10	4	40.00	1000-1600
OPU18	GAGGTCCACA	60	15	7	46.67	500-1500
	Total		336	204		
	Range		8-20	2-15	59.99	400-3000
	Average		12.44	7.5		

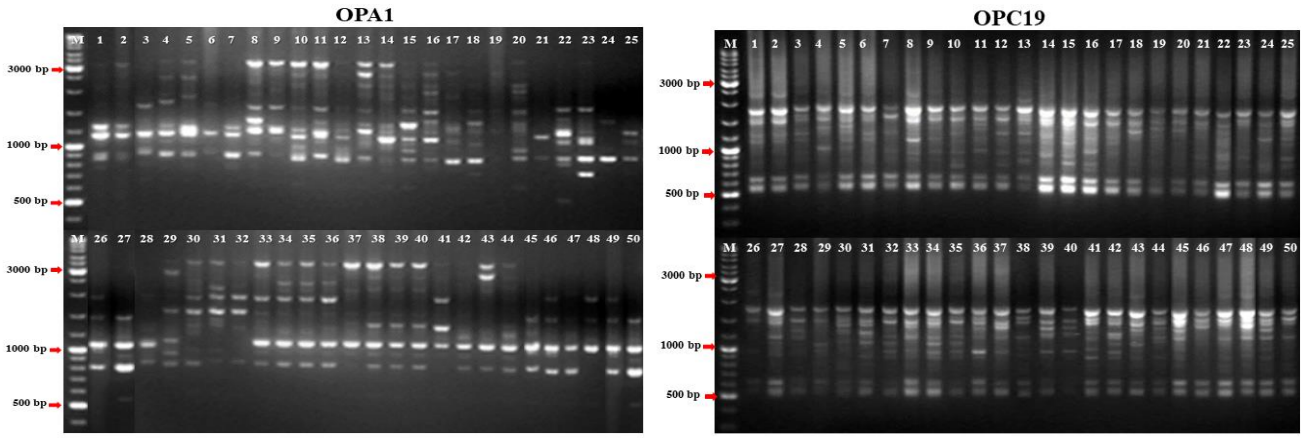

Figure 3. RAPD fingerprints of 50 wild *Phalaenopsis* accessions from the northeastern of Thailand using. A. OPA1. B. OPC19 primers. M refers to GeneRuler^{TM} DNA Ladder Mix (Fermentas) and lane with a numeric number (1-50) refers to the wild *Phalaenopsis* accessions in this study. 1: RO4, 2: RO10, 3: NK4, 4: NK6, 5:NK7, 6: NK8, 7: NK9, 8: CM3, 9: CM8, 10: CM11, 11: CM12, 12: CM13, 13: CM16, 14: CM17, 15: PK4, 16:P K8, 17: DB6, 18: DB8, 19: MDD1, 20: MDD2, 21: MDD3, 22: MDD6, 23: MDD10, 24: MDD11, 25: MDD15, 26: MDD17, 27: MDD20, 28: MDD22, 29: MDD26, 30: CM9, 31: MV4, 32: MV8, 33: MVP2, 34: MVP19, 35: MVP24, 36: MVP26, 37: MVP27, 38: MVP30, 39: MVP39, 40: MVP42, 41: PT1, 42: PT2, 43: PT4, 44: PT5, 45: MDM7, 46: MDM14, 47: MDM15, 48: MDM18, 49: MDM21, 50: MDM22

DNA fingerprinting using DNA markers has become an essential tool for estimating genetic diversity for parental selection in plant breeding programs, germplasm utilization and management, monitoring and managing genetic erosion, and erasing duplicates from germplasm collections (Fay 2018; Nadeem et al. 2018; Tikendra et al. 2019). Genetic similarity information at the DNA level generated by RAPD markers offer a more accurate characterization of germplasm collections compared to morphological information. As a result, RAPD analysis provides a cost-effective molecular tool for well-informed environmental management of endangered species. Furthermore, genetic relationship evaluation using RAPD markers is in good agreement with pedigree and RFLP data (Babu et al. 2021). RAPDs have been successfully applied in genetic relatedness or diversity estimation of several orchids, such as *Cymbidium* (Obara-Okeyo and Kako 1998), *Phalaenopsis* (Been et al. 2002; Goh et al. 2005; Niknejad et al. 2009, Mursyidin et al. 2022), *Vanilla* (Besse et al. 2004), and jewel orchid (Tran et al. 2022).

Genetic diversity

The Nei's genetic diversity of the two *Phalaenopsis* species (h^e) ranged from 0.3279 to 0.3386. Both species exhibited a high level of variability, with *P. pulcherrima* having 95.1% polymorphic bands and *P. ubonensis* having 97.06% polymorphic bands. The observed and effective number of alleles per locus of *P. pulcherrima* were 1.970 and 1.538, respectively, and for *P. ubonensis* were 1.951 and 1.562, respectively (Table 3).

A genetic similarity matrix produced from the 204 RAPD markers using Dice's coefficients ranged from 0.43 to 0.98 with an average of 0.69, indicating a relatively high level of genetic relationships among these accessions (Supplementary Table 1). The highest genetic similarity coefficient (0.98) was measured between accessions MVP30, MVP39 and MVP42. These three *P. pulcherrima* accessions were collected from Pho Sai district, Ubon Ratchathani province and they are morphologically and geographically close to each other, consequently, hybridization within these populations may have occurred in this area. The lowest genetic similarity coefficient (0.43) was measured between accessions NK9 (Phupalanyai, Buntharik district, Ubon Ratchathani province) and CM9 (Chong Meg, Sirindhorn district, Ubon Ratchathani province). NK9 belongs to *P. ubonensis*, while CM9 belongs to *P. pulcherrima*. Both species differ in terms of ploidy, resulting in distinctions in their morphological characters. *P. ubonensis* has larger leaves and stems, and is also taller than *P. pulcherrima*, with an average height of around 21 cm compared to approximately 13 cm in *P. pulcherrima*. Additionally, these two species display apparent differences in flower morphology, including size, characteristics, and color. Flowers of *P. ubonensis* are significantly larger than those of *P. pulcherrima* and their flower color ranges from light purple to dark purple, whereas *P. pulcherrima* exhibits flower color diversity varying from white to dark purple. Moreover, the karyotypes of *P. pulcherrima* and *P. ubonensis* were also different. *P. pulcherrima* is a diploid orchid *(*2n = 2x = 38) with chromosome patterns of 6 metacentric (M) + 24 submetacentric (Sm) + 8 Acrocentric (A). Whereas *P. ubonensis* is a tetraploid orchid (2n = 4x = 76) with a chromosome pattern of 12M + 48Sm + 16A (Kao et al. 2001; Rungratchakanon et al. 2013). Normal bivalents in meiosis of *P. ubonensis* suggest that it was an allotetraploid species and its meiotic behavior suggested that *P. ubonensis* did not evolve from the autoduplication of *P. pulcherrima* chromosomes (Phonyiam et al. 2010; Rungratchakanon et al. 2013). Therefore, morphological differences and polyploidization might be one of the important factors for the observed genetic distance of these two *Phalaenopsis* species.

Cluster and principle component analyses

In general, a dendrogram generated by UPGMA separated these 50 wild *Phalaenopsis* accessions into 2 clusters, namely cluster I consists of *P. ubonensis* (Daeng Ubon) accessions and cluster II consists of *P. pulcherrima* (Ma wing) accessions (Figure 4). The clustering of these orchid accessions is in good correspondence with their morphological classification and ploidy level. However, despite having similar morphological characters, the tetraploid species (*P. ubonensis*) exhibits larger structures compared to the diploid species (*P. pulcherrima*). The *P. pulcherrima* cluster consisted of two subclusters, subcluster i (II-i) comprised of 11 *Phalaenopsis* accessions from Ubon Ratchathani, whereas subcluster ii (II-ii) comprised 10 *Phalaenopsis* accessions from Mukdahan. The accessions NK9 and CM9 from Phupalanyai subdistrict, Buntharik district, Ubon Ratchathani province and Chong Meg subdistrict, Sirindhorn district, Ubon Ratchathani province, respectively, did not cluster with other accessions. These two accessions displayed the lowest genetic similarity coefficient (0.43) indicating that they might originated and grown in different ecological areas. It is worth noting that CM9 was obtained from local people near the border of Thailand, close to Laos, where wild orchid smuggling is known to occur along the natural border along the Mekong River. Therefore, the genetic make up of these accessions might be different from the others.

Table 3. Genetic diversity of *Phalaenopsis* species in the present study

Parameters	*P. pulcherrima*	*P. ubonensis*
Number of loci	204	204
Number of polymorphic bands	198	194
Percentage of polymorphic bands (%)	97.06	95.10
Observed number of alleles per locus	1.970	1.951
Effective number of alleles per locus	1.538	1.562
Expected heterozygosity	0.3386	0.3279

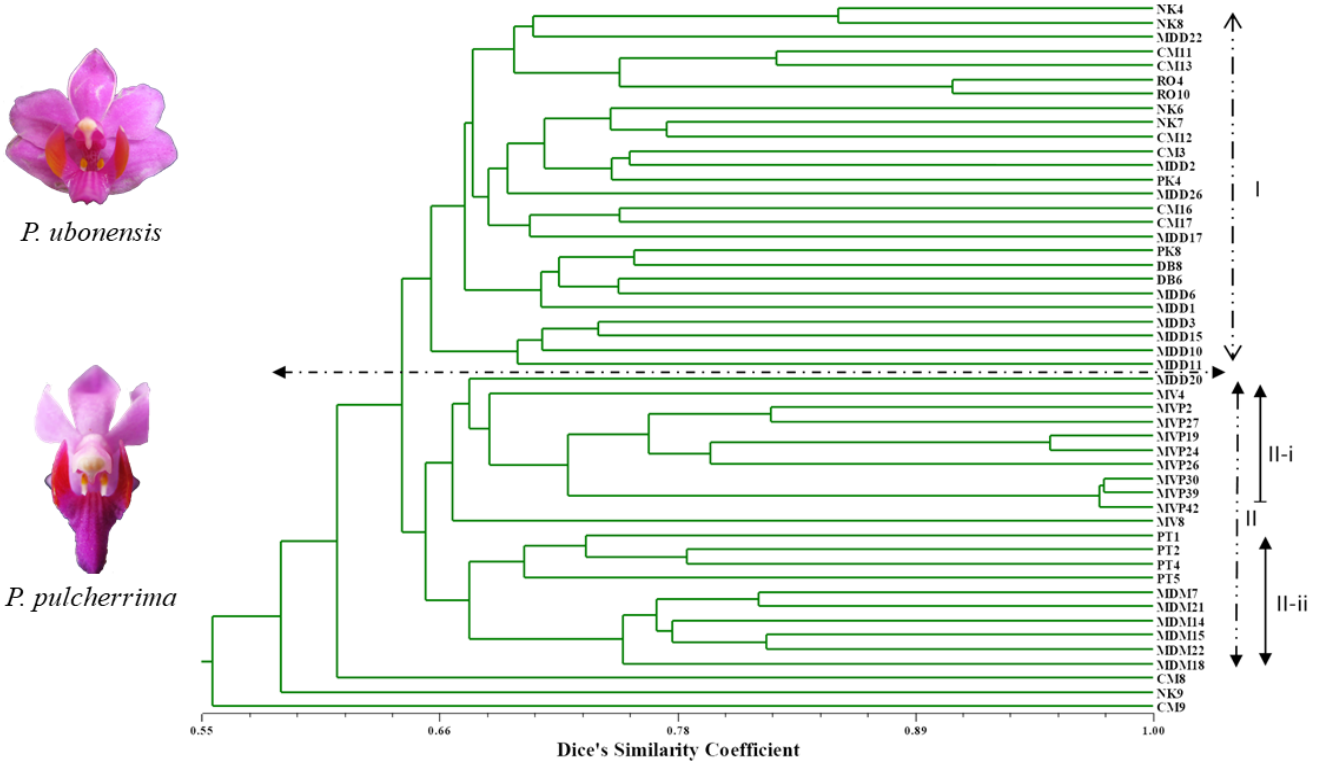

Figure 4. A dendrogram generated by UPGMA cluster analysis of Dice's similarity coefficient based on 204 bands showing genetic relationships among the 50 wild *Phalaenopsis* accessions

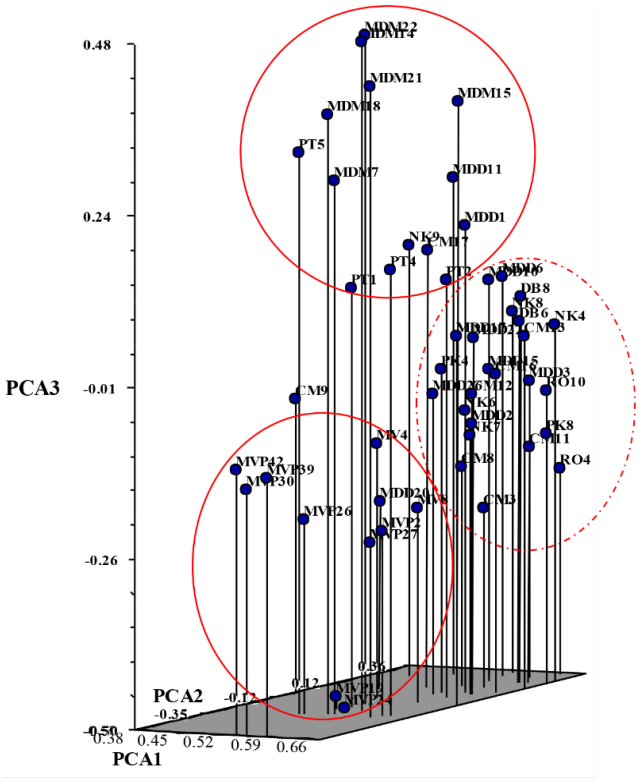

Figure 5. Principle coordinate plot of 50 wild *Phalaenopsis* accessions from northeastern Thailand estimated with 204 RAPD markers using Dice's similarity matrix

Associations among these wild *Phalaenopsis* orchids were also revealed by principal component analysis (PCA) (Figure 5). The first three factors in the PCA accounted for 43.71% of the total variation of the estimated genetic similarity. Clustering patterns based on PCA corresponded well with morphological classification and the UPGMA dendrogram constructed with RAPD markers. A relatively high cophenetic correlation (r = 0.75) was found between the RAPD data matrix and the cophenetic matrix, indicating a good fit for the cluster analysis.

The genetic diversity of these wild *Phalaenopsis* orchids (formerly known as *Doritis*) based on RAPD markers from our study, was found to agree with the *Doritis* genetic diversity report of Jantasuriyarat et al. (2012) which was performed using EST-SSR markers. The results from this study demonstrated that RAPD markers are useful for *Phalaenopsis* species identification and genetic diversity evaluation.

Phalaenopsis pulcherrima and *P. ubonensis* are monopodial lithophytic species primarily found in tropical lowland forests at elevations of 150-400 m above sea level. *P. ubonensis* is distributed only in Thailand and Laos (Christenson 2001), while the native range of *P. pulcherrima* is Assusm to Hainan and W. Malesia. According Sweet (1980), these two species were traditionally treated to the genus *Doritis*, but later they were merged into the genus *Phalaenopsis* by Christenson (2001) and confirmed with molecular and morphological evidence (Goh et al. 2005; Tsai et al. 2010). A phylogeny of *Phalaenopsis* was reconstructed by Padolina et al. (2005) using three chloroplast

markers, namely *matK*, *atpH-F*, and *trnD-E*. Results of Bayesian and maximum parsimony analyses supported the placement of the species of *Doritis* and *Kingidium* into the genus *Phalaenopsis*, as proposed in a revision of the genus by Christenson (2001). To date, the genus *Phalaenopsis* comprises approximately 66 species worldwide and is divided into 5 subgenera, namely *Proboscidioides*, *Aphyllae*, *Parishianae*, *Phalaenopsis*, and *Polychilos*. The classification is mainly based on plant size and floral morphology (flower color and pigmentation patterns, lip and callus structure, number of pollinia, and deciduousness). Among them, subgenus *Phalaenopsis* was further divided into 4 sections, namely *Phalaenopsis*, *Stauroglottis*, *Deliciosae*, and *Esmeralda* (Goh et al. 2005; Padolina et al. 2005; Tsai et al. 2010; Lin et al. 2015). The wild *Phalaenopsis* orchids (*P. pulcherrima* and *P. ubonensis*) in this study were classified under section *Esmeralda* and they are protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) appendix II. This means that they may become threatened with extinction and are therefore subject to international conservation measures.

The present study suggests that the genetic diversity pattern of wild *Phalaenopsis* accessions in the northeastern of Thailand shows a moderate level of relatedness. The lower RAPD diversity occurring in these species is more likely to be a consequence of habitat destruction and fragmentation, leading to genetic drift and limited gene flow among populations. Additionally, over-collection of wild individuals has played a crucial role in contributing to the scarcity and vulnerable status of wild *Phalaenopsis* species. As a result, the number and sizes of the subsisting populations have tremendously declined, leading to further loss of genetic diversity and alteration of population genetic structure (Fay 2018; Pykälä 2019; Chung et al. 2023). The findings from this study indicate an urgent need for the preservation and conservation of this orchid species in Thailand. Conservation strategies both in situ and ex situ should be further studied to identify the best approaches for preserving these wild *Phalaenopsis* species. Additionally, raising awareness among local communities about the critical genetic situation of these threatened wild orchids and involving local authorities in framing policies and strategies for proper plant management are highly recommended.

In conclusion, genetic diversity of 50 wild *Phalaenopsis* accessions in the northeastern of Thailand has been revealed using 204 polymorphic bands. These orchid accessions displayed moderate relationships. In general, the dendrogram generated by UPGMA cluster analysis divided these wild orchids into 2 clusters, namely cluster I consists of *P. ubonensis* accessions and cluster II consists of *P. pulcherrima* accessions. Cluster division corresponds well with their morphological characters and ploidy level. RAPD markers show efficacy in distinguishing these two wild *Phalaenopsis* species. The results of our study provide valuable information for the conservation management of these wild orchids in northeastern of Thailand.

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