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Contributed Paper

Genetic Relationships Between *Globba expansa* (Zingiberaceae) and Other Closely Related Taxa in Thailand Using HAT-RAPD Marker Analysis

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Abstract

Globba expansa Wall. ex Horan. is the most well-known and widespread species of the subsection *Nudae* K.Larsen in mainland Southeast Asia. Several sympatric species with morphological similarities have also been described through its distribution range, therefore creating difficulties in identification among their closely related taxa. To clarify their interspecific relationship, HAT-RAPD marker analysis was carried out for three confusing taxa in this subsection: *G. expansa*, *G. flagellaris* K.Larsen, and *G. macrochila* Sangvir. & M.F.Newman. Seventeen decamer primers were used to generate RAPD products and then to reconstruct a UPGMA dendrogram. Their species recognitions were confirmed since all four accessions of *G. expansa* were grouped together and placed sisterly to *G. flagellaris* in the UPGMA tree. A UBC61 primer gave a unique 200-basepair band for *G. expansa* suggesting that HAT-RAPD could be an effective assay for *Globba* species identifications. Useful morphological characters to discriminate each species, particularly labellum and staminodes, to discriminate each species were also discussed in this paper.

Keywords: DNA marker, *Globba expansa* Wall. ex Horan., HAT-RAPD, Thailand, Zingberaceae

1. Introduction

Globba L. is in the family Zingiberaceae comprising about 100 species. In Thailand, Larsen and Larsen reported that there are 41 species throughout the country [1]. This genus has been used in Thai culture as an ornamental plant. It is also a symbolic flower used during the 'Tak Bat Dok Mai' festival, a famous Buddhist ritual in Saraburi province, Central part of Thailand at which people offer a bunch of *Globba* flowers to monks. This festival takes place annually in the early rainy season, in the flowering period of *Globba*.

Globba expansa Wall. ex Horan. is one of the best-known species of *Globba*. It is

classified in *Globba* section *Nudae* K.Larsen subsection *Nudae*. *Globba expansa* is remarkable by its large habit, erect, conical inflorescence, and elongate cincinni [2]. It is also the most widespread species of subsection *Nudae*, distributed from Myanmar to the north of Thailand throughout Laos, covering most of the range of the subsection [2]. Nonetheless, *G. expansa* has been less studied than might be expected, especially in terms of molecular research. Only one report of the molecular phylogeny of *G. expansa* has been published, but as a member of the genus [3].

To gain a better understanding of the taxonomic relationships of *G. expansa* and its closely related taxa in subsection *Nudae*, we performed a phylogenetic analysis using internal transcribed spacers (ITS) nucleotide sequences of ribosomal DNA (Sangvirotjanapat *et al.*, in prep.). This failed to reveal clearly the relationships between *G. expansa*, *G. flagellaris* and *G. macrochila*. Their ITS sequences were identical leading to an unresolved clade (unpublished data). Likewise, the floral morphology of these three species was rather similar. They all have pure orange flowers so using floral parts to differentiate between them has to be done carefully [2].

As in other gingers, PCR-based marker analysis could be implemented to resolve the phylogenetic complexity of *G. expansa* and its closely related taxa in subsection *Nudae*. The Random Amplified Polymorphic DNA (RAPD) technique was reported to be an effective method to reveal genetic diversity within and between species of many Zingiberaceae genera. For instance, RAPD arbitrary primers were successfully used to determine species of *Curcuma* [4], *Hedychium* [5], *Mantisia* [6], and *Zingiber* [7]. The varieties of *Curcuma longa* [8] and other Zingiberalean genera, such as *Musa acuminata* [9] and *Costus speciosus* [10], have also been investigated using the RAPD technique. Moreover, High Annealing Temperature-RAPD (HAT-RAPD) was developed to produce more stable and distinctive banding patterns than the conventional RAPD technique [11]. With a higher annealing temperature of at least 46°C instead of 35°C-38°C, HAT-RAPD fragments gave better marker results in studies of *Hedychium* [5] and *Musa* [12].

In this research, we used HAT-RAPD technique to solve the questions (1) whether the RAPD technique would be an effective tool to distinguish complex *Globba* species in subsection *Nudae*; (2) whether *G. expansa* samples collected from different localities in Thailand are genetically uniform; (3) whether the morphological characteristics correspond to the RAPD results.

2. MATERIALS AND METHODS

2.1 Plant Materials and DNA Extraction

Seven accessions of three *Globba* species distributed in Thailand, *G. expansa*, *G. flagellaris*, and *G. macrochila,* were chosen for study (see their collecting localities in Figure1). All plant materials were obtained from the living collections of the Royal Botanic Garden Edinburgh (RBGE) and Queen Sirikit Botanic Garden (QSBG). Fresh flowers of each accession were carefully dissected and identified following the key to species of Sangvirotjanapat *et al.* [2]. Voucher specimens were made and deposited at the herbaria of Royal Botanic Garden Edinburgh (E) and Queen Sirikit Botanic Garden (QBG). See Table 1 for a list of voucher numbers and original localities. Leaf material was placed in silica-gel for dehydration before DNA extraction.

2.2 DNA Extraction and HAT-PCR Amplification

The total genomic DNA was extracted from the dried leaf specimens. Leaf tissue, approximately 25 mg, was frozen and ground to fine powder in liquid nitrogen using a mortar and pestle. The tissue powder was extracted immediately using a Geneaid® Genomic

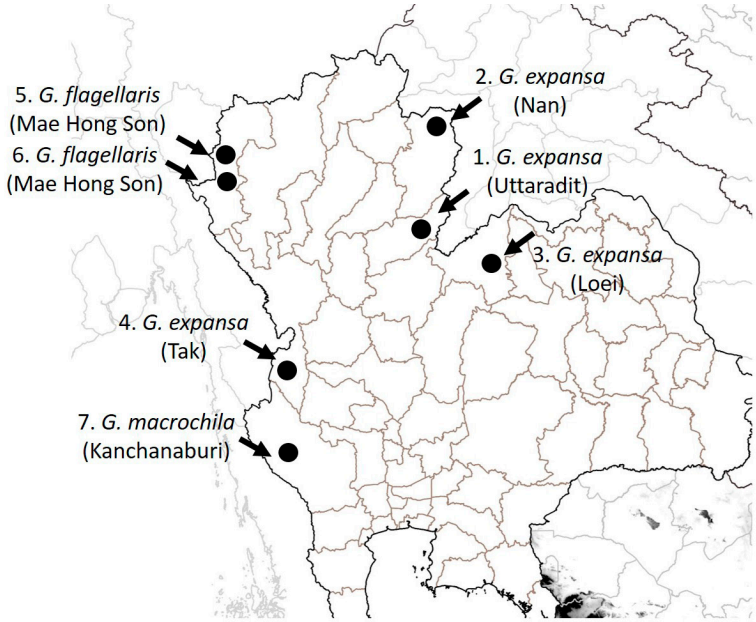

Figure 1. Collecting localities of the plant samples. Names in parentheses are provinces in Thailand.

Table 1. List of *Globba* plant materials.

Sample no.	Scientific name	Voucher number/herbarium code	Locality
1	*G. expansa* Wall ex Horan.	*MFN 2624* (QBG**)	Uttaradit
2	*G. expansa* Wall ex Horan.	*S. Sangvirotjanapat 612* (QBG)	Nan
3	*G. expansa* Wall ex Horan.	*S. Sangvirotjanapat 726* (QBG)	Loei
4	*G. expansa* Wall ex Horan.	*PN 2210* (QBG)	Tak
5	*G. flagellaris* K.Larsen	*MFN&JLS 2048* (E*)	Mae Hong Son
6	*G. flagellaris* K.Larsen	*S. Sangvirotjanapat 692* (QBG)	Mae Hong Son
7	*G. macrochila* Sangvir & M.F.Newman	*S. Sangvirotjanapat 795* (QBG)	Kanchanaburi

*Herbarium of Royal Botanic Garden Edinburgh: E
**Herbarium of Queen Sirikit Botanic Garden: QBG

DNA Mini Kit (Geneaid, Taiwan) following the manufacturer's protocol. The yield of extracted genomic DNA was estimated by 1.0% (w/v) agarose gel electrophoresis stained with ethidium bromide, and visualised with ultraviolet (UV) rays.

HAT-RAPD amplification was performed using a HotStart *Taq* DNA polymerase enzyme (Qiagen, Germany) following this 25 µl reaction of final concentration of: 1xPCR buffer, 8mM of dNTP, 2.5 µM of RAPD primers (University of British Columbia and Operon Technologies, Almeda, California), 2.5 unit of Hotstart *Taq* DNA polymerase and approximately 50 ng of genomic DNA template. The primer names and their nucleotide sequences are shown in

Table 3. The amplification conditions were: initial denaturation of 15 minutes at 95°C, followed by 35 cycles of 1 minute denaturation at 95°C, 45 seconds annealing at 55°C, and 2 minutes extension at 72°C, and a 10 minutes final extension at 72°C. The RAPD-PCR products were examined by 2% agarose gel electrophoresis at 100 Volt in 1xTBE for 45 minutes, stained with ethidium bromide, and photographed under UV illumination.

2.3 RAPD Analysis

An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram was constructed using PAUP*b4.0 software [13], with Nei-Li distance and 1,000 replicate bootstrap-supporting test. The amplified DNA fragments from each RAPD primer were scored as presence '(1)' and absence '(0)' bands. Total and polymorphic amplified PCR bands were counted from each gel result. The Polymorphic Information Content (PIC) value of each RAPD primer was calculated following the equation of Roldan-Ruiz *et al.* [14]. Resolving power (Rp) was calculated according to Prevost and Wilkinson [15].

3. Results

3.1 Floral Morphology Study

The seven accessions were identified into three species using floral morphology. The parts of the flower used to distinguish the species were the lateral staminodes and labellum which differ in size and shape as shown in Table 2 and Figure 2A–C. The four samples recognised as *G. expansa* originated from Uttaradit, Nan, Loei, and Tak. They had oblong lateral staminodes and a labellum length of 11–12 mm. These parts of the four samples showed slight differences in their size but otherwise resembled each other. In particular, the sample from Tak showed no significant difference from the others. Two samples from Mae Hong Son corresponded to *G. flagellaris* by their floral morphology as

Table 2. Comparison the differences of floral morphology of three *Globba* species, *G. expansa*, *G. flagellaris*, and *G. macrochila* from seven accessions of this study.

Characteristics		Scientific name/ voucher specimens		
		G. expansa	***G. flagellaris***	***G. macrochila***
		MFN 2624 ***S. Sangvirotjanapat 612*** ***S. Sangvirotjanapat 726*** ***PN 2210***	***MFN&JLS 2048*** ***S. Sangvirotjanapat 692***	***S. Sangvirotjanapat 795***
1.	Floral size	3.4-3.6 cm	3.3-3.6 cm	3-3.6 cm
2.	Colour	Orange, yellowish orange	Orange	Orange
3.	Lateral staminodes			
	-shape	oblong to narrowly obovate	obovate	narrowly obovate
	-size	11–13 × 3–5 mm	12–15 × 5–9 mm	12 × 7 mm
	-apex	apex round	apex round	apex acute
4.	Labellum			
	-shape	triangular	triangular	triangular
	-size	11–12 × 4–5 mm	10–12 × 4–7 mm	19 × 4 mm
	-spot	absent	absent	absent

Table 3. RAPD primers and their sequences, amplified bands and PIC values after HAT-RAPD amplification of all seven *Globba* samples. The mean of band per primer was 12.7 bands.

Primer no.	Primer name	Nucleotides sequence	Approx. fragment size (bp)	Total band	Number of polymorphic bands	Polymorphism (%)	PIC value	Resolving power (Rp)
1	UBC 25	ACA GGG GTG A	200 – 1,000	9	9	100	0.35	4.57
2	UBC 31	CCC CCC TTA G	200 – 1,500	15	12	80	0.28	6.00
3	UBC 32	CCG GCC TTC A	200 – 1,500	14	14	100	0.36	7.43
4	UBC 33	CCG GCT GGA A	200 – >1,500	11	10	90	0.30	4.86
5	UBC 43	GAG CAC CAG G	100 – >1,500	8	7	87.5	0.30	3.14
6	UBC 61	GAG CAC TAG C	200 – 1,500	13	12	92.3	0.36	6.86
7	UBC 62	GAG CC GGG A	200 – 1,500	10	10	100	0.39	5.71
8	UBC 63	GAG CTC GTG T	300 – 1,500	13	13	100	0.33	5.71
9	UBC 71	GAG GGC GAG G	200 – 1,500	15	15	100	0.33	6.86
10	UBC 72	GAG GTC CAG A	450 – >1,500	8	8	100	0.34	3.71
11	UBC 73	GGG CAC GCG A	200 – 1,500	18	16	88.9	0.33	8.86
12	UBC 74	GGG GCC TTA A	100 – 1,500	13	10	76.9	0.24	4.29
13	UBC 75	TGG ACC GGT G	150 – >1,500	14	14	100	0.37	7.43
14	UBC 76	TTA ACC CCG G	100 – >1,500	16	14	87.5	0.34	8.29
15	UBC 78	TTC CCC GAC C	200 – 1,500	11	7	63.6	0.19	2.57
16	UBC 79	TTC CCC GCC C	200 – 1,500	11	11	100	0.35	5.71
17	OPC-08	TTC CCC GTC G	250 – >1,500	17	15	88.2	0.34	8.26
		Total	100 –>1,500	216	197			
		Average		12.7	11.6	90.94	0.32	5.90

the lateral staminodes were obovate (12–15 × 5–9 mm) and the labellum was not longer than the lateral staminodes. The single sample from Kanchanaburi was *G. macrochila*. It had narrowly obovate lateral staminodes and a greatly elongated labellum (19 mm long) which was about twice as long as the lateral staminodes (Table 2.). The other characteristics of the flower had small differences which were not enough to permit species identification.

3.2 RAPD Polymorphism

From 22 RAPD primers preliminarily screened, 17 primers showed highly polymorphic fragment profiles and were then chosen for further analysis (Table 3). Two hundred and sixteen bands were obtained with lengths from approximately 100 to > 1,500 basepairs. The mean of band per primer was 12.7 bands. Of all 216 bands, 197 were found to be polymorphic. The average of polymorphic bands was 11.59 bands. Primer UBC73 had the highest number of amplified polymorphic bands (16 bands) with a range of 200 to 1,500 basepairs. On the other hand, primers UBC43 and UBC72 produced the lowest number of bands (7 bands), ranging from 100 to 1,500 basepairs and 450 to >1,500 basepairs, respectively. The PIC values of these 17 screened primers were from 0.19 of UBC78 primer to 0.39 of UBC62 primer. The average of PIC value was 0.32. The Resolving power (Rp) values were from 2.57 of UBC 78 primer to 8.86 of UBC73 primer while the average Rp was 5.90 (Table 3). Interestingly, primer UBC61 could distinguish *G. expansa* specifically with a unique 200 basepairs band (Figure 3). In addition, UBC61 and UBC62 primers could specify *G. expansa* sample from Tak province with approximately 700 basepair and 200 basepair bands, respectively.

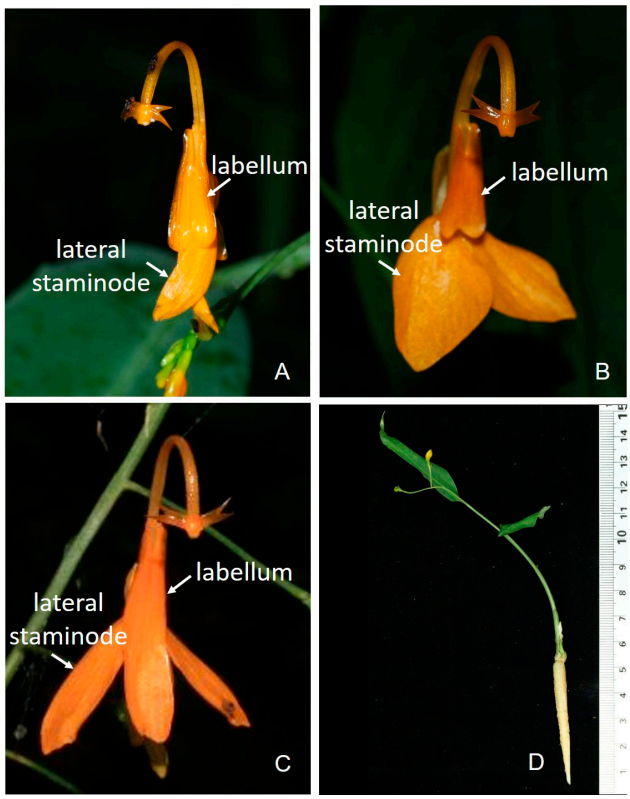

Figure 2. Floral characteristics of three *Globba* species. (A-C) flower of *G. expansa*, *G. flagellaris*, and *G. macrochila*, respectively. (D) a bulbil of *G. expansa* producing leafy shoot and inflorescence. Photos by Sunisa Sangvirotjanapat.

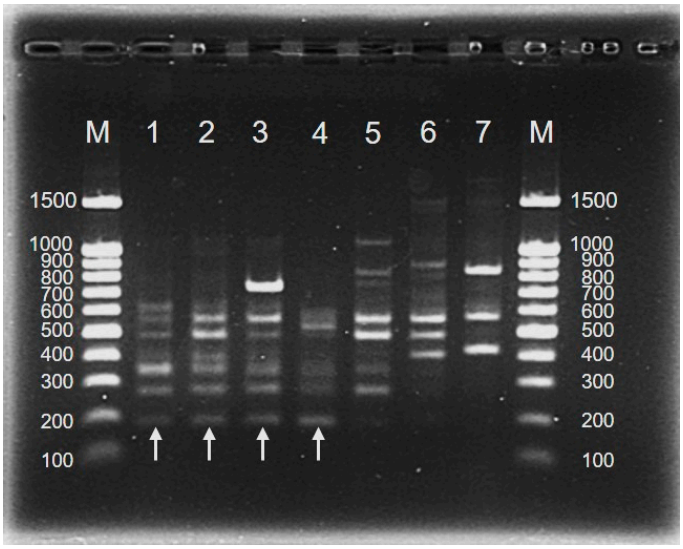

Figure 3. HAT-RAPD products of seven accessions of *Globba* amplified by primer UBC61. The specific bands for *G. expansa* are indicated by white arrows. Lane M= 100-bp DNA ladder marker; lanes 1–4 = *G. expansa*, lanes 5–6 = *G. flagellaris*, lane 7 = *G. macrochila*. Numbers of lanes related to plant accessions and collecting locality in Figure 1.

To analyse the genetic variation within the four accessions of *G. expansa*, the polymorphism of each primer was also calculated to percentage (Table 4). It ranged from monomorphism of primers UBC 72 and UBC78 to 100% polymorphism of primers UBC25, UBC32, and UBC63. The PIC values were from 0.00 of UBC72 and UBC78 primers to 0.43 of UBC25. The average PIC values was 0.23. Similarly, the Rp values of the results from these four *G. expansa* accessions were from 0.00 of the primer UBC72 and UBC78 primers to 6.50 of UBC75 primer with an average of 2.76 (Table 4).

3.3 UPGMA Result

The UPGMA dendrogram of our HAT-RAPD analysis generated from 17 primers revealed a highly-supported cluster of four *G. expansa* accessions collected from different localities in the north of Thailand (Figure 4). They were grouped monophyletically with 100% bootstrap supporting value. Within this *G. expansa* cluster, the two samples from Uttaradit and Nan provinces were paired strongly with 94% bootstrap support. The two accessions of *G. flagellaris* from Mae Hong Son also grouped together with 89% bootstrap. *Globba macrochila* from Kanchanaburi province was basally separated from the other six samples.

Table 4. Band polymorphism of four *G. expansa* samples from different localities in Thailand after HAT-RAPD analysis with 17 primers. The mean of band per primer was 7.4 bands.

Primer no.	Primer name	Total bands	Number of polymorphic bands	Polymorphism (%)	PIC value	Resolving power (Rp)
1	UBC 25	5	5	100	0.43	3.50
2	UBC 31	10	3	30	0.11	1.50
3	UBC 32	7	7	100	0.41	4.50
4	UBC 33	8	2	25	0.09	1.00
5	UBC 43	5	3	60	0.23	1.50
6	UBC 61	8	3	37.5	0.16	2.00
7	UBC 62	7	6	85.7	0.32	3.00
8	UBC 63	5	5	100	0.4	3.00
9	UBC 71	8	6	75	0.31	4.00
10	UBC 72	3	0	0.00	0.00	0.00
11	UBC 73	10	4	40	0.19	3.50
12	UBC 74	7	3	42.9	0.18	2.00
13	UBC 75	10	7	70	0.34	6.50
14	UBC 76	12	6	50	0.2	4.00
15	UBC 78	6	0	0	0	0.00
16	UBC 79	5	4	80	0.38	3.50
17	OPC-08	10	5	50	0.2	3.50
Total		126	69			
Average		7.4	4.1	55.7	0.23	2.76

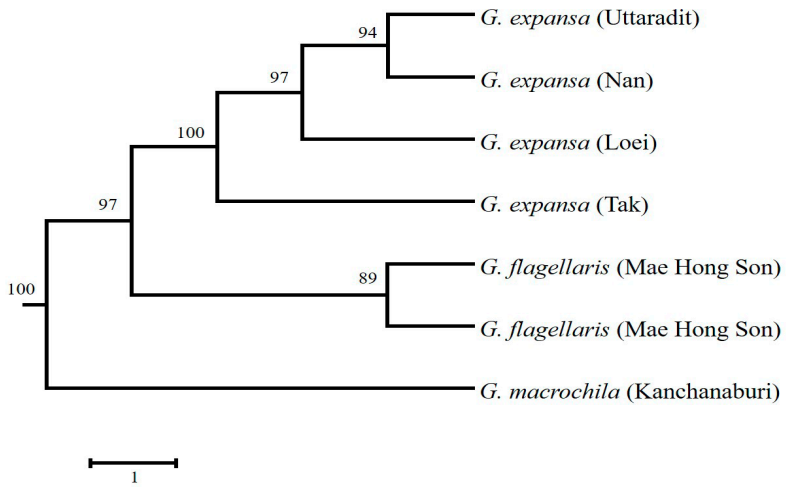

Figure 4. UPGMA dendrogram supporting genetic relationship of *G. expansa, G. flagellaris,* and *G. macrochila* from HAT-RAPD analysis. Numbers above each branch are %bootstrap supporting values.

4. DISCUSSION

From our genetic study of seven *Globba* accessions collected in Thailand, the HAT-RAPD profiles seemed to be very useful to distinguish *G. expansa* from the closely related species, *G. flagellaris* and *G. macrochila*. The method could reveal the genetic relationships between these three species with higher resolution than the ITS nucleotide sequences, which were found to be identical among the three species (unpublished data). Such ITS data cannot be used to investigate the circumscription of members of a species complex so other DNA regions must be added to the analysis. By contrast, the HAT-RAPD results in this study could solve the ITS problem and corresponded well to the different morphological traits of the species studied [2]. The primer UBC62 could be an effective primer to detect genetic polymorphism between these seven *Globba* species with PIC value = 0.39 (see Table 3). Likewise, the UBC73 primer could be the best to detect genetic variation between the plant individuals as its resolving power was the highest (Rp value = 8.86). From the result, the primer UBC25 was found to effectively detect genetic polymorphism within this species with its PIC value was high as 0.43 (see Table 4).

Previously, DNA fragment analysis had been proposed as an alternative method for species identification of several plants [4–12]. The HAT-RAPD technique was reported to be very useful for detecting genetic differences in the family Zingiberaceae. For instance, the HAT-RAPD DNA profiles of the genus *Hedychium* [5] were found to agree well with ITS sequence results [16] and then could support species delimitation. As in our work, most of the supporting values of clusters in their HAT-RAPD profiles were rather high.

Another study in the genus *Musa*, also demonstrated that the HAT-RAPD technique can distinguish cultivars of edible bananas following their parental genomes [12]. Moreover, the RAPD method was introduced to prove the taxonomic status of the genus *Euantha* (Orchidaceae) which had been described by its cytology and morphology. In this case, the result suggested that *Euantha* should remain in *Vanda* because it was nested in the *Vanda*

cluster on the dendrogram [17].

From our results, all accessions of *G. expansa* from four provinces were grouped together with 100% bootstrap (Figure 4), a good level of support to elucidate the species. Although the floral morphology of these plants was found to be identical (Table 2.), the sub-groupings of these *G. expansa* specimens also corresponded to their collecting localities (Figure 4). *Globba expansa* number 1 and 2, collected from Uttaradit and Nan provinces were paired together. Then this pair was grouped with *G. expansa* number 3 from Loei province. While the sample no. 4 from Tak was slightly separated from the other three. This would suggest an impact of geographical distribution to genetic variation of the species. Although all four provinces are in the Indo-Burmese floristic region [18], Uttaradit-Nan-Loei provinces are located in the Northeast as they are located in the Luang Prabang Range on the border of Laos PDR. However, Tak province is on the western side of the country. Since it is partially sit in the Tenessarim Range adjacent to Myanmar. We thus proposed that the genetic diversity among *G. expansa* accessions from different areas may have been driven by specific ecological factors such as microclimate and local insect pollinators. Additional specimens of *G. expansa* from other provinces in Thailand and also from the Mon state of Myanmar, which is the type locality of the species, would give an even clearer understanding of the effects of genetic variation and geographical factors.

Plant-pollinator interactions play a crucial role in co-evolution relationships since they can bring beneficial adaptations to the morphology of plant species [19]. From a morphological point of view, the Zingiberaceae shows very diverse floral traits. Their petaloid staminodes are an important floral characteristic as they form the labellum and staminodes with two different functions in the pollination system. The pollinators land on the labellum that acts as a platform [20-22] while the petal-like staminodes may attract pollinators by their colour [19]. All species in *Globba* subsection *Nudae* have pure yellow or orange flowers and we would expect that their pollinators are bees. Moreover, the visual perception of bees is in the yellow range of the spectrum [19]. In the case of *G. expansa*, it has floral characteristics which match to bees in genus *Amegilla*. From our observations in the field, there were at least two *Amegilla* species which could be pollinators of *G. expansa*. Both have similar body sizes and two of them were identified as *A. fimbriata* while the other could be a new species of *Amegilla* (unpublished data). When an *Amegilla* bee lands on the labellum to suck nectar, its body will be touched by the anther. The range of pollinator service of *Amegilla* bees could have an impact on an extent of occurrence of *G. expansa*. More study needs to be done on this.

Although *G. expansa* is a widespread species, the results of our HAT-RAPD study reveal that it has low genetic diversity. From Table 3, the polymorphism of the four *G. expansa* specimens was as low as 54% which was close to what was previously found in other *Globba* species. For example, *G. spathulata,* a species of restricted habitat in India had 57% polymorphism whereas *G. wengeri* showed the lowest of polymorphism at 37% [6]. We suggest that such low genetic diversity of *G. expansa* in Thailand may be caused by its specifically vegetative propagules called 'bulbils' (Figure 2D). Generally, the bulbils of *G. expansa* are produced in the late rainy season and grow as new plantlets in the next growing season. Sometimes they even grow while still attached to the mother plant. Therefore, the low genetic diversity of *G. expansa* may result from the specific condition of individual plants in populations whether they came from sexual or asexual propagation. This raises a conservation question since any species with low genetic variation would be expected to have

a reduced ability to cope up with environmental alterations [23].

The UPGMA tree from our HAT-RAPD analysis also indicated that *G. expansa* would be more closely related to *G. flagellaris* than to *G. macrochila* (Figure 4). Most species of *Globba* subsection *Nudae* have similar habit and flower colour but differ somewhat in the size and shape of their staminodes. Morphologically, *G. expansa* and *G. flagellaris* have an equal labellum size but the lateral staminodes of *G. expansa* are much narrower (Figure 2A and 2B). On the other hand, *G. macrochila* which was placed separately in the UPGMA tree has a longer labellum than the other two species (Figure 2C). Therefore, the labellum may be another useful criterion for species identification in subsection *Nudae*. The labellum and staminode characteristics have been used taxonomically for other Zingiberaceae genera, such as to separate *Borneocola* from *Scaphochlamys* [24] and to identify species within *Amomum* [25, 26].

5. Conclusions

In this study, the genetic relationship between *Globba expansa*, *G. flagellaris*, and *G. macrochila* was illustrated by using HAT-RAPD markers. The results obtained from the UPGMA dendrogram tree suggested that the three species should be recognised separately as different species and *G. expansa* was found to be closer to *G. flagellaris* than *G. macrochila*. Moreover, the grouping pattern of four accessions of *G. expansa* could reflect the influence of the distances between their growing localities. In addition, this HAT-RAPD result also corresponds well to their morphological characteristics and suggested that lateral staminodes and labellum could be very useful for a correct identification of the three species. HAT-RAPD is therefore an effective molecular technique to reveal genetic differences between *Globba* species and confirm their species identification.

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