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MORPHOLOGICAL CHARACTERIZATION OF WILD *Rhynchostylis gigantea* IN THAILAND

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ABSTRACT

The diversity of 49 living wild *Rhynchostylis gigantea* accessions collected from 22 locations from six regions throughout Thailand were morphologically characterized. The 42 characters, consisting of 12 quantitative and 30 qualitative characters were analyzed at Kasetsart University, Kamphaeng Saen Campus, Thailand, from 2012-2015. The quantitative characters mainly consisted of flower characters; i.e. size of flower, sepals and petals. Thirty qualitative characters were phenotypic characters of the whole plant that were characterized individually by the modified DOA Test Guidelines of Vanda and Vanda Hybrid Descriptor. Only 34 characters (22 qualitative and 12 quantitative traits) could be used for multivariate and clustering analysis. The orchid accessions could be grouped into two groups based on the geography of Thailand, with the members of the first group located in the low-land and the second group in the mountainous area.

Key words: orchid, diversity, characterization, morphology, clustering analysis

INTRODUCTION

Inhabitant orchid diversity is now a crisis worldwide due to several constraints, such as a lack of social awareness, over-exploitation, deforestation and urbanization (Koopowitz et al. 2003). In Thailand, Thitiprasert et al. (2007) reported that at least 175 orchid species are endangered, including orchid species of the genus *Rhynchostylis*. Under the tribe Vandeae, sub tribe Aeridinae, and family Orchidaceae (Dressler 1993), the genus *Rhynchostylis* is a small genus consisting of only four species. Three species, *R. gigantea, R. retusa* and *R. coelestis* are reported as endemic in southeast Asian countries, such as Thailand, Laos PDR, and Myanmar. Among the three species, *R. gigantea* is the best known. Being considered as an inhabitant endangered species, the commercially grown genus *Rhynchostylis;* however, has a high commercial value used for orchid export in Thailand due to available *in vitro* seed germination. In terms of the number of orchids exported from Thailand, as reported by Plant Varieties Protection Division, Ministry of Agriculture, Thailand, commercial hybrids of *R. gigantea* is in the highest demand compared with the other native orchid species. Artificial pollination of the rare alba, rubra forms and the regular form of this species, together with the success of *in vitro* seed germination, gave a wide array of flower colors that catch the eyes of consumers, thus bringing out a high exports per year. To maintain a balance between in-habitat protection and commercialization of this orchid species, knowledge of inhabitant diversity is necessary.

To determine *R. gigantea* diversity, morphological characterization has always been the first attempt due to its simplicity. Examples of orchids investigated on morphological variability include populations of *Epipactis helleborin* (Ehlers et al. 2002), epiphytic orchid species on a small oceanic island (Mallet et al. 2014), and the variance among populations of tropical orchid with a restricted gene flow (Tremblay 1997). Evolutionary studies based on morphological characterization were also reported in the subtribe Aeridina (Hidayat et al. 2006) and in *Liparis resupinata* (Tetsana et al. 2014).

However, for wild *R. gigantea*, a diversity study has never been reported. In this study, we investigated the morphological variation of 49 living wild *R. gigantea* collected throughout the floristic regions of Thailand. The number of twelve quantitative and twenty-two qualitative characters were characterized in order to understand the correlation and distribution of this species in Thai natural habitats.

MATERIALS AND METHODS

Plant samples

Forty-nine living wild *R. gigantea* were collected mainly from their primary natural habitat in Thailand, together with a secondary collection from plants grown in the Horticultural Research Center, Department of Agriculture, Ministry of Agriculture (DOA, Thailand), wherein their natural habitats were confirmed. The collected samples were grouped into 5 populations (Table 1), based on the floristic region of Thailand, as described by Smitinand (1958). These populations were the North (N), North-eastern (NE), East (E), South-western (SW), and Peninsular (PEN) populations. A group collected from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) rescue centers under DOA, Thailand was also included, and thus denoted as a group with ‘uncertain origin’. The outgroup samples were *R. retusa* and *R. coelestis*, since they belong to the same genus. All collected plants were maintained under a 50% shaded greenhouse at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand, from 2012-2015.

Morphological characterization

The number of 42 qualitative and quantitative characters, including 8 characters from stems and leaves, 2 characters of inflorescence, and 32 characters of flowers, were characterized. Each accession was evaluated individually by the modified DOA Test Guidelines of Vanda and Vanda Hybrid Descriptor (http://www.doa.go.th/ pvp/images/stories/ form_dus/27%20rb_vanda.pdf).

Qualitative traits characterization

The 30 qualitative characters from both vegetative and reproductive parts were characterized.

***Vegetative characters*:** Vegetative characters were evaluated during the first year the sample was collected. These characters included leaf shape (assessed by measuring the ratio of the length and width of the leaf), leaf apices, leaf cross section, angle at the top of leaf, twisting of the leaf apex, leaf margins, leaf variegation on the abaxial side, and color of the leaf base.

Floral characters: Due to the habit of ‘once a year’ flowering during December and February for this species, the floral characters, hence, were obtained during 2 flowering seasons, one from 2012-2013 and another from 2013-2014. Three flowers from the top, center, and bottom positions of an inflorescence indicated by arrows in the Fig.1A were characterized. The qualitative traits characterized included flower arrangement, inflorescence orientation, flower form, shape of dorsal sepal, shape of dorsal sepal apiece, cross section of dorsal sepal, twisting of dorsal sepal, wave in dorsal sepal, blushing on dorsal sepal, shape of lateral sepal, shape of lateral sepal apiece, cross section of lateral sepal, twisting of lateral sepal, wave in lateral sepal, blushing on lateral sepal, shape of petal, shape of petal apiece, cross section of petal, twisting of petal, wave in petal, blushing on petal, and lip character.

RESULTS AND DISCUSSION

Morphological variance of wild type *R. gigantea* in Thailand was determined and characterized through the modified Vanda descriptor. However, as the plant samples were collected from various natural habitats (Table 1), they were not only varied in age and size, but also some other phenotypes.

Table 1. Samples used for this study and the climatic conditions of their origin.

Provincial code	Region[1/]	/ Province	Sample size	Climatic zone [2/]
1	N	Chiang Rai	2	T1-H2
2	N	Chiang Mai	1	T1-H1
3	N	Lamphun	6	T1-H1
4	N	Tak	3	T1-H1
5	N	Sukothai	1	T2-H1
6	N	Phitsanulok	3	T2-H1
7	N	Kamphaeng Phet	2	T2-H1
8	NE	Loei	3	T1-H1
9	NE	Khon Kaen	3	T2-H1
10	NE	Sakon Nakhon	6	T2-H2
11	NE	Nakhon Phanom	2*	T1-H2
12	NE	Mukdahan	1	T2-H2
14	NE	Ubon Ratchathani	3*	T2-H2
15	E	Sri Sa Ket	2	T2-H2
17	E	Nakhon Ratchasima	1	T2-H1
18	SW	Kanchanaburi	3**	T2-H2
19	SW	Ratchaburi	1	T3-H2
20	SW	Petchaburi	1	T3-H3
21	SW	Prachuap Kriri Khan	1*	T3-H3
22	PEN	Chumphon	4	T3-H3

[1/] The abbreviation of region: North (N), North-eastern (NE), East (E), South-western (SW), and Peninsular (PEN).

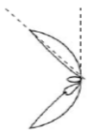
[2/] The climatic zone of Thailand according to Khedari et al. (2002) was divided into 3 zones based on temperature, with, T1=12-38 ºC, T2=16-38 ºC, and T3=20-38ºC, and into 4 zones based on relative humidity with, H1=30-100%, H2=41=100%, H3=50-100% and H4=59-100%.

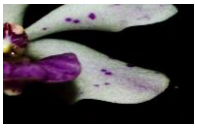
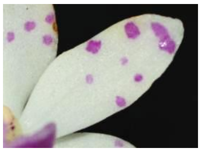
* The sample from CITES rescue center that may have another origin elsewhere.

** Two samples from Kanchanaburi and one sample from CITES rescue center.

Phenotypes that were highly correlated with physiological parameters were explained by Zotz et al. (2001), including plant height, leaf color, leaf texture, length of inflorescence, and number of flowers in an inflorescence. Therefore, in this report, these characters were disregarded and thus, 42 characters (8 characters from stems and leaves, 2 characters based on inflorescence, and 32 floral characters) were characterized. Among these 42 characters, 30 were qualitative while 12 were quantitative. The selected qualitative characters are listed in Table 2.

Table 2. Vegetative and floral characteristics of *Rhynchostylis gigantea* modified from DOA's Vanda descriptor.

Characteristics	Code	Scales of Characterization
Leaf shape	T1	(1) average of L/W ratio =3-6:1 (2) average of L/W ratio =7-10:1 (3) average of L/W ratio =>12:1 (4) average of L/W ratio = 21:1
Leaf apices	T2	 (0) Acute (1) Oblique (2) Emerginate (3) Praemorse (4) Tridenlate
Cross section of leaf	T3	 (0) Flat  (1) Carinate
Angle at the top of leaf	T4	 (0) <90º (1) 90º (2) >90º
Twisting of leaf apex	T5	 (0) Absent  (1) Present
Leaf margins	T6	 (0) Entire  (1) Undulate/Wavy
Inflorescence orientation	T10	 (1) Erect  (1) Horizontal  (2) Pendulous
Flower form	T11	 Incurved  (1) Semi-incurved  (2) Flat

Shape of sepal and petal apices	T13, T19, T25	 Acute	 (1) Obtuse	 (2) Cuspidate	 (3) Orbicular
Cross section of sepal and petal	T14, T26	 Margin incurved	 (1) Margin		
Blushing on dorsal sepal	T17	(1) Absent		(1) Present	
Cross section of sepal	T20	 Assemetry	 (1) Opposite		
Wavy lateral sepal	T22	Absent		(1) Present	
Blushing on lateral sepal	T23	(0) Absent		(1) Present	
Twisting of petal	T27	(0) Absent		(1) Present	
Wave in petal	T28	(0) Absent		(1) Present	

Variance analysis of quantitative characters

All quantitative characters fell in the size of flower. Flowers were sampled from 3 different positions; namely from the top, middle and bottom, of the inflorescence (Fig. 1A). Twelve quantitative characters of wild *R. gigantea* flower and floral component were measured. The characters of each flower that were measured are in Fig.1B. Multivariate Analysis of Variance (MANOVA) analysis showed highly significant differences among regions ($P< 0.01$) (data not shown). Morphological variance is assumed to be the result of diverse environmental conditions and/or habitat heterogeneity (Wagner et al. 2015).

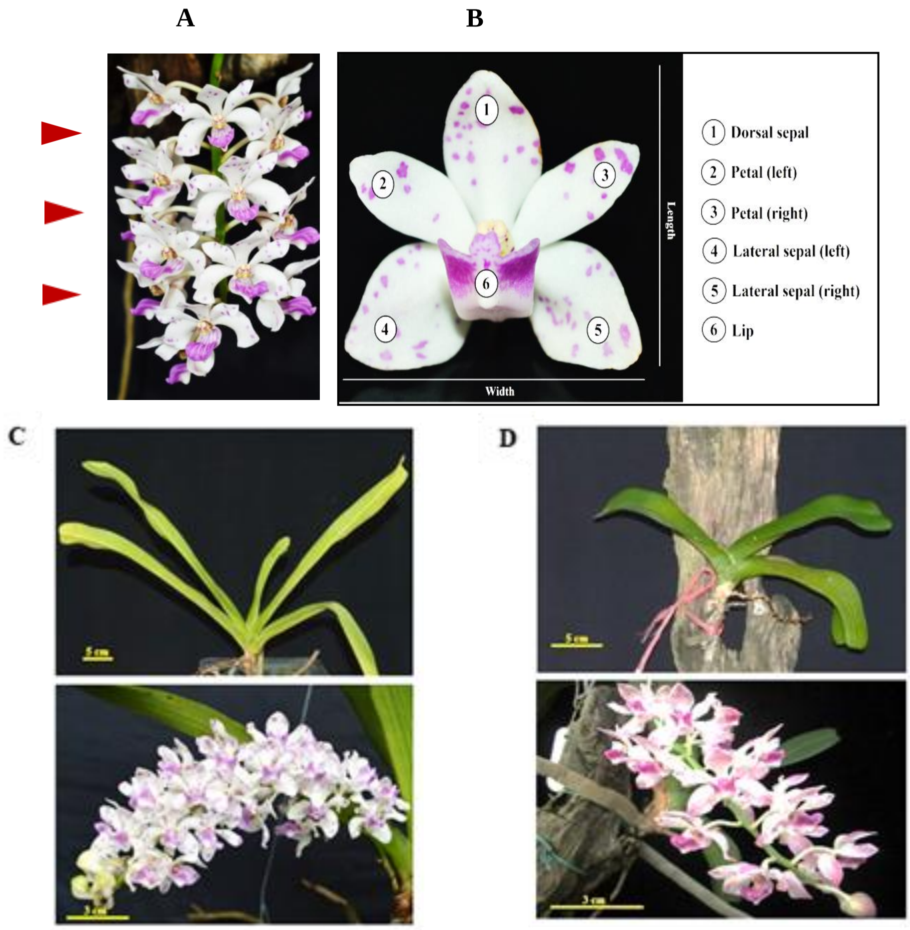


Fig. 1. Wild *Rhynchostylis gigantea* distributed in Thailand. The position of an inflorescence of collected flowers for morphological characterization (A) and flower structure (B). The characteristic of the stem, inflorescence and floral representative of group I (C) and group II (D) according to UPGMA analysis in Fig. 3.

Analysis of variance for each trait according to natural habitat showed high significant difference in floral size and dorsal sepal width ($P<0.05$) (Table 3). Sample from northern and south-western regions had the largest floral size and dorsal sepal width relative to samples from other regions. Whilst, smaller floral size in width was present from samples from the peninsular region. A narrow dorsal sepal was present in accessions collected from the Eastern and peninsular regions. This indicated that *R. gigantea* accessions, based on the population studied, had a low amount of variability in terms of size of floral-part characteristics.

The low variability in these samples may be due to a narrow genetic base of *R. gigantea* samples collected, based on morphological characteristics. Variation among *R. gigantea* accessions, however, was highly observed from 22 qualitatively floral-characteristics out of the 30 morphological floral traits (flower arrangement, inflorescence orientation, flower form, shape of dorsal sepal apices, cross section of dorsal sepal, blushing on dorsal sepal, shape of lateral sepal apices, cross section of lateral sepal, wave in lateral sepal, blushing on lateral sepal, shape of petal apices, twisting of petal, wave in petal, blushing on petal, and lip characteristics). A similar finding in terms of the high variation of floral characteristics was also reported by Wongsa et al. (2013) in a *R. gigantea* population.

Table 3. Mean value (mm), standard error, and *P-value* of ANOVA based on 12 quantitative traits from 49 *Rhynchostylis gigantea* accessions.

Character[2/]		**Location of Origin[1/]**					*P-value*
		N	**NE**	**E**	**SW**	**PEN**	
FS	W	28.5±0.4 ^{a}	28.±0.5 ^{a}	27.4±0.9 ^{a}	29.0±0.7 ^{a}	24.4±0.6 ^{b}	0.0004
	L	24.6±0.4 ^{a}	23.7±0.4 ^{ab}	21.9±0.2 ^{bc}	24.0±0.7 ^{a}	21.4±0.9 ^{c}	0.0028
DS	W	9.5±0.1 ^{a}	9.0±0.2 ^{ab}	8.7±0.2 ^{b}	9.5±0.2 ^{a}	8.9±0.4 ^{ab}	0.0307
	L	14.8±0.3	14.7±0.3	13.7±0.4	14.2±0.5	13.6±0.6	0.1515
LSL	W	9.3±0.1	9.1±0.2	9.0±0.2	9.2±0.2	8.5±0.3	0.2543
	L	14.8±0.2	14.8±0.3	13.8±0.4	14.8±0.4	13.7±0.4	0.1853
LSR	W	9.3±0.2	9.1±0.2	9.0±0.2	9.0±0.5	8.5±0.4	0.3392
	L	14.7±0.2	14.7±0.3	13.6±0.4	14.6±0.5	13.6±0.5	0.0739
PL	W	5.3±0.1	5.4±0.2	5.1±0.2	5.4±0.1	4.9±0.3	0.3506
	L	13.6±0.2	14.1±0.3	12.9±0.3	13.7±0.5	13.5±0.5	0.3292
PR	W	5.1±0.1	5.3±0.2	5.0±0.2	5.5±0.2	4.7±0.3	0.2310
	L	13.7±0.2	14.3±0.3	13.2±0.2	13.9±0.4	13.5±0.5	0.2442

[1/] Abbreviations of location of origin: N=North, NE = North-eastern, E=East, SW=South-western, PEN=Peninsular, and U=Uncertain origin.

[2/] Abbreviations of floral characters: FS=Flower size, DS=Dorsal sepal, LSL=Lateral sepal (left), LSR=Lateral sepal (right), PL=Petal (left), PR=Petal (right), W=Width of flower size, and L=Length of flower size.

Evaluation of quantitative characters

Characterization of 30 qualitative characters revealed that 22 of them were polymorphic while the rest were monomorphic. The number of 8 qualitative characters that were monomorphic consisted of 1) shape of dorsal sepal, 2) twisting of dorsal sepal, 3) wave in dorsal sepal, 4) shape of lateral sepal, 5) twisting of lateral sepal, 6) shape of petal, 7) cross section of petal and 8) blushing on petal. Therefore, only 22 qualitative traits and 12 quantitative traits from 49 *R. gigantea* samples were subjected to principal component analysis and cluster analysis.

Principle Component Analysis (PCA)

PCA is a technique that identifies the traits contributing most on the variation within a group of varieties or genotypes. Complex data are transformed from a number of relative traits into a smaller number of variable as PCs. The PCA shows that the first four components with an eigenvalue greater than 1.0 contributed about to 80.75% of the total variation (Table 4). PC1, accounted for 59.56% of the total variation, was mostly determined by the negative value of all quantitative characters and a positive value for leaf shape, leaf apices, and cross section of dorsal sepal. The highest loadings in PC1 indicated the importance of this component. These traits carried the largest portion of its variability. PC2, contributed 10.60% of the total variation, was mostly determined by a positive value of quantitative traits and a qualitative trait, such as twisting of leaf apex, leaf margin, shape of lateral sepal, petal and cross section of dorsal sepal. PC3 accounted for 5.65%, and was mostly determined by a positive value of floral sepal length, lip character, twisting of petal, and a negative value of sepal and petal width, leaf apex, and leaf margin characteristics.

The correlation coefficient between any two traits is approximated by a cosine of the angle between their vectors (Dehghani et al. 2008). A strong positive association among LSLW, LSRW, DSW, SLL, SLW and SRW; among LSRL, DSL, and LSLL among T3, T10, T11, T17, T22, T23, T27, T28 and T30; between T5 and T6, and among T1, T2, T8, T9, T14 and T19 (Table 2.) was shown in Fig. 2, as indicated by the small obtuse angles between their vectors. There was a negative correlation between groups T3 and T1, as indicated by the angle of approximately 180 degrees.

Table 4. The Eigenvalues, proportion of variation, and cumulative variations across the axis of the first ten principal components.

Principal component	Eigen value	Variation (%)	Cumulative variation (%)
1	20.24	59.56	59.56
2	3.60	10.60	70.17
3	1.92	5.65	75.82
4	1.68	4.93	80.75
5	0.99	2.91	83.66
6	0.77	2.25	85.91
7	0.60	1.77	87.69
8	0.47	1.39	89.07
9	0.45	1.33	90.40
10	0.39	1.16	91.56

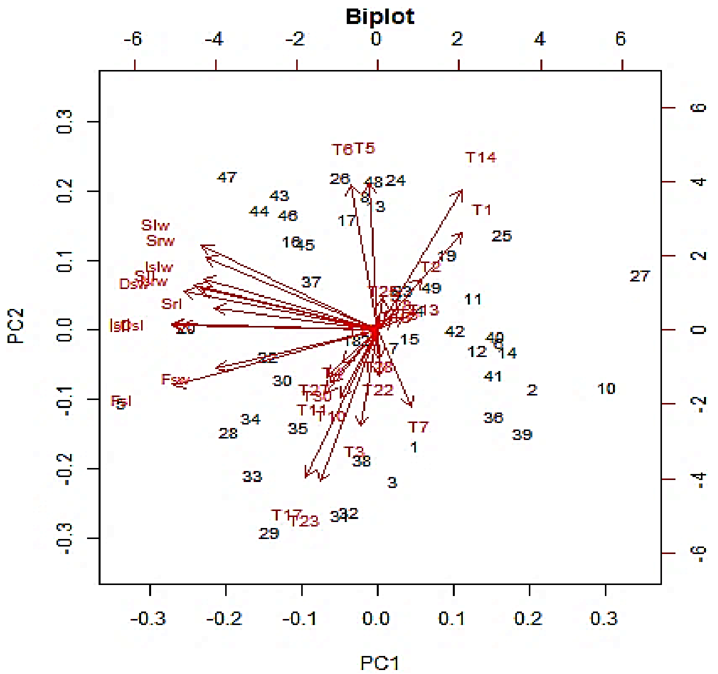


Fig. 2. Two dimensions of PCA analysis showing a relation among 49 accessions of wild *Rhynchostylis gigantea* collected in Thailand. Data from 22 qualitative and 12 quantitative characters were analyzed. The symbols (T..) are qualitative traits as explained in Table 2.

Clustering analysis using Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

Clustering analysis was performed on 49 wild *R. gigantea* accessions through an UPGMA approach. *R. retusa* and *R. colestis* were used as the outgroup samples. *R. retusa* and *R. colestis* clearly separated from the ingroup samples of *R. gigantea* (Fig. 3). This was in agreement with the morphological classification of this genus by Seidenfaden and Wood (1992) and Eng-Soon (2005).

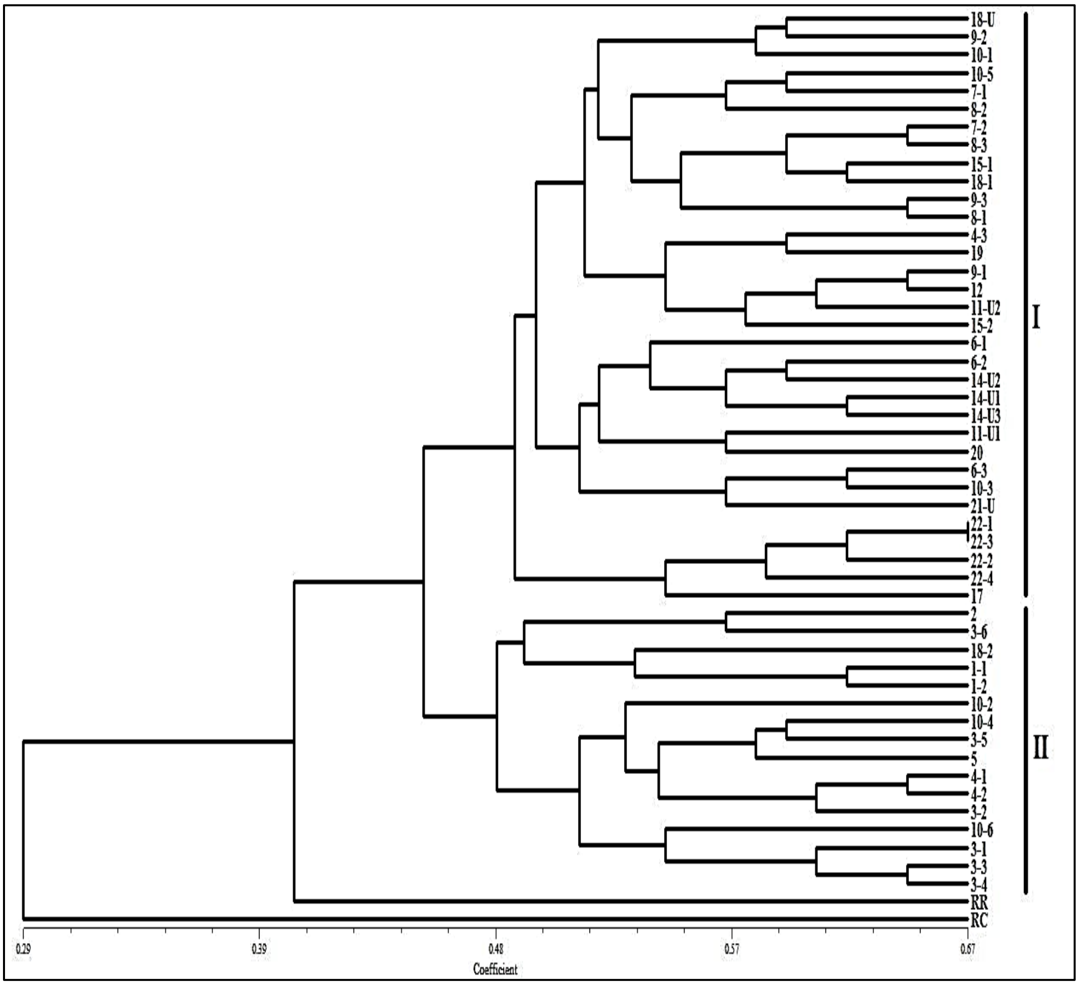


Fig. 3. Clustering analysis by UPGMA of wild *Rhynchostylis gigantea* distributed in Thailand generated by all characters.

For the ingroup accessions of wild *R. gigantea*, results revealed that the coefficient similarity was between 0.286 and 0.667. They were divided into two groups (Fig. 2A), corresponding to the geography of Thailand (Fig. 4), rather than the provincial location of their origin. The first group (Fig.1C) consisted of 33 accessions from different geographic regions, except the Northern region. The random pattern of distribution of accessions indicated no association between diversity of morphological trait and geography. The characteristics of this group included a leaf ratio of greater than 7:1 (97%) and a flat leaf cross section (76%). Inflorescence orientation observed were pendulous (82%) and horizontal (18%). No pink blushing was observed on the dorsal sepal (85%), lateral sepal (94%), and petals (100%). This group is widely distributed in the low-land areas of Thailand (Fig. 4).

The second group (Fig. 1D) consisted of 16 accessions from the Northern mountainous area of Thailand, including Chiang Rai, Chiang Mai, Lamphun, and Sukhothai provinces. The characteristics of this group, distinguished from the first group, include a leaf ratio of 3-7:1 (100%), craniate shape of leaf cross section (81.3%), non-twisting leaf apex (87.5%), pendulous inflorescence orientation (97.7%), pink blushing on the dorsal sepal (93.7%) and lateral sepal (87.5%) (Fig. 4).

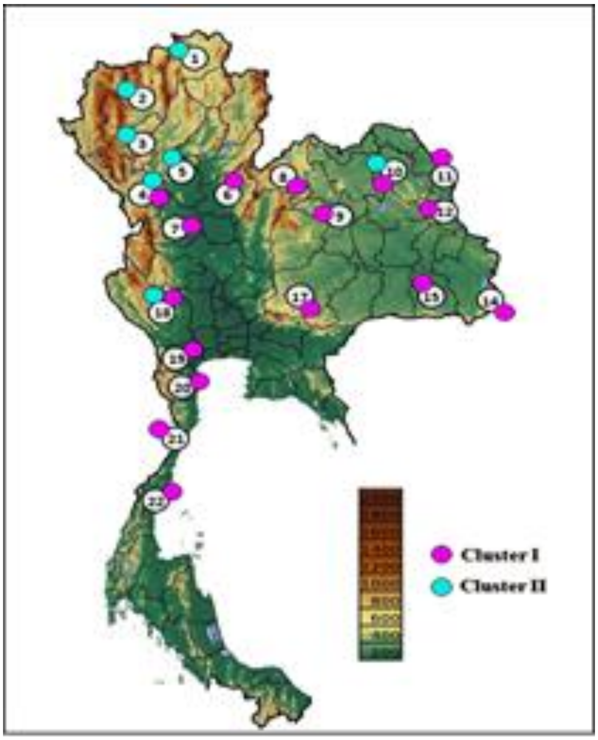


Fig. 4. The geographic maps of Thailand in correlation with the grouping of accessions.

In 2002, Khedari et al. discussed the climatic area of Thailand, which associated the altitude gradient and climate condition. The correlation revealed that the mountainous area has a lower ambient temperature and relative humidity than the lowland area (Table 1 and Fig. 4). Differences in environmental conditions, temperature, relative humidity, light, and precipitation are important factors influencing plant physiological performance (Zotz et al. 2001), which affects morphological variation in orchid species (Morales et al. 2010; Blinova 2012). In many orchid species, the environmental gradient was reported as a contributor to phenotypic variation, such as *Dactylorhiza hatagirea* (Warghat et al. 2012) and *Tolumnia variegate* (Morales et al. 2010). In their natural habitat, epiphytic plants encounter severe environmental stress, such as intense light and water shortage. Some orchid species utilize the Crassulacean Acid Metabolism (CAM) as a system for survival under severe environmental conditions. Some species have evolved to change their physiological process in order to adapt to their environment (Adiban and Ainuddin 2011). As a result, structures, such as shoots, leaf number and area, have been altered for survival (Blinova 2012; Willems and Ellers 1996).

Tremblay (1997) and Morales et al. (2010) reported that not only the macroclimate, but also the microclimate, influence morphological variation. In terms of natural distribution, *R. gigantea* is a vascular epiphyte which grows on their host trees, and are distributed throughout deciduous forests at an average of 200-1000 m above sea level and in coastal forests on limestone habitat (Pridgeon et al. 2014). Hoverer, an epiphyte-host interaction was also reported as associated with morphological variation (Callaway et al. 2002). Host trees facilitate not only to support epiphytes but also provide habitat. Host structure mainly influences variability in epiphyte performance. Different host traits, such as whole tree architecture, bark texture, bark chemical, leaf density, and host age, as well as substrate stability, microclimate, nutrition support, and toxicity (Wagner et al. 2015) contribute to the morphological variation of several orchid species, such as *Liparis resupinata* (Tetsana et al. 2014), *Caladenia catenata* (Morrison and Weston 1985), *Epidendrum* and *Tolumnia variegate* (Pinheiro and Cozzolino 2013). Moreover, season is another factor affecting morphological variation in epiphytes (Einzmann et al. 2014; Ackerman et al. 2011). These factors could therefore explain differences in *R. gigantea* population distribution in Thailand.

Genetic diversity associated with altitude gradient has been described in many plant species (Ohsawa and Ide 2008), such as *Cattley liliputana*, (Leles et al. 2015). In the contrast, Mallet et al. (2014) reported that genetic variation of an epiphytic orchid species, *Jumellea rossii*, was affected by habitat heterogeneity rather than by geographic distance. However, a diversity study on *R. retusa* in India revealed genetic similarity with altitude gradient (Parab and Krishnan 2008).

Considering the accessions obtained from CITES rescue centers, morphological characters were not grouped according to the locations of CITES rescue centers. For example, the accession 18-U collected from the rescue center at Kachanaburi (SW region) clustered with the same subgroup of *R. gigantea* accessions from Khon Kaen (9-2) and Sakon Nakorn (10-1), which were collected from the provinces in the North-eastern region of Thailand with a distance of 500-700 kilometers. Similar results were also found for other accessions collected from other CITES rescue centers as well. This may be due mainly to the illegal relocation of wild species by the vendors, which were reported were from neighboring countries (Phelps 2015). The trade network route between point of harvest and final market place was complex and might be transferred through several intermediary areas.

CONCLUSION

The diversity of *Rhynchostylis gigantea* accessions collected from 22 locations across five regions of Thailand was investigated. From a total of 42 morphological characters (30 qualitative and 12 quantitative traits), only 34 characters (22 qualitative and 12 quantitative traits) were used for multivariate and clustering analysis. The orchid accessions were grouped into two based on the geographical area of Thailand. The first group is found in the low-land while the second group is found on the mountainous area. These results suggest that diverse environmental conditions and/or habitat heterogeneity cause phenotypic variation. However, based on these morphological characteristics genetic diversity of *R. gigantea* was low, therefore, molecular analysis should be employed to obtain further information.

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