

Full Length Research Paper

DNA barcoding of the *Cymbidium* species (Orchidaceae) in Thailand

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The objective of the research is to achieve molecular markers for an economical, ornamental plant group in agriculture worldwide. These plants, *Cymbidium aloifolium*, *Cymbidium atropurpureum*, *Cymbidium bicolor*, *Cymbidium chloranthum*, *Cymbidium dayanum*, *Cymbidium devonianum*, *Cymbidium ensifolium*, *Cymbidium finlaysonianum*, *Cymbidium haematodes*, *Cymbidium insigne*, *Cymbidium lancifolium*, *Cymbidium lowianum*, *Cymbidium mastersii*, *Cymbidium munronianum*, *Cymbidium rectum*, *Cymbidium roseum*, *Cymbidium sinense*, *Cymbidium tigrinum*, and *Cymbidium tracyanum* in Thailand have been explored, collected and identified. DNA barcoding for a species specific marker was performed in order to provide further rapid, accurate, and automatable species identification for plants lacking flowers or having incomplete morphological characteristics and for the young plants that are massively grown on orchid cutting flower farms. The sequences of four standard regions as barcodes were tested for genetic distances. The genetic distances, means nucleotide variations in tag sequences based on *rpoB*, *rpoC1*, *matK*, and *trnH-psbA* spacer region sequences of the 19 species ranging from 0.012 to 0.546, 0.018 to 0.546, 0.052 to 0.385, and 0.026 to 0.528, respectively. DNA barcoding shows promise for molecular-based identification of *Cymbidium* species. The sequences have been deposited in GenBank.

Key words: *Cymbidium*, *matK*, *rpoB*, *rpoC1*, species diversity, *trnH-psbA*.

INTRODUCTION

Cymbidium Sw. is a popular group of orchids with significant commercial importance. Their flowers can come in all shades of colors with varying attractive shapes, and can be of long lasting quality and of large size (Obara-Okeyo and Kako, 1998). The members of this genus have a range of distribution from Northwest India to China and Japan, south through the Malay Peninsula to Northern and Eastern Australia (Choi et al.,

2006). *Cymbidium haematodes* Lindl. was once classified to be a subspecies of *Cymbidium ensifolium* (L.) Sw. by Du Puy and Cribb (1988). Currently, it is recognized as a species, *Cymbidium haematodes* by Liu et al. (2009). Also, *Cymbidium siamense* Rolfe and Downie was rearranged to be *C. ensifolium* by Du Puy and Cribb (1988), but was included to be *C. haematodes* by Liu et al. (2009). Many people have reported that 20 *Cymbidium* species can be found in Thailand. They are namely *Cymbidium aloifolium* (L.) Sw., *Cymbidium atropurpureum* (Lindl.) Rolfe, *C. bicolor* Lindl., *Cymbidium chloranthum* Lindl., *Cymbidium cyperifolium* Lindl., and *Cymbidium dayanum* Rchb.f., *Cymbidium devonianum*

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Paxton., *C. ensifolium* (L.) Sw., *Cymbidium finlaysonianum* Lindl., *Cymbidium insigne* Rolfe, *Cymbidium lancifolium* Hook., *Cymbidium lowianum* Rchb.f., *Cymbidium macrorhizon* Lindl., *Cymbidium mastersii* Griffith and Lindl., *Cymbidium rectum* Ridl., *Cymbidium roseum* J.J. Sm., *Cymbidium sinense* (Jacks) Willd., *Cymbidium tigrinum* Parish and Hook., *Cymbidium tracyanum* L. Castle, *Cymbidium wadae* Yukawa (Vaddhanaphuti, 1992; Thaithong, 2000; Yukawa, 2002; Sitthisatjadharn, 2006; Kaenratana, 2009).

DNA barcoding is a popular method for species identification that can be used in samples which have a short region of reliable DNA or even in those samples that have highly degraded DNA such as those found in processed foods, in fossil remains, or herbarium specimens. However, Taberlet et al. (2006) have claimed that this study in most cases only allows for the identification of specimens to a family, but does not allow for the identification of genera or species. Nevertheless, there have been many studies of the DNA barcoding in plants (Hebert et al., 2003). Since then there have been many studies that have tested standard regions in plant groups aiming to provide rapid, accurate, and automatable species identification by using a standardized DNA region as a tag (Hebert et al., 2005). Chase et al. (2007) proposed to use two options, the three regions combined for a standard protocol for barcoding all land plants namely *rpoB*, *rpoC1*, *matK*, and *trnH-psbA* spacer regions. Newmaster et al. (2008) proposed to use *matK* and *trnH-psbA* to identify plants in Myristicaceae.

Finally, Hollingsworth et al. (2009) at the Consortium for the Barcode of Life (CBOL), the plant working group, recommended *rbcl* and *matK* to be used as the core DNA barcode for land plants. We performed DNA barcoding on species of the genus *Cymbidium* in Thailand. DNA barcodes with *rpoB*, *rpoC1*, *matK*, and *trnH-psbA* spacer regions of each species were performed to further test rapid, accurate, and automatable species identification in the plants that lack flowers or have incomplete morphological characteristics and in immature plants.

MATERIALS AND METHODS

Plant materials

Cymbidium species were explored, collected and identified following Du Puy and Cribb (1988), Kaenratana (2009), and Liu et al. (2009). Short descriptions are given and photos are demonstrated. Voucher specimens were prepared and kept at BK.

Molecular performing

All collected samples were field grown and were studied with DNA extraction, DNA barcoding amplification, and DNA barcoding

sequence analysis. The DNA barcoding was done following *rpoB*, *rpoC1*, *matK*, and *trnH-psbA* spacer regions allowing for two options:

- 1) *rpoC1*, *matK*, and *trnH-psbA* spacer regions, and
- 2) *rpoB*, *matK*, and *trnH-psbA* spacer regions following Chase et al. (2007).

DNA extraction

Total genomic DNA was extracted using the Plant Genomic DNA Extraction Kit (RBC Bioscience). Extracted DNA was run on a 0.8% agarose gel electrophoresis stained with ethidium bromide. The quality and quantity of DNA samples were determined by a gel documenting instrument. DNA samples were diluted to a final concentration of 20 ng/μl, and these dilutions were used as DNA templates in the PCR reactions.

DNA barcode amplification

For DNA barcoding of the species-specific samples quoted earlier, PCR were performed using primer pairs 5'-AAGTGCATTGTTGGAAGTGG-3' and 5'-CCGTATGTGAAAAGAAGTATA-3' of *rpoB*, 5'-GTGGATACACTTCTTGATAATGG-3' and 5'-TGAGAAAACATAAGTAAACGGGC-3' of *rpoC1*, 5'-TAATTTACGATCAATTCATTC-3' and 5'-GTTCTAGCACAAAGAAAGTCCG-3' of *matK*, and 5'-GTTATGCATGAACGTAATGCTC-3' and 5'-CGCGCATGGTGGATTACAATCC-3' of *trnH-psbA* (<http://www.kew.org/barcoding/update.html>; 28 January 2009). The reaction mixture was done in 25 μl consisting of GoTaq Green Master mix (Promega), 0.25 μM each primer, and 10 ng DNA template. The reaction mixture was incubated at 94°C for 1 min and amplification was performed with the following thermal cycles: 35 cycles of denaturation for 30 s at 94°C, 40 s annealing temperature at 53°C, 40 s extension at 72°C, and 5 min final extension at 72°C. The amplified products were detected by 1.2% agarose gel electrophoresis in TAE buffer and were visualized with ethidium bromide staining.

DNA sequence analysis

The amplified specific fragments of the studied samples were sequenced and the sequences were submitted to the GenBank database. Sequence alignment was done by using MEGA software Version 5 (Tamura et al., 2011) and the genetic distances were calculated by this software.

RESULTS

Investigation and identification

All *Cymbidium* species are used ornamentally. From the investigation, 19 species were identified. The total number of voucher specimens has been deposited at BK as collector numbers A. Chaveerach 701 to 719. The list of taxa and GenBank accession numbers are shown in Table 1. Concise descriptions including short morphological characteristics, habits, habitats, and distribution

Table 1. GenBank accession numbers of the four barcoding regions of all studied *Cymbidium* species.

Plants	Voucher number	GenBank accession no.			
		<i>rpoB</i>	<i>rpoC1</i>	<i>mat K</i>	<i>trnH - psbA</i>
<i>C. aloifolium</i>	A. Chaveerach 701	HM137074	HM053600	HM137055	FJ527771
<i>C. atropurpureum</i>	A. Chaveerach 702	HM137080	HM053606	HM137061	HM008994
<i>C. bicolor</i>	A. Chaveerach 703	HM137066	GU990532	GU990531	FJ527762
<i>C. chloranthum</i>	A. Chaveerach 704	HM137065	HM053592	HM137047	FJ527761
<i>C. dayanum</i>	A. Chaveerach 705	HM137064	HM053591	HM137046	FJ527760
<i>C. devonianum</i>	A. Chaveerach 706	HM137072	HM053598	HM137053	FJ527769
<i>C. ensifolium</i>	A. Chaveerach 707	HM137069	HM053595	HM137050	FJ527765
<i>C. finlaysonianum</i>	A. Chaveerach 708	HM137067	HM053593	HM137048	FJ527763
<i>C. haematodes</i>	A. Chaveerach 709	HM137082	HM053608	HM137063	HM008996
<i>C. haematodes (C. siamense)</i>	A. Chaveerach 709	JN412744	JN412743	JN412742	JN412741
<i>C. insigne</i>	A. Chaveerach 710	HM137071	HM053597	HM137052	FJ527768
<i>C. lancifolium</i>	A. Chaveerach 711	HM137077	HM053603	HM137058	HM008991
<i>C. lowianum</i>	A. Chaveerach 712	HM137076	HM053602	HM137057	HM008990
<i>C. mastersii</i>	A. Chaveerach 713	HM137078	HM053604	HM137059	HM008992
<i>C. munronianum</i>	A. Chaveerach 714	HM137081	HM053607	HM137062	HM008995
<i>C. rectum</i>	A. Chaveerach 715	HM137070	HM053596	HM137051	FJ527767
<i>C. roseum</i>	A. Chaveerach 716	HM137079	HM053605	HM137060	HM008993
<i>C. sinense</i>	A. Chaveerach 717	HM137075	HM053601	HM137056	HM008989
<i>C. tigrinum</i>	A. Chaveerach 718	HM137073	HM053599	HM137054	FJ527770
<i>C. tracyanum</i>	A. Chaveerach 719	HM137068	HM053594	HM137049	FJ527764

areas are as follows Figure 1:

***C. aloifolium* (L.) Sw. (Swartz, O. Nov. Act. Soc. Sci. Upsal. ser 2, 6:73. 1799):** This species enjoys hot and humid weather. This epiphyte has a large number of upright acute roots and xerophytes. Plants are highly variable in size with some as large as *C. finlaysonianum* at 1 m tall and others are as short as 25 cm. Leaves are erect, thick and coriaceous, ranging from 1 to 6.3 cm wide to 25 to 100 cm long. Inflorescences are pendulous, ranging from 30 to 90 cm long and having 14 to 50 flowers on each rachis. The flowers are 3 to 5 cm in diameter and range in color from red mixed with brown to brown mixed with yellowish hues. Flowering takes place from April to July. Distribution: Through Thailand, 0 to 1500 alt.

***C. atropurpureum* (Lindl.) Rolfe (Rolfe, R.A. Orchid Rev. 11:190-191. 1903):** These medium to large-sized plants are epiphytes and lithophytes. Leaves are erect, thick, glossy, and coriaceous and range in size from 3 to 4.5 cm wide to 50 to 90 cm long. Inflorescences are pendulous, ranging from 28 to 75 cm long and having 10 to 33 flowers on each rachis. The flowers are 3.5 to 4.5 cm in diameter and range in color from dark red mixed with brown to brown mixed with green. Flowering takes place from March-May. Distribution: On trees or rocks in the evergreen forests of Southern Thailand such as Yala,

and Narathiwat Provinces, 0 to 2,200 alt.

***C. bicolor* Lindl. (Lindley, J. Gen. Sp. Orchid. Pl. 164. 1833; Lindley, J. Bot. Reg. 25: misc. 47. 1839):** Its vegetative parts look very like *C. aloifolium* and it is virtually impossible to distinguish between these two species without having flowers. However, *C. bicolor* is generally smaller than *C. aloifolium* including crown, leaf, and the number and size of the flowers. They are epiphytic and xerophytic. The leaves are erect, thick, glossy, and coriaceous ranging in size from 0.8 to 3 cm wide to 20 to 70 cm long. Inflorescences are pendulous ranging from 10 to 50 cm long and having 5 to 26 flowers on each rachis. The flowers are 2.5 to 4.5 cm in diameter, and the petals are dark red at the center and yellow or light green at the edges. Flowering takes place between October-March. Distribution: On trees through Thailand, 0 to 1,500 alt.

***C. chloranthum* Lindl. (Lindley, J. Bot. Reg. 29:68.1843):** The species is an attractive small *Cymbidium*. Its specific characteristic is a change in flower color from green mixed with yellow to reddish pink when flowers have been pollinated or when the anther caps have been removed. The change in flower color is also pronounced in *Cymbidium insigne*. It is an epiphyte with thick, arching leaves, ranging from 3 to 4 cm wide to 40 to 60 cm long. Inflorescences are erect to arching



Figure 1. All *Cymbidium* species found for barcoding construction, species showing natural habits, inflorescences and flowers namely: *C. aloifolium* (A1, A2); *C. atropurpureum* (B1, B2); *C. bicolor* (C1, C2); *C. chloranthum* (D1, D2); *C. dayanum* (E); *C. devonianum* (F1, F2, F3); *C. ensifolium* (G); *C. finlaysonianum* (H1, H2); *C. haematodes* (I1, I2); *C. insigne* (J1, J2); *C. lancifolium* (K1, K2); *C. lowianum* (L1, L2); *C. mastersii* (M1, M2); *C. munronianum* (N1, N2); *C. rectum* (O1, O2); *C. roseum* (P1, P2); *C. sinense* (Q); *C. tigrinum* (R1, R2); *C. tracyanum* (S1, S2).

ranging from 30 to 47 cm long and having 15 to 40 flowers on each rachis. Flowers are about 3 cm in diameter and are green in color mixed with yellow. Flowering is not fixed in any month, but it can always be found in bloom from June to November. Distribution: On trees in evergreen forests of Southern Thailand, such as

Chumporn, Distribution: Nakornsrihammarach and Pattanee Provinces, 250 to 1,000 alt.

***C. dayanum* Rchb. (Reichenbach, H.G. Gard. Chron.710. 1869):** It is a small to medium sized *Cymbidium* with the beautiful feature of long and narrow,

but durable leaves. The plant is an epiphyte. The leaves are thick, dark green and arching and range from 0.7 to 2.2 cm in width to 40 to 115 cm in length. Inflorescences are pendulous ranging from 18 to 30 cm long and having 5 to 20 flowers on each rachis. The flowers are 3 to 4.5 cm in diameter and are either white with a red line at the middle and with red lips or all parts are red. Flowering can take place all year round, but most flowering takes place in August and September. Distribution: On trees in evergreen forests of Southern Thailand such as Chumporn, Nakornsrihammarach and Pattanee Provinces, starting from 800 alt. or more.

***C. devonianum* Paxton. (Paxton, J. Mag. Bot. 10: 97-98, + fig. 1843):** It is a small one *Cymbidium* species and has wider leaves that differ from the others. It is an epiphyte, a terrestrial plant and a xerophyte. Leaves are lorate, erect or slightly arching, thick and semi-coriaceous and range from 3.5 to 6.2 cm wide to 20 to 49 cm long. Inflorescences are pendulous ranging from 15 to 44 cm in length and having 15 to 50 flowers ranging from 2.5 to 3.5 cm in diameter. The flowers are brown in color mixed with orange and green with the exception of the lips which range from violet to dark red. Flowering takes place from March to June. Distribution: Endemic species at Phuluang Mountain, Loei Province, Northeastern Thailand, 1,400 alt. It grows on sandy soil mixed with leaf litter and in partial shade provided by the flowering montane scrub.

***C. ensifolium* (L.) Sw. (Swartz, O. Nov. Act. Soc. Sci. Upsal. 6:77. 1799):** It is a small one *Cymbidium* species terrestrial in habit. Leaves are arching with a width ranging from 0.8 to 2.5 cm and a length ranging from 29 to 94 cm. Inflorescences are erect and are usually shorter than the leaves measuring 15 to 67 cm in length. There are 3 to 9 flowers on each rachis which are 3 to 5 cm in diameter. The flowers are white mixed with yellow and there are red longitudinal lines along the sepals and petals. Flowering takes place near the end of summer to early winter (about May to November in Thailand). Distribution: Terrestrial growing in rather heavy leaf litter in both deciduous and evergreen forests at Doi Chiang Dao National Park, Chiang Mai Province, Doi Khun Tan National Park, Lumpang Province, Northern Thailand; Phukradung National Park, Loei Province, Northeastern Thailand, 1200 to 1900 alt.

***C. finlaysonianum* Lindl. (Lindley, J. Gen. Sp. Orchid. Pl. 164. 1833):** The species is a rather large member of the *Cymbidium* species. It is epiphytic and xerophytic. Leaves are thick and coriaceous ranging from 3 to 6 cm wide to 36 to 100 cm long. Inflorescences are pendulous measuring 30 to 140 cm long and having a suitable distance between flowers. On each rachis there are 25 to 40 flowers, which are 4.0 to 5.7 cm in diameter. The

colors range from brown mixed with yellow to brown mixed with green. Flowering takes place from March to June, but this species can bloom sporadically throughout the year. Distribution: Epiphyte found on tree branches or trunks in partial shade or in open locations of lowland forests in Chumporn and Nakornsrihammarach Provinces, Southern Thailand, 0 to 500 alt.

***C. haematodes* Lindl. (Lindley, J. Gen. Sp. Orchid. Pl. 162. 1834):** This terrestrial species has arching, leathery leaves ranging from 1.1 to 1.3 cm in width to 35 to 45 cm in length. Inflorescences are erect extending from the leaf shoot and measure 45 to 50 cm in length. There are 8 to 9 flowers on each rachis measuring 3 to 5 cm in diameter. The color of the flowers is yellowish-green mixed with violet. Flowering takes place from September to January. Distribution: Border of Thailand and Laos, terrestrial found in rather heavy leaf litter in deciduous forests, Northeastern Thailand, 200 to 250 alt.

***C. insigne* Rolfe. (Rolfe, R.A.Gard. Chron. ser. 3, 35-387. 1904):** This is a terrestrial plant that is found in shallow soil and on sandy ground. It is a medium-sized member of *Cymbidium*. It has lorate leaves, membranous arching, and an acute apex. Leaves range from 0.7 to 1.8 cm in width to 40 to 100 cm in length. Inflorescences are erect and measure from 100 to 150 cm in length. On each rachis there are 5 to 23 flowers, which are 7 to 9 cm in diameter and are located near the ends of the inflorescences. Colors range from white to white mixed with pink. The lips can display red lines, distributed red points, or can display no color points at all. Flowering takes place from January to April. Distribution: On shallow, sandy ground with minimal soil or humus and under the broken shade of ericaceous shrubs such as *Rhododendron* species in lower montane scrubs, endemic species at Phuluang Mountain, Loei Province, Northeastern Thailand, 1,450 alt.

***C. lancifolium* Hook. (Hooker, W.J. Exot. Flora. 1: t.51. 1823):** The plant is a small-sized *Cymbidium* species. It is terrestrial with a stem supported by roots above ground. The leaves are lorate and range from 1.9 to 5.5 cm wide to 9 to 50 cm long. Inflorescences are erect measuring 7 to 35 cm long and have 4 to 8 flowers distanced on the rachis. Flowers are 2.5 to 5 cm in diameter and are light green, light yellow, or light orange in color with red lines along the sepals and petals. Flowering takes place between April and October. Distribution: Terrestrial with heavy leaf litter in evergreen forests of Phaduowdai, Khao Yai National Park, Nakornratchasima Province, Northeastern Thailand at 1,600 alt.

***C. lowianum* Rchb.f. (Reichenbach, H.G. Gard. Chron. n.s. 11: 332, 404, t.56.1879):** It is a large-sized member of *Cymbidium* with large flowers that are very long

lasting. It is an epiphyte. Leaves are arching and are 3.5 cm wide and measuring up to 90 cm long. The inflorescences gently arch and are finally pendulous. The rachis is large, strong, and displays beautiful arching. There are 12 to 40 flowers measuring 8 to 10 cm in diameter on each rachis. The flowers are yellowish-green with a dominant v-band of red on the lip. Flowering takes place from January to June. Distribution: Epiphyte on tall trees in damp, shady evergreen or mixed forests at Doi Pahompok National Park, Doi Inthanon National Park, Chiang Mai Province, Northern Thailand, about 1,600 alt.

***C. mastersii* Griffith and Lindl. (Griffith, W. & Lindley, J. Bot Reg. 31: t.50. 1845):** The species is a medium-sized member of *Cymbidium*. It is an epiphyte and a lithophyte. Leaves are arching and measure about 1.8 cm in width and up to 64 cm in length. Inflorescences ranging from 20 to 30 cm in length are arching and are finally pendulous. On each rachis there are 2 to 10 flowers measuring approximately 6 cm in diameter. Colorations are white or pinkish-white having red points distributed on the lip. Flowering is from September to December. Distribution: Epiphyte on tall trees and rocks in damp and shady evergreen or mixed forests at 900 to 2500 alt in Doi Inthanon National Park in Chiang Mai Province, Northern Thailand, Phluangmountain, Loei Province, Northeastern Thailand.

***Cymbidium munronianum* King and Pantling (King, G. and Pantling, R. J. Asiatic Soc. Bengal. 64: 338. 1895):** *C. munronianum* is a small *Cymbidium* species and a terrestrial plant. Leaves are erect and glossy ranging from 1.8 to 2.7 cm wide to 60 to 80 cm long. Inflorescences are erect measuring up to 60 cm and having 9 to 16 flowers with a diameter of 2.5 to 3.5 cm on each rachis. The flowers range from white mixed with yellow to white mixed with green. There are red lines along the sepals and petals. Flowering takes place during August and September. Distribution: Northern border areas of Thailand and Myanmar at about 500 alt.

***C. rectum* Ridl. (Ridley, H.N. J. Roy. Asiat. Soc. Str. Br. 82: 198. 1920):** The species is a rather small *Cymbidium* species. It is a rare and endangered species due to habitat loss and is unable to withstand drought conditions. It is an epiphyte that grows on trees. The leaves, which are narrow linear, slightly arching, v-shaped, glossy, and coriaceous, measure from 0.8 to 1.4 cm wide to 20 to 60 cm long. Inflorescences are upright or sub-erect, measure 20 to 40 cm in length, and have 8 to 17 flowers on each rachis. The flowers, which measure 3 to 4 cm in diameter, are yellowish mixed with brownish in color and usually have large brownish lines along the sepals and petals. Flowering takes place from September-December. Distribution: Grows in shade on tree trunks, tree stumps, and fallen trees and does not

usually grow higher than 80 cm from the ground in swamps, swampy forests and se-open (?) tropical forests at 450 to 800 alt. of Krabi Province, Southern Thailand.

***C. roseum* J.J.Sm. (Smith, J.J. Orch. Java 475. 1905; Smith, J.J. Bull.Jard. Bot. Buitenzorg. ser. 3, 6: t.11. 1924):** It is a rare *Cymbidium* species with a small to medium size. It is a lithophyte. Leaves are erect, thick, and rigid measuring from 2.2 to 2.7 cm in width to 20 to 40 cm in length and having unequal tip lobes. Inflorescences are erect and finally arching measuring 19 to 30 cm in length and having 2 to 5 flowers on each rachis. Flowers are 5 to 6 cm in diameter and are white or light pink in color. Flowering takes place from August to December. Distribution: Growing on exposed rocks or cliffs with minimal shade in Krabi Province, Southern Thailand at 1,000 to 1400 alt.

***C. sinense* (Jacks) Willd. (Willdenow, C. Sp. Pl., ed.4: 111. 1805):** The plant is a medium-sized *Cymbidium* species. It is a terrestrial species. The leaves are dark green, erect, and glossy and range from 1.5 to 3.2 cm wide to 40 to 103 cm long. Inflorescences are erect and are 40 to 80 cm long with 8 to 26 flowers on each rachis. Flowers measure approximately 5 cm in diameter and are reddish-brown in color or are green mixed with brown. Flowering takes place from November to March. Distribution: Grows on the ground with rather heavy leaf litter in both deciduous and evergreen forests and is usually found in partially shaded to heavily shaded locations near small ravines at 250-2,300 alt. in Chiang Mai Province, Northern Thailand.

***C. tigrinum* Parish and Hook. (Parish, C. & Hooker, W.J. Bot. Mag. 90: t.5457. 1864):** It is a small *Cymbidium* species and is both a lithophyte and an epiphyte. Its arching leaves are thin and lorate measuring up to 3.3 cm wide and up to 22 cm long. Inflorescences measuring 12 to 23 cm long are sub-erect or arching and have 2 to 5 flowers on each rachis. Flowers are from 4 to 5 cm in diameter. The color is green mixed with yellow and the lips are white with red lines and violet. Flowering takes place from March to July. Distribution: Grows on exposed rock and cliffs without shade. In Thailand the plants were found as epiphytes on mossy branches of tall, shaded trees and on *Rhododendron lyi* in cloud forests near the Thai-Laos border, at 2,106 alt. of Phu Soi-dao National Park, Chiang Mai Province, Northern Thailand.

***C. tracyanum* L. Castle (Castle, J.L. J. Hort. ser. 3, 21: 513. 1890):** This species is a large-sized *Cymbidium* species and is an epiphyte with a large number of upright acute roots. The bush looks very much like *C. lowianum* and can be separated by its flowers. Its arching leaves are large and rigid measuring 3.5 to 4 cm in width and up

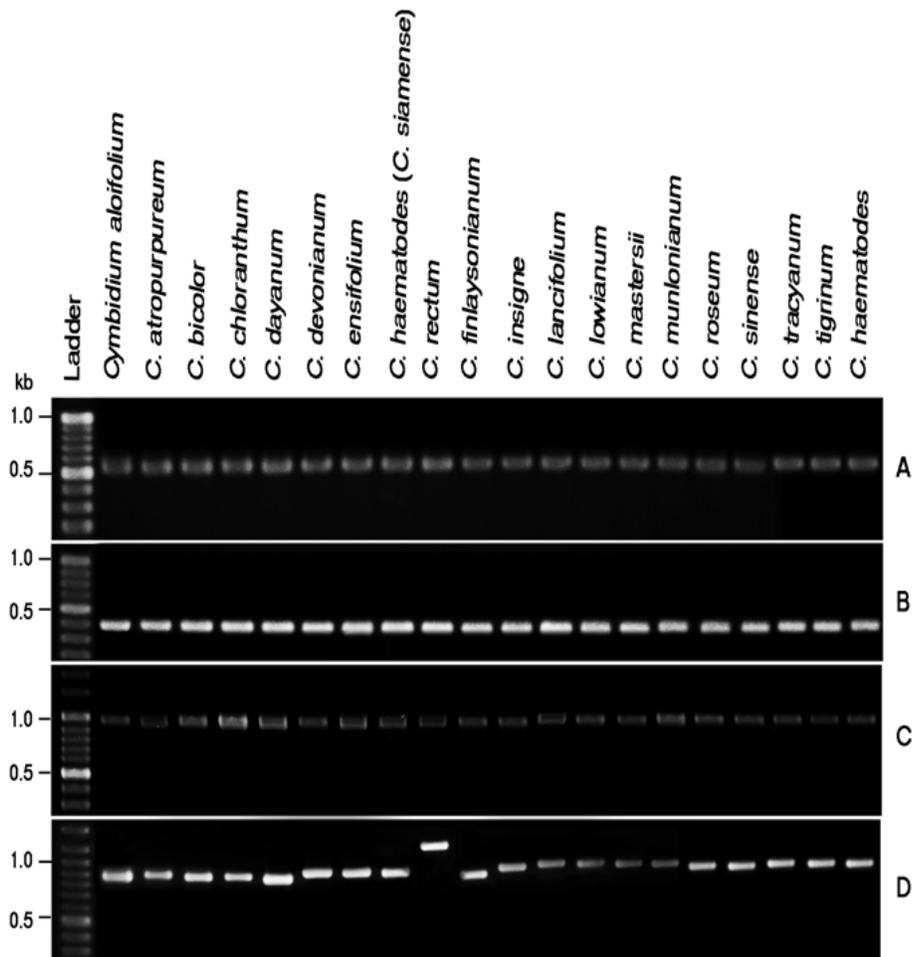


Figure 2. DNA barcode fragments of all *Cymbidium* species in standard regions *rpoB* (A), *rpoC1* (B), *matK* (C), and *trnH-psbA* (D).

to 95 cm in length. Inflorescences are erect or arching, and measure up to 130 cm long with 10 to 20 flowers on each rachis. Flowers, which are 12 to 15 cm in diameter, are yellow mixed with green and have brown lines along the sepals and petals. Flowering takes place from September-January. Distribution: Grows in moist, shady areas, on tree trunks, and on fallen trees in evergreen or mixed forests of Doi Inthanon National Park, Chiang Mai Province, Northern Thailand, and in Tak Province, Western Thailand, at 1200-1900 alt.

Molecular marker as barcode treatment and sequence analysis

DNA extraction has been successful in all samples. DNA barcodes have been successfully created with four standard regions in all studied samples as shown in Figure 2. These were separated to be two options: 1) *rpoC1*, *matK*, and *trnH-psbA* spacer regions and 2) *rpoB*,

matK, and *trnH-psbA*. Being a species-specific marker, the DNA barcode of each species was performed for further rapid, accurate, and automatable species identification. The sequences of the four standard regions as barcodes are namely the *rpoC1*, *matK*, and *trnH-psbA* spacer regions, and *rpoB*, *matK* and *trnH-psbA* were tested for genetic distances using MEGA software Version 5. The genetic distances of all species are shown in Tables 2 to 5. The genetic distance levels are 0.012 to 0.546 (*C. tracyanum* and *C. roseum*-*C. haematodes* (*C. siamense*) and *C. ensifolium*) for *rpoB*, 0.012 to 0.546 (*C. tracyanum* and *C. roseum*; *C. haematodes* (*C. siamense*) and *C. ensifolium*) for *rpoC1*, 0.052 to 0.385 (*C. finlaysonianum* and *C. bicolor*; *C. mastersii* and *C. dayanum*) for *matK*, and 0.026-0.528 (*C. roceum* and *C. mastersii*; *C. haematodes* (*C. siamense*), and *C. aloifolium*) for *trnH-psbA* spacer region. These tag sequences were submitted to the GenBank database. The GenBank accession numbers are listed in Table 1.

Table 2. Genetic distances of the studied species from the sequence analysis of the *rpoB* region.

Studied species	<i>C. aloifolium</i>	<i>C. atropurpureum</i>	<i>C. bicolor</i>	<i>C. chloranthum</i>	<i>C. dayanum</i>	<i>C. devonianum</i>	<i>C. ensifolium</i>	<i>C. haematodes</i>	<i>C. haematodes(C. siamense)</i>	<i>C. finlaysonianum</i>	<i>C. insigne</i>	<i>C. lancifolium</i>	<i>C. lowianum</i>	<i>C. mastersii</i>	<i>C. munronianum</i>	<i>C. rectum</i>	<i>C. roseum</i>	<i>C. sinense</i>	<i>C. tracyanum</i>	<i>C. tigrinum</i>
<i>Cymbidium aloifolium</i>	0.000																			
<i>C. atropurpureum</i>	0.072	0.000																		
<i>C. bicolor</i>	0.042	0.081	0.000																	
<i>C. chloranthum</i>	0.066	0.087	0.057	0.000																
<i>C. dayanum</i>	0.158	0.185	0.155	0.155	0.000															
<i>C. devonianum</i>	0.045	0.066	0.045	0.036	0.134	0.000														
<i>C. ensifolium</i>	0.042	0.066	0.045	0.036	0.146	0.018	0.000													
<i>C. haematodes</i>	0.063	0.093	0.066	0.051	0.161	0.051	0.045	0.000												
<i>C. haematodes(C. siamense)</i>	0.531	0.540	0.525	0.534	0.534	0.537	0.546	0.540	0.000											
<i>C. finlaysonianum</i>	0.057	0.081	0.045	0.063	0.158	0.048	0.048	0.069	0.540	0.000										
<i>C. insigne</i>	0.051	0.057	0.063	0.057	0.161	0.036	0.036	0.066	0.537	0.066	0.000									
<i>C. lancifolium</i>	0.030	0.087	0.042	0.042	0.161	0.045	0.039	0.045	0.525	0.057	0.063	0.000								
<i>C. lowianum</i>	0.069	0.090	0.057	0.072	0.164	0.060	0.060	0.072	0.537	0.036	0.078	0.063	0.000							
<i>C. mastersii</i>	0.087	0.113	0.087	0.081	0.158	0.066	0.072	0.078	0.543	0.090	0.078	0.093	0.099	0.000						
<i>C. munronianum</i>	0.063	0.087	0.069	0.066	0.164	0.057	0.054	0.048	0.540	0.066	0.060	0.063	0.078	0.063	0.000					
<i>C. rectum</i>	0.045	0.072	0.033	0.057	0.146	0.036	0.036	0.051	0.537	0.018	0.054	0.045	0.030	0.078	0.060	0.000				
<i>C. roseum</i>	0.036	0.057	0.036	0.030	0.137	0.009	0.009	0.042	0.537	0.039	0.027	0.036	0.051	0.063	0.051	0.027	0.000			
<i>C. sinense</i>	0.078	0.104	0.078	0.090	0.176	0.075	0.075	0.084	0.522	0.075	0.081	0.072	0.084	0.107	0.078	0.063	0.072	0.000		
<i>C. tracyanum</i>	0.042	0.069	0.042	0.042	0.143	0.021	0.021	0.036	0.546	0.045	0.033	0.042	0.057	0.051	0.039	0.033	0.012	0.072	0.000	
<i>C. tigrinum</i>	0.078	0.104	0.084	0.084	0.167	0.072	0.075	0.069	0.561	0.075	0.075	0.084	0.090	0.081	0.051	0.063	0.066	0.099	0.054	0.000

DISCUSSION

From the investigation, the authors found 19 species. We have not seen *C. cyperifolium* Wall.

exLindl., *C. macrorhizon* Lindl., or *C. wadae* Yukawa. However, we found two more species, *C. haematodes* Lindl. and *C. munronianum* King & Pantl., which had not been included in the earlier

reports. Their reproduction is by autogamous seed formation. However, seedlings are germinated by tissue culture because they have no endosperm. Since all *Cymbidium* species are

Table 3. Genetic distances of the studied species from the sequence analysis of the *rpoC1* region.

Studied species	<i>C. aloifolium</i>	<i>C. atropurpureum</i>	<i>C. bicolor</i>	<i>C. chloranthum</i>	<i>C. dayanum</i>	<i>C. devonianum</i>	<i>C. ensifolium</i>	<i>C. haematodes</i>	<i>C. haematodes(C. siamense)</i>	<i>C. finlaysonianum</i>	<i>C. insigne</i>	<i>C. lancifolium</i>	<i>C. lowianum</i>	<i>C. mastersii</i>	<i>C. munronianum</i>	<i>C. rectum</i>	<i>C. roseum</i>	<i>C. sinense</i>	<i>C. tracyanum</i>	<i>C. tigrinum</i>
<i>Cymbidium aloifolium</i>	0.000																			
<i>C. atropurpureum</i>	0.072	0.000																		
<i>C. bicolor</i>	0.042	0.081	0.000																	
<i>C. chloranthum</i>	0.066	0.087	0.057	0.000																
<i>C. dayanum</i>	0.158	0.185	0.155	0.155	0.000															
<i>C. devonianum</i>	0.045	0.066	0.045	0.036	0.134	0.000														
<i>C. ensifolium</i>	0.042	0.066	0.045	0.036	0.146	0.018	0.000													
<i>C. haematodes</i>	0.063	0.093	0.066	0.051	0.161	0.051	0.045	0.000												
<i>C. haematodes(C. siamense)</i>	0.531	0.540	0.525	0.534	0.534	0.537	0.546	0.540	0.000											
<i>C. finlaysonianum</i>	0.057	0.081	0.045	0.063	0.158	0.048	0.048	0.069	0.540	0.000										
<i>C. insigne</i>	0.051	0.057	0.063	0.057	0.161	0.036	0.036	0.066	0.537	0.066	0.000									
<i>C. lancifolium</i>	0.030	0.087	0.042	0.042	0.161	0.045	0.039	0.045	0.525	0.057	0.063	0.000								
<i>C. lowianum</i>	0.069	0.090	0.057	0.072	0.164	0.060	0.060	0.072	0.537	0.036	0.078	0.063	0.000							
<i>C. mastersii</i>	0.087	0.113	0.087	0.081	0.158	0.066	0.072	0.078	0.543	0.090	0.078	0.093	0.099	0.000						
<i>C. munronianum</i>	0.063	0.087	0.069	0.066	0.164	0.057	0.054	0.048	0.540	0.066	0.060	0.063	0.078	0.063	0.000					
<i>C. rectum</i>	0.045	0.072	0.033	0.057	0.146	0.036	0.036	0.051	0.537	0.018	0.054	0.045	0.030	0.078	0.060	0.000				
<i>C. roseum</i>	0.036	0.057	0.036	0.030	0.137	0.009	0.009	0.042	0.537	0.039	0.027	0.036	0.051	0.063	0.051	0.027	0.000			
<i>C. sinense</i>	0.078	0.104	0.078	0.090	0.176	0.075	0.075	0.084	0.522	0.075	0.081	0.072	0.084	0.107	0.078	0.063	0.072	0.000		
<i>C. tracyanum</i>	0.042	0.069	0.042	0.042	0.143	0.021	0.021	0.036	0.546	0.045	0.033	0.042	0.057	0.051	0.039	0.033	0.012	0.072	0.000	
<i>C. tigrinum</i>	0.078	0.104	0.084	0.084	0.167	0.072	0.075	0.069	0.561	0.075	0.075	0.084	0.090	0.081	0.051	0.063	0.066	0.099	0.054	0.000

economical, ornamental plants and can be sold both as potted plants and as cut flowers, they, therefore, need specific markers for rapid, automatable, and accurate species identification,

especially for the immature plants that are massively grown on orchid farms. The DNA barcoding has served the purpose. After morphological identification, the species-specific

cards called barcodes were done to support further rapid, accurate, and automatable species identification. Apart from the young plants, DNA barcodes can also be used to verify the plants

Table 4. Genetic distances of the studied species from the sequence analysis of the *matK* region.

Studied species	<i>C. aloifolium</i>	<i>C. atropurpureum</i>	<i>C. bicolor</i>	<i>C. chloranthum</i>	<i>C. dayanum</i>	<i>C. devonianum</i>	<i>C. ensifolium</i>	<i>C. haematodes</i>	<i>C. haematodes(C. siamense)</i>	<i>C. finlaysonianum</i>	<i>C. insigne</i>	<i>C. lancifolium</i>	<i>C. lowianum</i>	<i>C. mastersii</i>	<i>C. munronianum</i>	<i>C. rectum</i>	<i>C. roseum</i>	<i>C. sinense</i>	<i>C. tracyanum</i>	<i>C. tigrinum</i>	
<i>Cymbidium aloifolium</i>	0.000																				
<i>C. atropurpureum</i>	0.073	0.000																			
<i>C. bicolor</i>	0.073	0.074	0.000																		
<i>C. chloranthum</i>	0.192	0.176	0.167	0.000																	
<i>C. dayanum</i>	0.277	0.275	0.261	0.308	0.000																
<i>C. devonianum</i>	0.134	0.124	0.113	0.190	0.279	0.000															
<i>C. ensifolium</i>	0.214	0.188	0.185	0.230	0.272	0.171	0.000														
<i>C. haematodes</i>	0.169	0.146	0.129	0.200	0.275	0.143	0.209	0.000													
<i>C. haematodes</i> (<i>C. siamense</i>)	0.120	0.124	0.115	0.181	0.265	0.122	0.171	0.146	0.000												
<i>C. finlaysonianum</i>	0.068	0.070	0.052	0.183	0.272	0.110	0.195	0.148	0.115	0.000											
<i>C. insigne</i>	0.153	0.146	0.129	0.197	0.275	0.131	0.207	0.113	0.134	0.141	0.000										
<i>C. lancifolium</i>	0.136	0.136	0.124	0.188	0.284	0.059	0.190	0.146	0.136	0.120	0.155	0.000									
<i>C. lowianum</i>	0.113	0.106	0.089	0.171	0.268	0.122	0.185	0.131	0.117	0.094	0.134	0.127	0.000								
<i>C. mastersii</i>	0.329	0.310	0.300	0.308	0.385	0.329	0.324	0.333	0.300	0.308	0.310	0.322	0.308	0.000							
<i>C. munronianum</i>	0.124	0.117	0.108	0.178	0.256	0.122	0.171	0.134	0.063	0.113	0.138	0.138	0.099	0.298	0.000						
<i>C. rectum</i>	0.101	0.087	0.080	0.169	0.270	0.110	0.169	0.150	0.094	0.085	0.136	0.122	0.108	0.315	0.106	0.000					
<i>C. roseum</i>	0.153	0.134	0.127	0.178	0.272	0.146	0.204	0.153	0.143	0.131	0.141	0.150	0.094	0.272	0.117	0.129	0.000				
<i>C. sinense</i>	0.167	0.157	0.141	0.192	0.279	0.148	0.164	0.176	0.146	0.150	0.174	0.157	0.146	0.303	0.150	0.136	0.157	0.000			
<i>C. tracyanum</i>	0.117	0.115	0.096	0.157	0.279	0.117	0.195	0.153	0.103	0.099	0.134	0.127	0.103	0.296	0.106	0.099	0.124	0.129	0.000		
<i>C. tigrinum</i>	0.171	0.143	0.143	0.202	0.284	0.164	0.242	0.164	0.169	0.155	0.171	0.169	0.131	0.308	0.155	0.160	0.106	0.188	0.150	0.000	

Table 5. Genetic distance of the studied species from the sequence analysis of the *trnH-psbA* region.

Studied species	<i>C. aloifolium</i>	<i>C. atropurpureum</i>	<i>C. bicolor</i>	<i>C. chloranthum</i>	<i>C. dayanum</i>	<i>C. devonianum</i>	<i>C. ensifolium</i>	<i>C. haematodes</i>	<i>C. haematodes(C. siamense)</i>	<i>C. finlaysonianum</i>	<i>C. insigne</i>	<i>C. lancifolium</i>	<i>C. lowianum</i>	<i>C. mastersii</i>	<i>C. munronianum</i>	<i>C. rectum</i>	<i>C. roseum</i>	<i>C. sinense</i>	<i>C. tracyanum</i>	<i>C. tigrinum</i>
<i>Cymbidium aloifolium</i>	0.000																			
<i>C. atropurpureum</i>	0.099	0.000																		
<i>C. bicolor</i>	0.097	0.089	0.000																	
<i>C. chloranthum</i>	0.107	0.087	0.101	0.000																
<i>C. dayanum</i>	0.101	0.099	0.111	0.105	0.000															
<i>C. devonianum</i>	0.107	0.077	0.115	0.087	0.107	0.000														
<i>C. ensifolium</i>	0.083	0.075	0.087	0.055	0.093	0.075	0.000													
<i>C. haematodes</i>	0.097	0.075	0.089	0.034	0.093	0.079	0.047	0.000												
<i>C. haematodes(C. siamense)</i>	0.528	0.512	0.516	0.522	0.508	0.520	0.508	0.518	0.000											
<i>C. finlaysonianum</i>	0.089	0.085	0.038	0.091	0.105	0.105	0.077	0.083	0.518	0.000										
<i>C. insigne</i>	0.121	0.105	0.117	0.099	0.123	0.109	0.081	0.093	0.518	0.101	0.000									
<i>C. lancifolium</i>	0.089	0.077	0.071	0.063	0.093	0.081	0.051	0.055	0.502	0.063	0.093	0.000								
<i>C. lowianum</i>	0.095	0.071	0.089	0.057	0.097	0.075	0.049	0.049	0.508	0.079	0.093	0.053	0.000							
<i>C. mastersii</i>	0.089	0.071	0.073	0.071	0.085	0.079	0.059	0.063	0.506	0.063	0.083	0.038	0.053	0.000						
<i>C. munronianum</i>	0.123	0.107	0.111	0.097	0.115	0.117	0.073	0.075	0.518	0.097	0.079	0.089	0.097	0.091	0.000					
<i>C. rectum</i>	0.128	0.128	0.109	0.121	0.146	0.142	0.111	0.115	0.520	0.097	0.134	0.109	0.111	0.101	0.132	0.000				
<i>C. roseum</i>	0.087	0.071	0.061	0.071	0.071	0.079	0.057	0.059	0.506	0.057	0.081	0.038	0.053	0.026	0.087	0.097	0.000			
<i>C. sinense</i>	0.089	0.079	0.089	0.067	0.095	0.077	0.055	0.063	0.502	0.075	0.087	0.055	0.065	0.055	0.093	0.115	0.049	0.000		
<i>C. tracyanum</i>	0.095	0.087	0.075	0.083	0.081	0.095	0.069	0.071	0.506	0.073	0.101	0.049	0.069	0.045	0.103	0.113	0.030	0.061	0.000	
<i>C. tigrinum</i>	0.099	0.093	0.087	0.093	0.081	0.097	0.079	0.083	0.512	0.081	0.109	0.057	0.081	0.051	0.111	0.123	0.043	0.067	0.043	0.000

lacking flowers or having incomplete morphological characteristics. DNA barcodes with the *trnH-psbA* spacer region, *matK*, *rpoC1* and *rpoB* for each species were performed following the

guidelines outlined by Chase et al. (2007) using two options: 1) the *trnH-psbA* spacer region, *matK*, and *rpoC1*, and 2) the *trnH-psbA* spacer region, *matK*, and *rpoB*. They proposed using

three regions of plastid DNA as the standard protocol for barcoding on land plants. The low levels of variation in the plastid DNA make three regions necessary. Therefore, the three regions

have been combined to create a standard protocol for the barcoding of all *Cymbidium* species.

The genetic distance levels here means the nucleotide variations in the standardized sequence regions between species of the genus *Cymbidium* in Thailand are 0.012 to 0.546 for *rpoB*, 0.012 to 0.546 for *rpoC1*, 0.052 to 0.385 for *matK*, and 0.026 to 0.528 for the *trnH-psbA* spacer region. All of the genetic distance values that were taken from these regions are standardized enough in the plant group that was studied. They are suitable regions which ideally show enough variation within them to discriminate between species. There are many advantages to the plastid regions, such as conserved gene order and high copy number in each cell providing for the easy retrieval of DNA for PCR and sequencing. Therefore, these DNA samples had PCR performed for barcoding with specific primers of the quoted three genes and a spacer region. In the studied *Cymbidium* sample group, the *trnH-psbA* spacer region was distinct and efficiently displayed different banding sizes in *C. rectum* and *C. finlaysonianum*. The other species showed a few distinguishable sizes as indicated in Figure 2. The efficiency of the region agrees with Hollingsworth et al. (2009) who proposed that the *trnH-psbA* region is a strong candidate for plant barcoding aside from core barcodes such as *rbcL* and *matK*.

It is possible to use small sample sizes in molecular studies as quoted by Hillis (1987). Molecular studies are usually much smaller than in morphological studies (often as small as a single individual) because the analyses of large sample sizes are often limited by the availability of specimens and/or the costs of the analysis. However, the studied samples were randomly collected which has led to providing realistic results. The DNA barcode, which is the specific marker for identifying plants using the standard sequences, usually uses only one individual sequence for a species used at the family, genus and species levels of identification and generally shows nucleotide variations in intra-specific and inter-specific species that are applicable whenever the tags are compared. The ultimate interpretation is dependent upon the researchers' knowledge of the included sequences. Therefore, when several individuals are performing the sequencing, there are disadvantages that make the process time consuming and expensive.

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