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Full Length Research Paper

Genetic analysis for identification, genomic template stability in hybrids and barcodes of the *Vanda* species (Orchidaceae) of Thailand

Tawatchai Tanee[1], Piyawadee Chadmuk[2], Runglawan Sudmoon[2], Arunrat Chaveerach[2]* and Kowit Noikotr[3]

[1]Faculty of Environment and Resource Studies, Mahasarakham University, Mahasarakham 44000, Thailand.
[2]Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.
[3]Department of Biology, Faculty of Science, Ramkhamhaeng University, Bangkok 10250, Thailand.

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Molecular data supporting morphological characters for identification and specific markers for ornamental *Vanda* species in Thailand can be achieved for economic means. The ten native Thai species that have been explored and identified are *Vanda bensonii*, *Vanda brunnea*, *Vanda coerulea*, *Vanda coerulescens*, *Vanda denisoniana*, *Vanda pumila*, *Vanda lilacina*, *Vanda liouvillei*, *Vanda testacea* and *Vanda tessellata*. Three unidentified species (*Vanda* sp. 1, *Vanda* sp. 2 and *Vanda* sp. 3) have been discovered. In addition, three hybrids, hybrid 1 (maternal A × *Vanda tessellata*), hybrid 2 (*Vanda denisoniana* × *Vanda bensonii*), and hybrid 3 (maternal B × paternal C), and two transferred species, *Holcoglossum kimballianum* (previously *Vanda kimballiana*) and *Papilionanthe teres* (previously *Vanda teres*) were included in genetic analysis by dendrogram constructed from random amplified polymorphic deoxyribonucleic acid (DNA) (RAPD) markers. The results indicate that identical species showed monophyletic group and genetic distances (D) that were between 0.15 to 0.17 which lead to the identification of *Vanda* sp. 1 as *Vanda bensonii* and *Vanda* sp. 2 as *Vanda brunnea* because different species give D as higher as 0.20 to 0.40 with divided ancestors. Genomic template stability (GTS) test of hybrids were calculated indicating the percentage of descendant characteristics from parents. The GTS values of hybrid 2 compared with maternal and paternal were 32.88 and 36.62, respectively. Regarding hybrid 1 and 3 for which maternal and / or paternal are unclear, the GTS values when compared to other identified species ranged from 20.34 to 36.84 and 23.19 to 45.98, respectively. Finally, the barcodes of all wild studied species were done by two core barcodes and the tag sequences were tested for nucleotide variations of 0.005 to 0.076 in *mat*K and 0.007 to 0.040 in *rbc*L regions. The sequences were deposited in GenBank database with accession numbers.

Key words: Genetic analysis, genomic template stability, *mat*K, *rbc*L, *Orchidaceae, Vand,*.

INTRODUCTION

Vanda is a genus in the family Orchidaceae with the most magnificent flowers which are very interesting for ornamentation. Many species are important in hybridization and produce flowers for the cut flower market. The genus has a monopodial growth habit with stems which vary considerably in size from miniature to several meters in length. The shape of the leaves show a high degree of variety ranging from flat, to typically broad, to ovoid leaves (strap-leaves), and to cylindrical (terete). Few to many flowers develop on the inflorescences. Most flowers have a yellow-brown color with brown markings, but they also appear in purplish blue, white, green, orange, red, and burgundy shades. *Vanda* species usually bloom every few months and the flowers are maintained for two to three weeks.

Based on leaf shape, the *Vanda* has been decidedly separated into two morphological groups: strap- and terete-leaved groups. Hybrids within each group showed

*Corresponding author. E-mail: raccha@kku.ac.th.

close homology of chromosomes and were fertile, whereas hybrids between the groups had low fertility due to the lack of chromosome homology (Storey, 1955). In addition, intergeneric hybrids between strap-leaved *Vanda* and strap-leaved *Ascocentrum* exhibited even stronger chromosomal homology than hybrids between the strap-leaved and terete-leaved *Vanda* (Shindo and Kamemoto, 1962). There are two species that have been transferred to the other genera namely: *Vanda kimballiana* which was reassigned to be *Holcoglossum kimballianum* (Garay, 1972) and *Vanda teres* relocated to be *Papilionanthe teres* (Garay, 1974). *Vanda coerulea* is a famous native species in Northern Thailand, which shows purplish-blue colored flowers, and has been used as parental stock for development of new hybrids (Sripotar, 2008) especially producing of interspecific and intergeneric hybrids. Therefore, *Vanda* has been encouraged in an economically important plant for the cut-flower and potted plant industries.

Regarding the aspect of biodiversity conservation, many *Vanda* species, especially *V. coerulea,* are endangered due to habitat destruction. Based on the fact that all orchids are listed in Appendix II of the Convention on International Trade in Endangered Species (CITES, 1973), the worldwide exportation of wild collected *V. coerulea* and other wild *Vanda* species is strictly prohibited.

Genetic analysis by molecular data leads to an invaluable knowledge which is profitable for identification, conservation, sustainable uses, breeding, etc. Recently, the development of molecular technology has provided new tools for the detection of genetic alteration in response to plant treatment by direct examination at the deoxyribonucleic acid (DNA) level, in both sequence and structure. Various molecular markers, for example, DNA fingerprints based on polymerase chain reaction (PCR) and DNA barcodes are essential to precisely identifying plant parts without flowers. The fingerprints have proven to be extremely variable and sensitive enough to differentiate cultivars and natural populations (Wolfe et al., 1998). These molecular markers are generally independent of environmental factors and are more numerous than phenotypic characters. As a result, they indicate clearer underlying variations in the genome.

Random amplified polymorphic DNA (RAPD), one of DNA fingerprinting methods, is generally used to effectively indicate genetic relationships by phylogenetic tree reconstruction as seen on many researches (Choi et al., 2006; Jain et al., 2007; Ram et al., 2008; Talebi et al., 2008; Yang et al., 2008; Subramanyam et al., 2009; Ince et al., 2010; Marouelli et al., 2010). Furthermore, RAPD bands can be scored for genomic template stability (GTS) evaluation to detect various types of DNA damage and mutations (rearrangement, point mutations, small insertions or deletions of DNA and polyploidy changes) which suggests that RAPD bands may potentially form the basis of novel biomarkers assays for detection of DNA damage and mutations in the cells of bacteria, plants and animals (Savva, 1998; Atienzar et al., 1999).

Gupta and Sarin (2009) used RAPD bands for GTS evaluation in *Hydrilla verticillata* and *Ceratophyllum demersum* treated with Cd, Hg and Cu to show DNA damage. Zhou et al. (2011) also used RAPD bands for GTS evaluation indicating DNA damage in *Euplotes vannus* (Protozoa, Ciliophora) induced by nitrofurazone in marine ciliates. After identification, DNA barcodes should be done for a species specific marker. There is much extensive research on DNA barcoding in plants beginning in 2003 by Dr. Paul D.N. Hebert, a population geneticist at the University of Guelph in Ontario (Hebert et al., 2003). Since then, there have been many studies testing the standard regions in plant groups aiming to provide rapid, accurate, and automatable species identification by using a standardized DNA region as a tag (Hebert and Gregory, 2005).

Chase et al. (2007) proposed to use two barcoding region options as a standard protocol for barcoding all land plants: the three combined regions of the *rpo*C1, *mat*K, and *trn*H-*psb*A intergenic spacers, or the *rpo*B, *mat*K and *trn*H-*psb*A regions. Newmaster et al. (2007) proposed to use *mat*K and *trn*H-*psb*A to identify plants in Myristicaceae. Finally, Hollingsworth et al. (2009) at the Consortium for the Barcode of life (CBOL) plant working group recommended *rbc*L+*mat*K as the core DNA barcode regions for land plants. Thailand has no *Vanda* species revision. Therefore, the authors need to investigate species diversity, especially for the wild species, and then make species specific markers by DNA barcodes. Additionally, genetic relationships to verify wild orchids, hybrids, and transferred species will need to be checked in order to make the data useful for genetic diversity conservation management, systematics, sources and hybridization programs in commercial production. Moreover, GTS will be calculated to detect various types of DNA changes in hybrids as compared to their parents.

MATERIALS AND METHODS

Species diversity treatment

All *Vanda* species can be grown in a commercial setting throughout Thailand. Therefore, the species diversity of wild *Vanda* has been investigated in both the wild and in commercial settings across all different regions/provinces/locations of Thailand over the period of one year (2010). We collected samples for the current research as follows: three synthetic hybrids, hybrid 1 (maternal A x *Vanda tessellata*), hybrid 2 (*Vanda denisoniana* × *Vanda bensonii*), and hybrid 3 (maternal B × paternal C); and two transferred species, *Holcoglossum kimballianum* and *Papilionanthe teres*. The outgroups in phylogeny reconstruction are *Luisia thailandica* Seidenf and *Ascocentrum miniatum* (Lindl.) Schltr. Identification was conducted according to Zenghong et al. (1993), Vaddhanaphuti (1997), Thaithong (1999), Sitthisatjadham (2006), and Chen and Bell (2009) methods. Voucher specimens were prepared and kept at BK.

Molecular performing

All collected samples was performed with DNA extraction, DNA fingerprinting, DNA barcoding amplification, and DNA banding and barcoding sequence analysis. The DNA barcoding was done following *mat*K and *rbc*L regions.

DNA extraction

Total genomic DNA was extracted using the Plant Genomic DNA Extraction Kit (RBC Bioscience). Extracted DNA was examined by subjecting it to 0.8% agarose gel electrophoresis stained with ethidium bromide. The quality and quantity of DNA were determined by a gel documenting instrument. Then, 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 fingerprinting by RAPD marker and dendrogram construction

Amplifications were carried out on each sample in 25 µl reactions consisting of GoTaq Green Master mix (Promega), 0.5 µM primer and 5 ng DNA template. 32 RAPD primers were screened and the sixteen primers that successfully amplified clear bands are as follows (5′ to 3′): TGCCGAGCTG, AATCGGGCTG, GGGTAACGCC, CAATCGCCGT, GGACCCTTAC, GGACTGCAGA, GGCGGTTGTC, TGGGCGTCAA, ACCGCCTGCT, AGCGAGCAAG, GAGCGTCGAA, CTGGCGAACT, GTTTCGCTCC, GGTGGTCAAG, GACCTACCAC, and AGGTCTTGGG. The reaction mixture was incubated at 94°C for 3 min and the amplification was performed with the following thermal cycles: 35 cycles of denaturation for 1 min at 94°C, 2 min annealing temperature at 40°C, 2 min at 72°C, and 7 min final extension at 72°C using a thermal cycler (Swift^{TM} Maxi Thermal Cycler, Esco Micro Pte. Ltd.). Amplification products were detected by 1.2% agarose gel electrophoresis in Tris-acetate EDTA (TAE) buffer and visualized using ethidium bromide staining. The resulting RAPD bands were used to construct a dendrogram using NTSYS-pc version 2.1 (Rohlf, 1998).

Genomic template stability (GTS) test

GTS is calculated by following equation: GTS (%) = (1-a/n) x 100; where, ‘a’ is the number of polymorphic bands detected in each treated sample, and ‘n’ is the number of total bands in the control (parents/other species in the genus). Polymorphism observed in RAPD profile included disappearance of a normal band and appearance of a new band when compared with control profile (set to 100%). Primers that did not produce changes in RAPD profiles or which were too difficult to score were not used in calculation (Atienzar et al., 1999).

DNA barcode amplification

For DNA barcoding of the species-specific samples quoted above, PCR was performed using primer pair’s 5′-ATGTCACCACA-AACAGAGACTAAAGC-3′ and 5′-GTAAAATCAAGTCCACCRCG-3′ for *rbc*L and 5′-TAATTTACGATCAATTCATTC-3′ and 5′-GTTCTAGCACAAGAAAGTCG-3′ for *mat*K (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 35 thermal cycles:denaturation for 30 s at 94°C, 40 s annealing temperature at 53°C, 40 s extension at 72°C, then followed by 5 min final extension at 72°C. The amplified products were detected by 1.2% agarose gel electrophoresis in TAE buffer and 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. The sequence alignment was done using MEGA5 software (Tamura et al., 2011) with unweighted pair-group method with arithmetic averages (UPGMA) and bootstrap approaches used.

RESULTS

Investigation and Identification

Vanda species have been investigated in all regions of Thailand in a period of one year (2011). The herbarium specimens at Bangkok Herbarium (BK) and forest Herbarium (BKF) have not yet been available. The investigation sites have covered almost all areas including the areas primarily recorded and our other explored areas. They are all widely distributed throughout Thailand and are seen in nearly all 76 provinces as commercial plants for cultivation and ornamentation. The taxonomic identification was investigated based on available references. A total of voucher specimens were deposited in BK under collector numbers A. Chaveerach 720 to A. Chaveerach 734. All are used ornamentally, and from the investigation, 10 species were identified. The recognized species are wild Thailand species including *Vanda bensonii* Bateman*, Vanda brunnea* Rchb.f., *Vanda coerulea* Griff. ex Lindl., *Vanda coerulescens Griff., Vanda denisoniana* Benson and Rchb.f., *Vanda pumila* Hook.f., *Vanda lilacina* Teijsm. and Binn., *Vanda liouvillei* Finet*, Vanda testacea* Rchb.f., and *Vanda tessellata* Hook. ex G.Don. Photos of some species are shown in Figure 1.

Molecular marker analysis

The 16 different polymorphism primers produced 1,443 bands ranging in size from 300 to 3000 bp in 22 samples (18 individuals of *Vanda* species, two individuals of transfer species and two individuals of outgroups). An example of RAPD banding pattern is shown in Figure 2. RAPD analysis was successfully used to distinctly separate species, with UPGMA method, on a different branch of the dendrogram (Figure 3) constructed using these RAPD bands. The dendrogram shows the high powered efficiency of the RAPD data used which clearly distinguishes the wild orchids, hybrids, transferred species, and outgroup species from each other with different three D levels (Table 1).

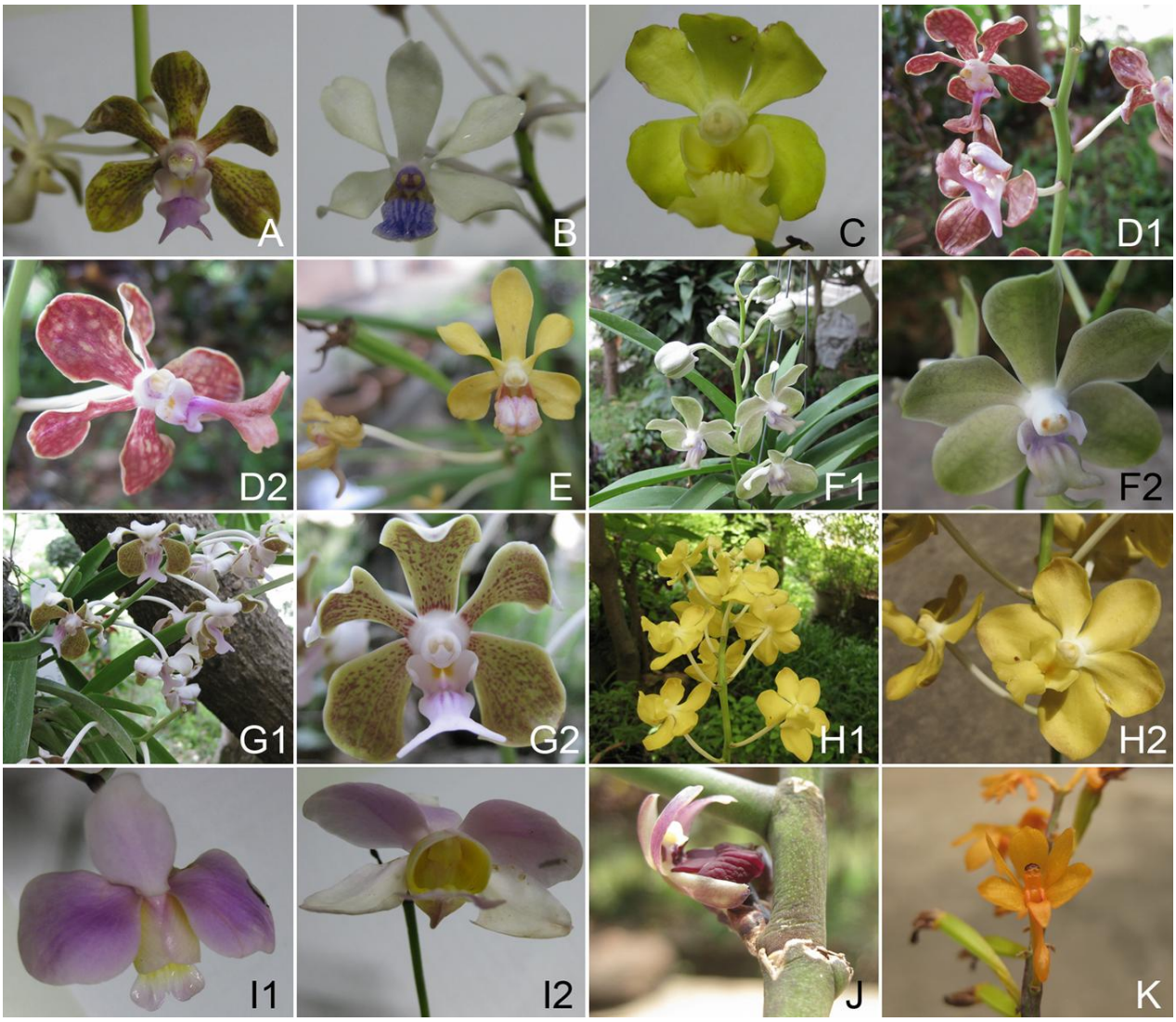

Figure 1. *Vanda* species including hybrids and outgroup species: **A.** *V. bensonii*; **B.** *V. coerulescens*; **C.** *V. denisoniana*; **D.** *V. liouvillei*; **E.** *V. testacea*; **F.** Hybrid 1; **G.** Hybrid 2; **H.** Hybrid 3; **I.** *Papilionanthe teres*; **J.** *Luisia thailandica*; **K.** *Ascocentrum miniatum*.

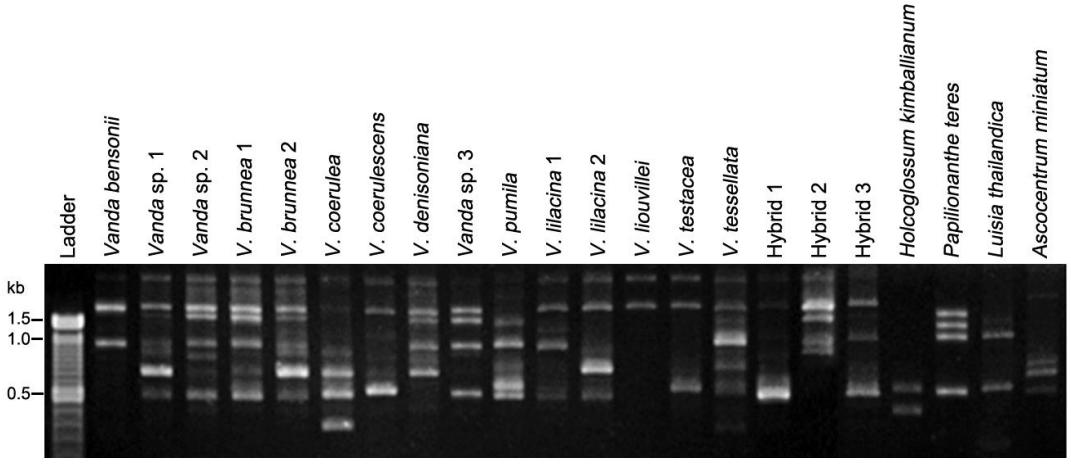

Figure 2. An example of RAPD banding patterns from primer ACCGCCTGCT.

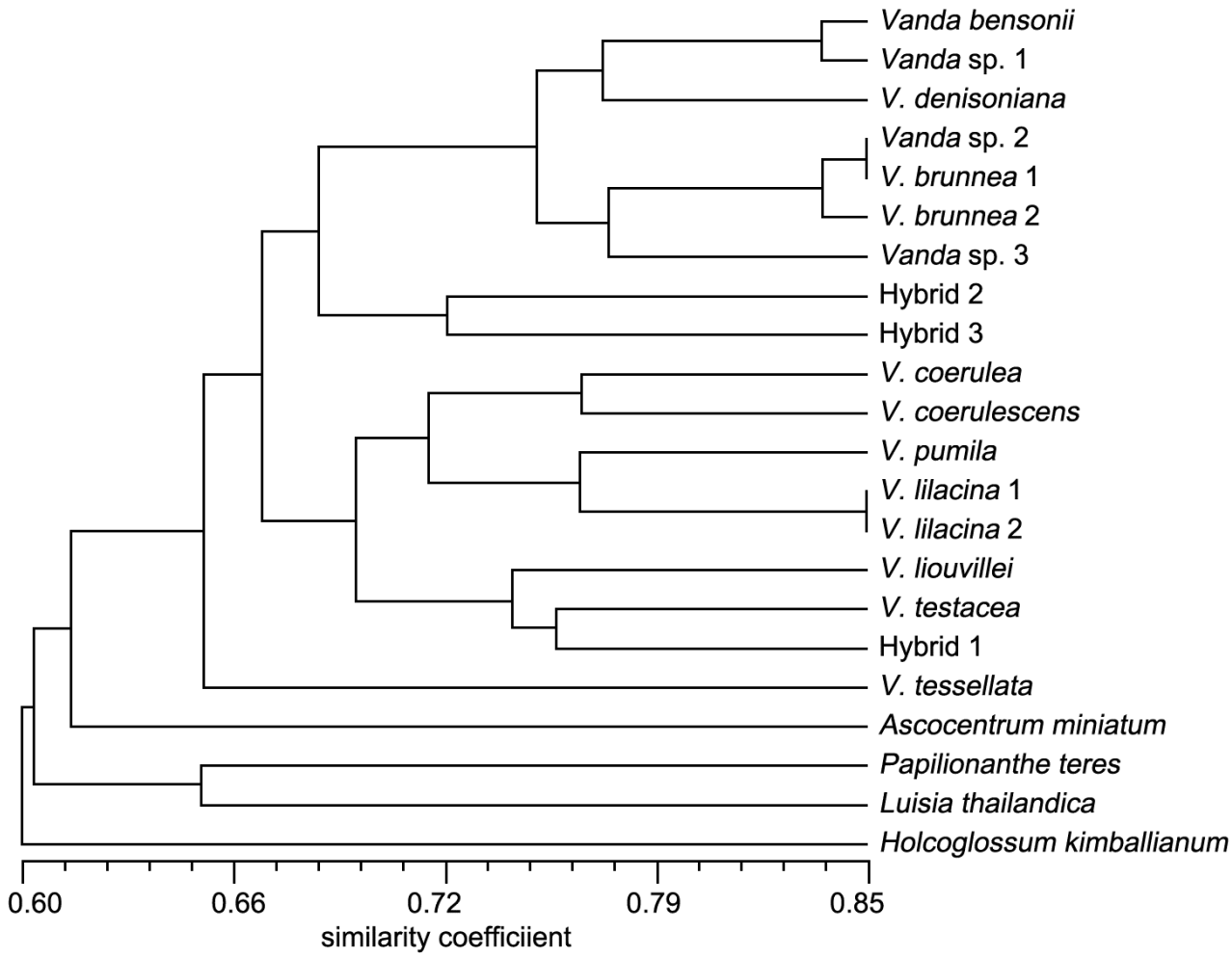

Figure 3. The dendrogram constructed from RAPD bands from 16 primers in 10 species of the *Vanda* by NTSYS-pc version 2.1 UPGMA.

The D level of wild species began with the values of 0.15 (*V. lilacina* 1-*V. lilacina* 2; *Vanda* sp. 2-*V. brunnea* 1), 0.16 (*V. brunnea* 1-*V. brunnea* 2), and 0.17 (*Vanda* sp. 2-*V. brunnea* 2). The higher D values of the wild species are from 0.20 (*Vanda* sp. 3-*V. brunnea* 2; *V. lilacina*-*V. pumila*) to 0.40 (*V. testacea*-*V. brunnea* 1). The highest D values were also available in hybrids, transferred species, and outgroup. They ranged between 0.26 (Hybrid 1-Hybrid 3) to 0.34 (Hybrid 1-Hybrid 2), and 0.33 (*A. miniatum*-*V. lilacina* 2) to 0.44 (*L. thailandica*-*V. bensonii*). GTS values of Hybrid 2 compared to its parents, *V. denisoniana* and *V. bensonii*, are 32.88 and 36.62, respectively. Also, the GTS of all hybrids has been calculated as shown in Table 2.

Barcode treatment and sequence analysis

DNA barcodes were successfully created with two standard regions called core barcodes, *mat*K and *rbc*L which are located in chloroplast genome in all studied samples as shown by Figure 4. The sequences were alignment tested for genetic distances, here were nucleotide variations, using MEGA5. In Tables 3 and 4, the genetic distances of all species are shown to provide the following values: 0.005 (*V. brunnea*-*V. bensonii*; *V. coerulescens*-*V. coerulea*) to 0.076 (*V. lilacina*-*V. denisoniana*; *V. liouvillei*-*V. denisoniana*) in *mat*K region and 0.007 (*V. liouvillei*-*V. bensonii*; *V. liouvillei*-*V. coerulescens*; *V. liouvillei*-*V. lilacina*) to 0.040 (*V. tessellata*-*V. coerulea*) in *rbc*L region.

These tag sequences were submitted to GenBank database under the accession numbers listed in Table 5.

DISCUSSION

Species diversity as explored by the authors of the genus *Vanda* in Thailand is 10 species according to Nanakorn and Indharamusika (1999). Also, Thaithong (1999) stated

Table 1. Genetic distance matrix of *Vanda* species analyzed from DNA fingerprint data from 16 RAPD profiles.

Vanda spp.	*V. bensonii*	*Vanda* sp. 1	*Vanda* sp. 2	*V. brunnea* 1	*V. brunnea* 2	*V. coerulea*	*V. coerulescens*	*V. denisoniana*	*Vanda* sp. 3	*V. pumila*	*V. lilacina* 1	*V. lilacina* 2	*V. liouvillei*	*V. testacea*	*V. tessellata*	Hybrid 1	Hybrid 2	Hybrid 3	*H. kimballianum*	*P. teres*	*L. thailandica*	*A. miniatum*
Vanda bensonii	0.00																					
Vanda sp. 1	0.17	0.00																				
Vanda sp. 2	0.24	0.21	0.00																			
V. brunnea 1	0.28	0.24	0.15	0.00																		
V. brunnea 2	0.26	0.24	0.17	0.16	0.00																	
V. coerulea	0.33	0.29	0.33	0.29	0.24	0.00																
V. coerulescens	0.36	0.29	0.37	0.32	0.35	0.24	0.00															
V. denisoniana	0.22	0.24	0.24	0.26	0.24	0.26	0.31	0.00														
Vanda sp. 3	0.28	0.24	0.27	0.22	0.20	0.26	0.31	0.26	0.00													
V. pumila	0.30	0.28	0.30	0.26	0.29	0.28	0.28	0.26	0.30	0.00												
V. lilacina 1	0.37	0.35	0.35	0.31	0.34	0.33	0.26	0.32	0.29	0.20	0.00											
V. lilacina 2	0.36	0.35	0.33	0.33	0.33	0.28	0.27	0.31	0.29	0.28	0.15	0.00										
V. liouvillei	0.35	0.35	0.39	0.39	0.38	0.37	0.33	0.31	0.26	0.33	0.29	0.30	0.00									
V. testacea	0.35	0.30	0.39	0.40	0.36	0.33	0.26	0.34	0.31	0.31	0.26	0.22	0.24	0.00								
V. tessellata	0.33	0.31	0.37	0.39	0.40	0.37	0.37	0.30	0.34	0.33	0.38	0.31	0.38	0.32	0.00							
Hybrid 1	0.35	0.30	0.39	0.40	0.35	0.31	0.29	0.33	0.30	0.29	0.33	0.30	0.27	0.24	0.29	0.00						
Hybrid 2	0.29	0.35	0.27	0.35	0.32	0.43	0.35	0.31	0.33	0.35	0.35	0.39	0.32	0.37	0.39	0.34	0.00					
Hybrid 3	0.31	0.34	0.30	0.30	0.31	0.36	0.33	0.30	0.26	0.31	0.31	0.31	0.29	0.32	0.29	0.26	0.27	0.00				
Holcoglossum kimballianum	0.40	0.37	0.38	0.41	0.43	0.43	0.38	0.36	0.37	0.42	0.40	0.40	0.37	0.40	0.43	0.38	0.45	0.38	0.00			
Papilionanthe teres	0.40	0.39	0.44	0.40	0.42	0.41	0.42	0.39	0.33	0.38	0.43	0.44	0.40	0.37	0.36	0.32	0.43	0.28	0.37	0.00		
Luisia thailandica	0.44	0.39	0.43	0.43	0.41	0.40	0.35	0.42	0.42	0.36	0.42	0.40	0.45	0.36	0.36	0.31	0.43	0.36	0.43	0.35	0.00	
Ascocentrum miniatum	0.37	0.37	0.44	0.44	0.43	0.43	0.38	0.42	0.40	0.34	0.35	0.33	0.37	0.35	0.43	0.34	0.41	0.33	0.41	0.44	0.43	0.00

there are 10 *Vanda* species; however, there are some species that have been transferred to the others. The *V. teres* was transferred to be *Papilionanthe teres*, and *V. kimballiana* was transferred to be *Holcoglossum kimballianum*. Additionally, Chen and Bell (2009) indicated that *V. denisoniana* is synonym of *V. brunnea* which disagrees with the results by dendrogram and genetic distance, 0.26 with *V. brunnea* 1 and 0.24 with *V. brunnea* 2. Therefore, they could not be identical species.

RAPD powerfully shows the best data suitable for the *Vanda* species analysis. The dendrogram constructed from banding patterns separated wild *Vanda* species from hybrids, and the other genera including the transferred species and the outgroup species. The identical species showed D between 0.15 to 0.17 by 0.15 in pairs of *V. lilacina* 1-*V. lilacina* 2 and *Vanda* sp. 2-*V. brunnea* 1, 0.16 in a pair of *V. brunnea* 1-*V. brunnea* 2, and 0.17 in a pair of *Vanda* sp. 2-*V. brunnea* 2. Therefore,

Table 2. Percentage of genomic template stability (GTS) of hybrids compared to each *Vanda* species calculated from numbers of band changes of 16 RAPD profiles.

Hybrid	*V. bensonii*	*Vanda* sp. 1	*Vanda* sp. 2	*V. brunnea* 1	*V. brunnea* 2	*V. coerulea*	*V. coerulescens*	*V. denisoniana*	*Vanda* sp. 3	*V. pumila*	*V. lilacina* 1	*V. lilacina* 2	*V. liouvillei*	*V. testacea*	*V. tessellata*
Hybrid 1	22.54	31.88	29.89	22.22	34.15	36.84	36.62	30.14	22.95	34.29	21.54	20.34	30.00	36.67	28.13
Hybrid 2	36.62*	20.29	50.57	32.10	39.02	13.16	22.54	32.88*	16.39	22.86	15.38	-3.39	16.67	3.33	6.25
Hybrid 3	30.99	23.19	45.98	41.98	41.46	26.32	28.17	35.62	32.79	31.43	24.62	16.95	23.33	16.67	28.13

*GTS values compared to parents of hybrid 2 whereas there is not a clue of parents for other two hybrids.

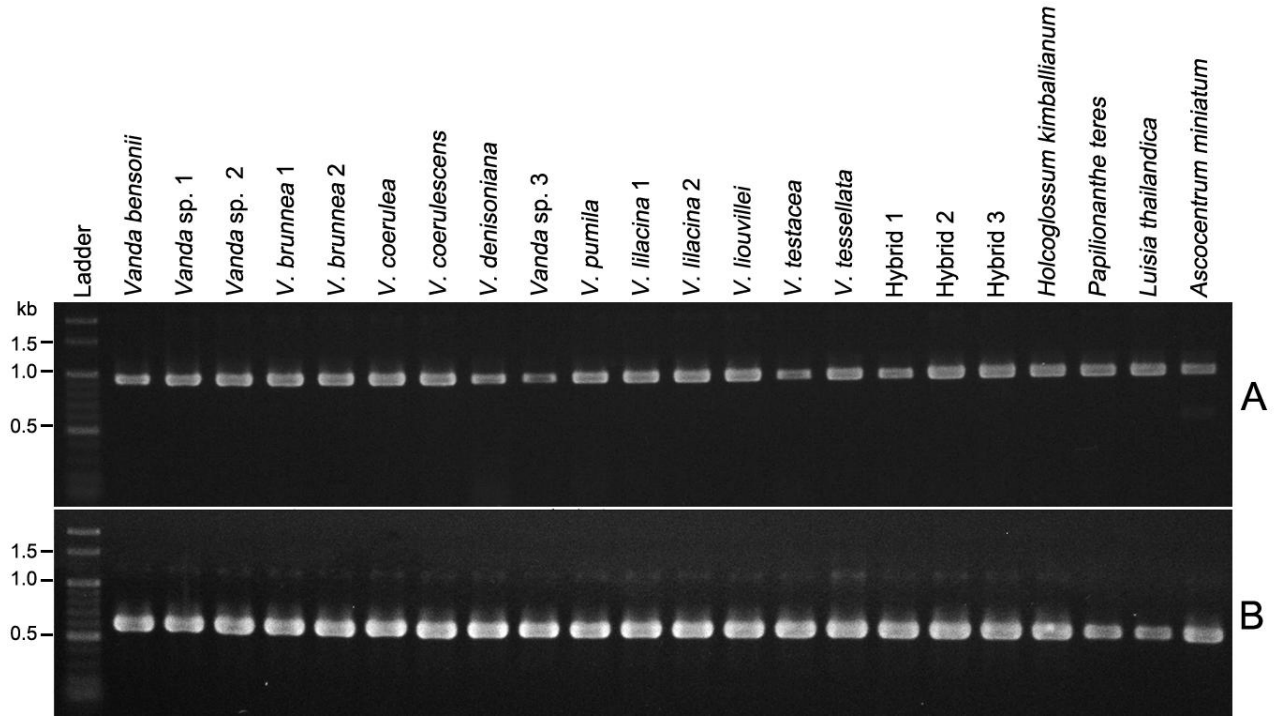

Figure 4. DNA barcode fragments as a species-specific marker from *mat*K (A) and *rbc*L (B) regions of the 10 *Vanda* species.

Vanda sp. 1 is *V. bensonii* with the D of 0.17 and *Vanda* sp. 2 is *V. brunnea* having the same D value.

The different species that showed the higher D sp. 3-*V. brunnea* 2 and *V. lilacina*-*V. pumila*, and indicating D of 0.40 are *V. testacea*-*V. brunnea* 1. As the results have shown, the *Vanda* sp. 3 remains unidentified, but most assuredly, it is a values of the wild species started from 0.20 to 0.40. The pairs indicating D of 0.20 are *Vanda* wild *Vanda* species belonging to a sister group of the *Vanda* species and is closely related to *V.*

Table 3. Genetic distance matrix based on nucleotide variations from sequence alignment of *mat*K region by MEGA5.

Vanda spp.	*V. bensonii*	*V. brunnea*	*V. coerulea*	*V. coerulescens*	*V. denisoniana*	*V. pumila*	*V. lilacina*	*V. liouvillei*	*V. testacea*	*V. tessellata*
Vanda bensonii	0.000	-	-	-	-	-	-	-	-	-
V. brunnea	0.005	0.000	-	-	-	-	-	-	-	-
V. coerulea	0.025	0.025	0.000	-	-	-	-	-	-	-
V. coerulescens	0.020	0.020	0.005	0.000	-	-	-	-	-	-
V. denisoniana	0.068	0.068	0.052	0.046	0.000	-	-	-	-	-
V. pumila	0.031	0.031	0.015	0.010	0.052	0.000	-	-	-	-
V. lilacina	0.046	0.046	0.038	0.033	0.076	0.038	0.000	-	-	-
V. liouvillei	0.052	0.052	0.044	0.041	0.076	0.046	0.071	0.000	-	-
V. testacea	0.041	0.041	0.025	0.020	0.049	0.020	0.054	0.052	0.000	
V. tessellata	0.044	0.044	0.028	0.023	0.065	0.033	0.036	0.052	0.044	0.000

Table 4. Genetic distance matrix based on nucleotide variations from sequence alignment of *rbc*L region by MEGA5.

Vanda spp.	*V. bensonii*	*V. brunnea*	*V. coerulea*	*V. coerulescens*	*V. denisoniana*	*V. pumila*	*V. lilacina*	*V. liouvillei*	*V. testacea*	*V. tessellata*
Vanda bensonii	0.000	-	-	-	-	-	-	-	-	-
V. brunnea	0.021	0.000	-	-	-	-	-	-	-	-
V. coerulea	0.025	0.030	0.000	-	-	-	-	-	-	-
V. coerulescens	0.011	0.021	0.028	0.000	-	-	-	-	-	-
V. denisoniana	0.033	0.033	0.042	0.030	0.000	-	-	-	-	-
V. pumila	0.016	0.025	0.028	0.014	0.030	0.000	-	-	-	-
V. lilacina	0.011	0.021	0.023	0.009	0.033	0.009	0.000	-	-	-
V. liouvillei	0.007	0.014	0.025	0.007	0.028	0.011	0.007	0.000	-	-
V. testacea	0.018	0.032	0.037	0.023	0.037	0.021	0.018	0.018	0.000	
V. tessellata	0.025	0.028	0.040	0.021	0.028	0.025	0.025	0.018	0.032	0.000

brunnea by the dendrogram. The authors are in need of its flowers. Also, *V. testacea* is a different species from hybrid 1 indicating D of 0.24. The highest D values are provided in hybrids, transferred species, and outgroups. The values are 0.26 to 0.34 in hybrids, 0.37 in transferred species, *H. kimballianum* and *P. teres*, and 0.43 in the two outgroup species, *L. thailandica* and *A. miniatum*. Additionally, the highest D values are also found in hybrids, transferred species and outgroup when compared to the all studied *Vanda* species as shown in Table 1. These highest values are in agreement with the systematic hypothesis of the polyphyletic group which is in different groups as different species and genera (Simpson, 2006).

Hybrids cannot be identified by the type of wild Vanda species their parents belong to. From these results, it can be assumed that the Vanda species has high genetic diversity and/or high genetic variation in the group affected by humans in planting, and more importantly, genetic variation in Vanda are mediated by hybridization; thus, hybridization process can potentially encourage the increase of genetic diversity. Additionally, multiple alleles may be the factor affecting the diversity, leading to gathering and rearrangements of genes from parents. Therefore, offspring and hybrids may receive and/or express shared characteristics among many species of *Vanda*

Table 5. Voucher specimen numbers and GenBank accession numbers of the two barcoding regions of the studied *Vanda* species.

Voucher specimen number	Species	GenBank accession numbers	
		***mat*K**	***rbc*L**
A. Chaveerach 720	*Vanda bensonii*	JN880434	JQ180382
A. Chaveerach 721	*V. brunnea*	JN880435	JQ180383
A. Chaveerach 722	*V. coerulea*	JN880436	JQ180384
A. Chaveerach 723	*V. coerulescens*	JN880437	JQ180385
A. Chaveerach 724	*V. denisoniana*	JN880438	JQ180386
A. Chaveerach 725	*V. pumila*	JN880439	JQ180387
A. Chaveerach 726	*V. lilacina*	JN880440	JQ180388
A. Chaveerach 727	*V. liouvillei*	JN880441	JQ180389
A. Chaveerach 728	*V. testacea*	JN880442	JQ180390
A. Chaveerach 729	*V. tessellata*	JN880443	JQ180391

group. Using GTS will be profitable for expecting characteristics received in the breeding program, which are useful for genetic diversity and conservation management, systematics, genetic resources, and hybridization programs in commercial *Vanda* production.

Since all *Vanda* species are ornamental plants that are economically profitable when sold as potted plants and as cut flowers, there need to be specific markers for further rapid, automatable, and accurate species identification, especially for the immature plants that are massively grown in orchid farms. The DNA barcoding has served the purpose. Therefore, after morphological and fingerprint identification, the species specific card called barcodes were done to support the evidence mentioned. Besides the immature plants, DNA barcodes can be used to verify the plants lacking flowers or those having incomplete morphological characteristics.

DNA barcodes with *mat*K and *rbc*L for each species were performed following the guidelines set by Hollingsworth et al. (2009). They proposed to use the two regions of plastid DNA as a standard protocol for the core barcoding on land plants. The low levels of variation in the plastid DNA make two regions necessary. So, the two regions have been combined to create a standard protocol for the barcoding of all *Vanda* species. The genetic distance, based on UPGMA method, levels as noted here means the nucleotide variations in the standardized sequence regions between species of the genus *Vanda* in Thailand. They are 0.005-0.076 in *mat*K region and 0.007-0.040 in *rbc*L region. All of the genetic distance values that were taken from these regions are standardized enough in the studied plant group. They are suitable regions which ideally show enough variation within them to discriminate among species.

There are many advantages to the plastid regions, such as conserved gene order and a high copy number in each cell to enable the easy retrieval of DNA for PCR and sequencing. Therefore, these DNA samples had PCR performed for barcoding with specific primers of the quoted two genes in the studied *Vanda* sample group. It is possible to use small sample sizes in molecular studies as quoted by Hillis (1987). The sizes in 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 expense of the analysis. However, the studied samples were randomly collected which has led to having realistic of genetic analysis results.

Molecular data is a study on whole genomes including expressed and unexpressed regions, accordingly, actually to limited variation in intraspecific and interspecific levels following the Weier et al. (1982) proposal. They stated that all operational taxonomic units, except for the barcode sequences of short sequence regions that have similarities between 85 to 100% might be recognized as part of the same species, while a criterion of 65% might be used for the genus level. 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 level of identification and generally have nucleotide variations in intraspecific and interspecific species, whenever applicable by tag comparison. The advantage of this marker is that it can be used to identify plant parts having incomplete morphological characters in which traditional taxonomy cannot be accomplished.

Abbreviations

RAPD, Random amplified polymorphic DNA; **GTS,** genomic template stability; **UPGMA,** unweighted pair-group method with arithmetic averages.

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