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Molecular Decoding of Identity of Some Commercially Important Vandaceous Orchids (Orchidaceae) Based on the Sequences of the Internal Transcribed Spacer (ITS) Sequences and Their Phylogeny

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Research Article

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Abstract

Indiscriminate, rampant collection and natural habitat destruction of Vandaceous orchids for their ornamental and medicinal value have resulted in threatened category to many of the important species and struggling for their survival. Unconfirmed commercially important Vandaceous orchids still continues as these orchids are similar in shaped, size and difficult to identify and classify, especially when they are not in flowering stage. To solve this problem molecular barcode can be an useful tool for rapid identification even from vegetative tissues for commercial purpose. The present study was carried out to test the discriminatory power of IT1 and IT2 region in barcoding and to ascertain their phylogenetic relationship among these 31 species (67) from 15 genera of vandaceous orchids. The sequences were aligned using ClustalW and genetic distances were computed using MEGA 7.0. Data analyses suggest that Internal Transcribed Spacer (ITS) of nuclear ribosomal DNA is a reliable marker which can be use as an efficient barcode to identify (species resolution at 95.52% by genetic distance, 79.40% by BLAST analysis and 95.52% by phylogenetic tree building method) and analyze their phylogenetic relationship among Vandaceous orchids.

Introduction

Vandaceous orchids belongs to family Orchidaceae, subfamily Epidendroideae, and subtribe Aeridinae (formerly Sarcanthinae), a monopodial group mostly epiphytes. This group of orchid is represented by over 1350 species from ~ 90 genera which are distributed predominantly throughout the warm-temperate and tropics of Asia, Australia, and the eastern Pacific Islands, which forms large proportion of tribe Vandeae [1]. Beside their strange shape, beautiful looks, longevity and highly attractive color of flowers, they are also known for their aesthetic/ornamental and medicinal importance [2–3]. Orchids are easy material for breeding programme and as a result there are many natural as well as designed orchid hybrids developed. Many of these species/varieties are morphologically similar when they are in vegetative phase; it is difficult to identify with certainty when they are not in flowering stage; even in certain species it is very difficult to identify precisely from the flowers if the specimen under question is a breed outcome of two closely related species. Till date many of these species are still collected from the wild for commercial purpose especially for illegal trade due to high market value. These collections from the wild in most of the times are in vegetative phase which lead to wrong identification. This indiscriminate collection because of the lack of effective method to check most of the species are pushed to rare, endangered and threatened category of many important species [4–9].

For identification and classification of different taxa, rapid species identification techniques like DNA barcoding have been undertaken [10–11] by different groups utilizing DNA region from the mitochondrial, plastid and nuclear genomes. Traditional morpho-phenology methods to identify Vandaceous species mostly based on phenotypic characters [12], but morphological characteristics are subjected to be affected by developmental and environmental [13–15]. Therefore, the DNA barcoding method was undertaken for checking these illicit practices by offering a fool proof method for their detection in any form and stage and this could indirectly help in safe utilization and genetic resource conservation.

Many past reports indicated that the spacer regions of genome can be used to infer phylogeny for identification of closely related taxa, detecting genetic variation among genera, species and within species [11, 16–18] and has become the most valuable region in plant molecular studies. Nuclear gene region has several advantages for being biparental genetically inherited and has high rates of base substitution, it exists in higher copy number and priming sites surrounding the 18S and 26S regions are highly conserved [19].

Nagaland is a home to over 60 Vandaceous species under 22 genera, which are the major ornamental crops as cut flower and potted plants and has a high demands in National as well as and International markets. For identification of these species at any stage of life and authenticate the difference between parent and the new hybrids which has similar morphological traits, with that in mind the present study was initiated for molecular characterization of variety, species certification of 31 commercially potential Vandaceous species from 15 genera.

Materials And Methods

Plant Materials

In the present study a total of 31 species from 15 genera of Vandaceous orchids (Subtribe: Aeridinae) were collected from different parts of Nagaland during the field survey. Morpho-taxonomic identification of the collected specimens was accomplished with the help of the available literatures (taxonomic keys) and with the help of the experts. The collected specimens are brought under cultivation in the Departmental Orchidarium and all corresponding voucher samples were deposited in the herbarium of the Department of Botany, Nagaland University, Lumami, India for future reference.

DNA Extraction, Amplification, and Sequencing

For isolation of DNA, fresh young/tender leaves were used and isolation was achieved following genomic DNA extraction protocols of Doyle and Doyle [20] and Kamba and Deb [21]. For cross checking of the results, for each species multiple specimens collected from different areas were considered i.e., a total 80 individuals of 31 species from 15 genera were studied (Table 1). The extracted genomic DNA from all the species were used for amplification of barcode genes ITS. The primers are those used by Tsai *et al.* [22] (IT1 5'-TCGTAACAAGGTTTCCGTAGGT-3' and IT2 5'-GTAAGTTTCTTCTCCTCCGCT-3'). For PCR reaction mixture contained 10X PCR buffer with 25mM $MgCl_2$, 2mM dNTPs, 10 µM of each primer and 20–30 ng of template DNA, 2 units of DNA polymerase. The final volume was adjusted to 20 µL with ddH_2O. Thermal cycle followed for ITS as: one cycle of DNA denaturation at 94°C for 5 min, followed by 35 cycles of 45 sec at 94°C, 45 sec at 52°C and 1 min at 72°C, with a final extension of 10 min at 72°C. Separation of amplicons was done by electrophoresis in 1.5% TBE agarose gels stained with EtBr (0.5lg ⁄ mL) and visualized under gel documentation system. The amplified products were then sending it for sequencing to ‘1st Base Laboratories Malaysia’ and ‘Chromous Biotech Pvt. Ltd. Bangalore, India’. Sequence quality checks were performed by BioEdit sequence alignment Editor Software and the

nucleotide sequence of ITS region for the Orchid species were subsequently submitted to NCBI GenBank (http://www.ncbi.nim.nih.gov/genbank/). The targeted DNA sequences of ITS and retrieved nucleotide sequences from the GenBank after blasting they were subjected to multiple sequence alignment with the ClustalW, a tool for multiple sequence alignment [23] through MEGA-7 software [24]. The nucleotide sequences of ITS regions for the candidate species were submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and accession numbers were obtained. Phylogenetic trees were constructed with Neighbor Joining (NJ) method in MEGA while, evolutionary distances were worked out using Kimura 2-parameter (K2P) distance as a model of substitution and running 1000 bootstrap replicates to calculate the relative support for the branches for the investigated species. The percentage of the replicate tree where the associated taxa grouped/clustered together in the bootstrap test projected next to the branches. The inter-specific K2P distances were determined for each representative species sequence.

Table 1
Collection details and GenBank accession numbers of submitted sequences

Sl. No.	Genus	Species	Herbarium Accession Number	Collection sites	Accession no.
1.	*Vanda*	1. *V. coerulea* Griff. ex Lindl.	NUBOT-JK-VC-01	Lumami	MG818987
				Lumami	MW362400
				Alichen	MW492905
		2. *V. bicolor* Griff.	NUBOT-JK-VB-02	Longkhum	MG822845
				Longkhum	MW368614
				Lumami	MW365331
		3. *V. ampullacea* (Roxb.) L.M. Gardiner	NUBOT-JK-VA-12	Khuzama	MN170562
		4. *V. stangeana* Rchb.f.	NUBOT-JK-VS-16	Jaluki	MG822848
				Jaluki	MW362402
		5. *V. testacea* (Lindl.) Rchb.f.	NUBOT-JK-VT-13	Jaluki	MN170573
				Punglwa A	MW425864
		6. *V. alpina* (Lindl.) Lindl.	NUBOT-JK-VA-18	Asukhomi	MN173057
				Asukhomi	MW362399
				Asukhomi	MW362401
		7. *V. pumila* Hook.f.	NUBOT-JK-VP-31	Asukhomi	MN517224
				Asukhomi	MW368597
				Asukhomi	MW493108
2.	*Cleisostoma*	1. *C. paniculatum* (Ker-Gawl.) Garay	NUBOT-JK-CR-15	Mongchen	MT422095
				Lumami	MW442838
		2. *C. simondii* (Gagnep.) Seidenf.	NUBOT-JK-CS-14	Khuzama	MG822849
				Khuzama	MW355894
				Khuzama	MW362366
		3. *C. williamsonii* (Rchb.f.) Garay	NUBOT-JK-CW-20	Phek	MN517118
				Phek	MW442840
3.	*Acampe*	1. *A. rigida* (Buch.-Ham. ex Sm.) P.F.Hunt	NUBOT-JK-AR-17	Jaluki	MN173056
				Jaluki	MW617314

Sl. No.	Genus	Species	Herbarium Accession Number	Collection sites	Accession no.
		2. *A. praemorsa* (Roxb.) Blatt. & McCann	NUBOT-JK-AP-21	Jaluki	MN517126
				Jaluki	MN170566
		3. *A. ochracea* (Lindl.) Hochr.	NUBOT-JK-AO-19	Lumami	MN170563
				Lumami	MW600257
				Lumami	MW600256
4.	*Gastrochilus*	1. *G. obliquus* var. *obliquus* (Lindl.) Kuntze	NUBOT-JK-GO-30	Jaluki	MN240429
		2. *G. calceolaris* (Buch.-Ham. ex Sm.) D.Don	NUBOT-JK-GC-09	Mongchen	MN517123
				Mongchen	MW475266
		3. *G.acutifolius* (Lindl.) Kuntze	NUBOT-JK-GA-22	Dikhu	MT225573
				Mongchen	MW475270
5.	*Papilionanthe*	1. *P. teres* (Roxb.) Schltr.	NUBOT-JK-PT-04	Lumami	MG821161
				Doyang Jaluki	MW362367
					MW362392
		2. *P. vandarum* (Rchb.f.) Garay	NUBOT-JK-PV-07	Zunheboto	MG821080
				Zunheboto	MW362394
				Zunheboto	MW362368
6.	*Arachnis*	1. *A. clarkei (Esmeralda)* (Rchb.f.) J.J.Sm.	NUBOT-JK-EC-10	Tuensang	MG820621
				Meinkong	MW452979
		2. *A. labrosa* (Lindl. & Paxton) Rchb.f.	NUBOT-JK-AL-06	Dikhu	MG820749
				Sema settsu	MW599843
7.	*Vandopsis*	1. *V. undulata* (Lindl.) J.J.Sm.	NUBOT-JK-VU-08	Zunheboto	MG786550
				Zunheboto	MW452980
8.	*Renanthera*	1. *R. imschootiana* Rolfe	NUBOT-JK-RI-11	Khuzama	MG820707
				Khuzama	MW599845
9.	*Aerides*	1. *A. odorata* Lour.	NUBOT-JK-AO-05	Sema Settsu Lumami	MG822846
					MW599844

Sl. No.	Genus	Species	Herbarium Accession Number	Collection sites	Accession no.
10.	*Rhynchostylis*	1. *R. retusa* (Linn.) Bl.	NUBOT-JK-RR-03	Mongchen	MG822847
				Lumami	MW475272
				Mongchen	MW475274
				Mongchen	MW475276
11.	*Sarcoglyphis*	1. *S. mirabilis* (Rchb.f.) Garay	NUBOT-JK-SM-23	Punglwa	MT416451
				Punglwa	MW475278
12.	*Smitinandia*	1. *S. micranta* (Lindl.) Holttum	NUBOT-JK-SM-29	Jaluki	MN170568
				Jaluki	MW617320
13.	*Thrixspermum*	1. *T. tsii* W.H.Chen & Y.M.Shui	NUBOT-JK-TT-28	Jaluki	MN170569
14.	*Phalaenopsis*	1. *P. braceana* (Hook.f.) E.A. Christenson	NUBOT-JK-PB-27	Longkhum	MT974319
		2. P. wilsonii Rolfe	NUBOT-JK-PW-26	Kithsakita	MG952632
		3. *P. hygrochila* (Veitch & Rchb.f.) Pfitzer *(Hygrochilus parishii)*	NUBOT-JK-PH-24	Jaluki	MN170567
				Jaluki	MW599846
				Mokokchung	MW617318
15.	*Stereochilus*	1. *S. laxus* (Rchb.f.) Garay	NUBOT-JK-SL-25	Kithsakita	MT178771

Results

The barcode loci ITS from the nuclear genome as DNA barcode was tested for molecular characterization 31 Vandaceous orchid species belonging to 15 genera (80 individuals) of the family Orchidaceae. Although 80 individuals were tested in the present study, successful amplication of targeted region was achieved in 67 individual. The amplification and sequencing success rate stand at 91.25% and 91.78% respectively. Among the investigated individuals, some species belonging to *Acampe, Aerides* and *Arachnis* species showed multiple bands of amplicons with the tested loci. The band having molecular weight nearest to that of the target locus was marked and eluted followed by sequencing. The 67 successful barcode sequences with 600-700bp length were generated successfully and submitted to NCBI GenBank (Table 1). Values of intra- and inter-specific divergence were calculated using genetic distance method.

Interspecific K2P distances were calculated and revealed an average inter-specific K2P distance of 0.150 with a range of 0-0.539. Out of the 31 species (67 individuals) analyzed 3 exhibited zero distance estimates with one or the other species. The maximum inter-specific K2P distance 0f 0.539 was observed between *Acampe rigida* and *Vanda alpina*. Parsimony informative sites for all 7 inter-specific genera were given in Table 2.

Table 2
Average Inter-specific K2P distances along with the number of species analyzed for 7 genera

Sl. No.	Genus	No. of species	Average Inter-specific K2P Distance (Range)	No. of variables	Parsimony informative sites
1	*Acampe*	3	0.187 (0-0.431)	231	145/784 nucleotides
2	*Arachnis*	2	0.032 (0-0.063)	32	9/716 nucleotides
3	*Cleisostoma*	3	0.041 (0-0.070)	67	46/744 nucleotides
4	*Gastrochilus*	3	0.054 (0-0.078)	71	26/749 nucleotides
5	*Papilionanthe*	2	0.054 (0-0.067)	45	31/778 nucleotides
6	*Phalaenopsis*	3	0.169 (0-0.262)	187	42/680 nucleotides
7	*Vanda*	7	0.119 (0-0.269)	320	218/816 nucleotides

The intra-specific variations were estimated for only those species, which were represented by more than one individual. The inter-specific K2P distances and species resolution analysis was carried out at the generic level only. At the generic level, the inter-specific variations among the species of a genus were calculated using the distance matrix prepared by aligning the sequences of all the accessions belonging to different species of a genus. The K2P distances and tree building methods were used to discriminate the congeneric species of a genus (Table 3).

Table 3
Intra-specific K2P distance for the candidate loci

Sl. No.	Species	Intra-specific K2P distances based on genetic distance method
1	*Vanda coerulea*	0-0.010
2	*Vanda bicolor*	0-0.089
3	*Vanda ampullacea*	-
4	*Vanda stangeana*	0-0.092
5	*Vanda testacea*	0-0.037
6	*Vanda alpina*	0-0.034
7	*Vanda pumila*	0-0.010
8	*Cleisostoma paniculatum*	0-0.008
9	*Cleisostoma simondii*	0-0.009
10	*Cleisostoma williamsonii*	0-0.001
11	*Acampe rigida*	0-0.012
12	*Acampe praemorsa*	0-0.050
13	*Acampe ochracea*	0-0.162
14	*Gastrochilus obliquus*	-
15	*Gastrochilus calceolaris*	0-0.007
16	*Gastrochilus acutifolius*	0-0.010
17	*Phalaenopsis wilsonii*	-
18	*Phalaenopsis braceana*	-
19	*Phalaenopsis hygrochila*	0-0.038
20	*Papilionanthe teres*	0-0.004
21	*Papilionanthe vandarum*	0-0.011
22	*Arachnis clarkei (Esmeralda)*	0-0.019
23	*Arachnis labrosa*	0-0.006
24	*Stereochilus laxus*	-
25	*Vandopsis undulata*	0-0.024
26	*Renanthera imschootiana*	0-0.002

Sl. No.	Species	Intra-specific K2P distances based on genetic distance method
27	*Aerides odorata*	0-0.008
28	*Rhynchostylis retusa*	0-0.034
29	*Sarcoglyphis mirabilis*	0-0.019
30	*Smitinandia micranta*	0-0.013
31	*Thrixspermum tsii*	-

Species Resolution

Species resolutions for ITS locus were calculated based on three methods *viz*., genetic distances, BLAST analysis and through phylogenetic tree method.

Distance Based Method

The distance matrix revealed 2 species pair that had zero distance estimates. The formation of these species pairs involved 3 species; therefore the *per cent* species resolution stands at 95.52%. The species pairs formed were: *Stereochilus laxus-Acampe praemorsa*; *Papilionanthe teres-P. teres*. (Table 4, Supplementary table: ITS pairwise genetic distance).

Blast Analysis

In the BLAST analysis, 55 sequences correctly matched with the sequences of their own species. Of the remaining 12 species that does not correctly matched/identified with their own species, 9 species are correctly identified up to genus level but not at the species level. Thus, the species resolution based on ITS locus using BLAST method was 79.40%. The BLAST analysis was also carried out to determined that the query/amplified sequence is of the targeted locus and it is observed that all the 67 ITS sequences generated in the present study were found to be only of the targeted locus and not contamination of fungi as they form symbiotic relationship with orchids/the host.

Phylogenetic Tree-Building

The aligned ITS sequences revealed that out of 848 nucleotide sites compared, 652 were variable sites of which 534 were parsimony-informative sites and 117 were singleton sites. The Neighbour joining tree was constructed with 1000 bootstraps replicates, revealed two different clusters comprising 3 species and thus resulting in 95.52% species resolution. The species clusters forms were (i) *Stereochilus laxus* and *Acampe praemorsa* (ii) *Papilionanthe teres* species. (Fig. 1: ITS tree). The individuals of 4 species *viz*., *Vanda pumila, V. alpina, Rhynchostylis retusa* and *Cleisostoma simondii* that had intra-specific variations however formed a single cluster with all the accessions of each of these four clustering together. One each individual of *Vanda testacea* and *Acampe ochracea* were clustered together with *V. coerulea* and *Hygrochilus parishii.* The species in which all the individuals clustered together in a single

clade are considered as identified species and those which clustered with the individuals of the other species were treated as unresolved species.

Discussion

DNA barcoding, a technique projected for rapid identification of unknown biological samples which uses short (known as 'Folmer' region which has 658 bp long, present at the 5' end of the *CO1* mitochondrial genome) and agreed upon DNA sequences [25–26]. Upon its first initial success in more than 200 allied *Lepidopteran* species, it has been considered as a powerful tool for identification of all eukaryotes at the species level. However, it was found that *CO1* gene was not suitable for plants because as such there is no region of genome, cytoplasm or nuclear that could be identified. The plant mitochondrial genes with low nucleotide substitutions and low evolutionary rates were considered unsuitable for barcodes of plants [27–30]. Therefore, the present study was initiated to check the applicability of nuclear genome (ITS) for identification, authentication of some Vandaceous orchids based on the earlier recommendation made by Plant Working Group of consortium for the Barcode of Life (CBOL) and Barcode of Life Database (BOLD) standard guidelines [30]. The sampled specimens (individuals) collected from different parts of Nagaland during the field survey were brought to the Department laboratory and stored at -20°C in a deep freezer to minimize the degradation of DNA and to preserve them till DNA was extracted. For DNA isolation, CTAB method [18] protocol was followed for some species. However, some orchid species accumulate mucilage (*Acampe, Aerides, Arachnis*) to conserve water and as food reserve [31]. The presence of high mucilage (polysaccharides and polyphenols) contents in such species was the major obstacle in DNA isolation and PCR amplification. Therefore, a modified CTAB method [19] in concentration and a step-wise manner was modified for those species which has high mucilage content.

The amplification success rate for ITS was 91.25% in the present study, a relatively higher as compared to other workers [28, 32–33]. A higher amplification rate of 97% was reported by Roy *et al*. [34] in the tested samples of 11 species of *Ficus* and 4 species of *Gossypium* and also Singh *et al*. [35] while testing the congeneric species of *Dendrobium* with 98.97% amplification success rates was reported.

Following amplification, the successful amplicons are packed, labeled and send it for sequencing after completing all the formalities/company instruction to various laboratories to sequence the desire size/targeted loci. The sequence success rate and the total number of barcode sequence generated was 91.78% and 67 respectively.

Intra and Inter-Specific Variations

For correct identification and generation of DNA barcodes for species, the assessment of intra and inter-specific variations is important [23–24, 36]. The minimum inter-specific variation is greater than the maximum intra-specific variation and the difference between the two is referred as 'barcode gap' [37]. The intra and inter-specific divergence were evaluated/expressed in terms of K2P distances as done by Chen *et al*. [38] and also Parveen [33]. The intra-specific variations were evaluated for 25 species that were

represented by more than one individual. Variable range of intra-specific distances was obtained in all different species. The minimum/lowest divergence among the investigated species was observed in *Cleisostoma williamsonii* while the maximum/highest divergence was observed in *Acampe ochracea*. While evaluating the inter-specific divergence for 7 genus that were represented by more than one species in a genus, it was observed that the genus *Arachnis* (2 species) with an average inter-specific K2P Distance of 0.032 (Ranges from 0-0.063) was the lowest while *Acampe* with 0.187 (ranges from 0-0.431) was the highest.

Species Discrimination Rates and Evaluation of DNA Barcodes

For evaluating species resolution and selecting DNA barcodes three methods were used *viz.*, genetic distance, phylogenetic tree method and BLAST analysis. The genetic distance employs the assessment of intra and inter-specific divergence. The intra and inter-specific divergence should not overlap in an ideal barcode. The difference/gap between the two specific divergences provides a perfect barcode which is referred as barcode gap [25–26, 36]. The phylogenetic tree method is constructed using sequences from the targeted locus and the percent species resolution was determined by cluster analysis [36, 39]. The species for which all the individuals clustered together in a single clade are considered as unequivocally identified species and those which clustered with the individuals of the other species were treated as unresolved. During the present analysis, the species resolution of the investigated loci calculated using both these methods showed different results.

The last method used for evaluating species discrimination is the BLAST analysis [40]. In this method, the unknown individual barcode sequence is search in the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for a very similar/identical sequence from the database, containing reference barcodes of correctly identified species.

In spite of low amplification rate, ITS showed more species discrimination rates of 90.90% and 95.45% by distance based method and phylogenetic tree method. At the genus level, ITS BLAST hits increases from 79.40-95.52%. The overall species discrimination rates are 89.15%. The high species discrimination ability of this region could be due to its high rate of evolution leading to genetic changes that allows differentiation of closely related congeneric species [28, 35, 41–42]. No out-group was use in the phylogenetic tree construction so as to find/compare the genetic closeness of this closely related species, which are similar in vegetative characters and difficult to identify based on morphological characters.

Conclusion

Present study demonstrated that the loci Internal Transcribed Spacer (ITS) are useful DNA barcode to identify Vandaceous Orchids. Moreover, a genetic relationship among this Vandaceous orchids was compared using the sequences and phylogeny was constructed. However, more Vandaceous species should be included in the future to verify whether the findings hold true when even more closely related

taxa are included. This study provided much useful genetic information about Vandaceous species, which will be useful for germplasm management and resource protection.

Declarations

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Conflicts of Interest/Competing Interest: Authors declare that there is no conflict of interest exist.

Availability of Data & Materials (data transparency): All raw data and materials are available and will be provided, if situation arises.

Code Availability (software application or custom code): Not applicable as all online free software used for the present study.

Authors Contribution: CRD has designed the work, arranged fund and facilities for the work, supervised the research work, corrected data interpretation and the paper. JK has executed the research work as a part of his Ph. D. programme, data analyzed, drafted the paper.

Ethical Approval: Not applicable/required.

Consent to Participate: Both authors consented to be the part of the paper with equal share of contribution.

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Similarity Test of the Manuscript: The text of the manuscript (excluding references and data) has been checked for 'Similarity/Plagiarism Check' using URKUND software (Analysis address: debchitta.naga@analysis.urkund.com, ID: D110089442) and resulted 7% similarity.

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Figures

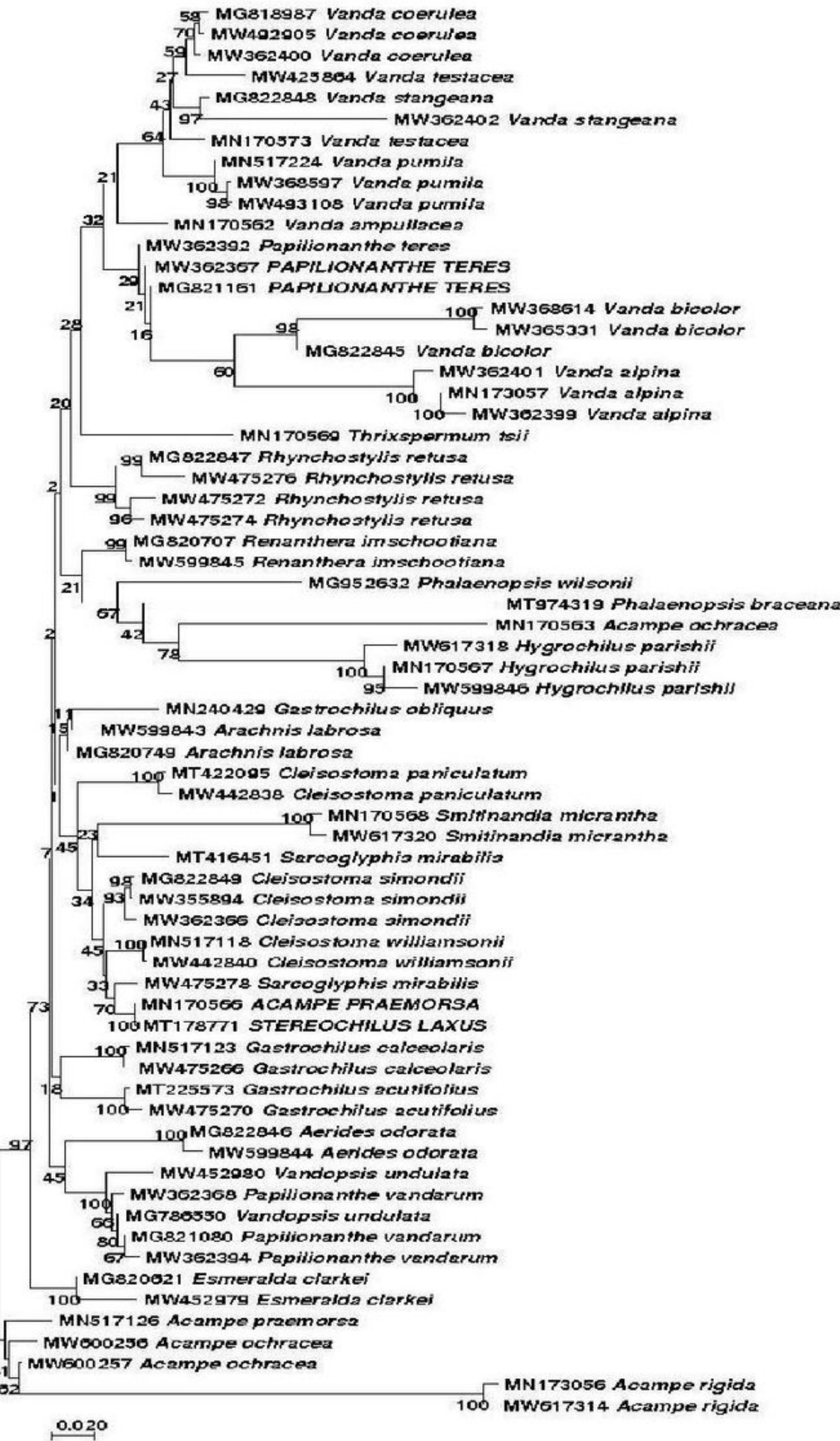

Figure 1

Neighbor joining tree of 67 species based on ITS sequences. The species showing zero inter and intra specific divergence are shown bold, capital and both as well.

Supplementary Files

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- Table4Supplementarymaterials.xlsx