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Evaluating the Effectiveness of Three DNA Bar code Loci to Classify Jewel Orchids Using *In silico* Approach

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ABSTRACT

Three DNA barcode loci consisting of ITS, *matK* and *rbcL* have been used intensively to discriminate jewel orchid species. Nevertheless, the discrimination ability of each locus is inconsistent among researches. Therefore, it is necessary to simultaneously check a large number of published DNA sequences to get an overview of the ability to recognize orchid varieties. In this study, total of 124 DNA sequences of these three loci from Genbank were evaluated for the discrimination ability on 114 species belonging to five genus of jewel orchids. The obtained data show the significant variability of barcode sequences at both taxonomy levels. Distinguishing ability descend from *matK*, ITS to *rbcL*. The obtained results suggest that the discrimination capacity of ITS, *matK* and *rbcL* DNA barcode loci are variable among different species of jewel orchid plants, in which *matK* and ITS loci reveal more potential for genetic classification at genus and species level of this herbal plants. In future, higher sequence number should be included in the analysis to give more reliable result. The information from this study could be useful in conservation and development programs of jewel orchid plants.

KEY WORDS: DNA BARCODE, ITS, JEWEL ORCHID, MATK, RBCL.

INTRODUCTION

Jewel orchid is a general term of different species belonging to Orchidaceae family. This plant can be found in large area from Southern China, Northeast India, Thailand, Vietnam, the Philippines, Malaysia, Indonesia and Myanmar. Beside ornamental use, jewel orchid is got more attention due to its high medical value. Different parts of plant are applied for treating numerous diseases such as abdominal pain, diabetes, nephritis, fever, hypertension, liver, and pleurisy (Du et al., 2008) since several medicinal compounds with strong biological activity have been identified in these plants (Liang et al, 1990; Lin et al., 1993; Panda et al., 2014). The term "jewel orchid" is used to refer to various species in Orchidaceae family having velvety brocade-like leaves with beautiful veins. Nevertheless, the medicinal and economical value are species dependent, thus, an accurate classification would be crucial importance as a basis for conservation and development. Traditionally, jewel orchids are discerned based on plant morphological characteristics such as leaves, stem structure, or flowers (Trinh et al., 2020).

While this method has been confirmed economically due to its ease and low cost, it is error-prone method due to identical external morphology features, large variable polymorphisms between adult and juvenile stages, and environmental factors as well as the plant growth development phases, all are leading to inaccuracy (Ahmedand and Mohamed, 2014). Furthermore, the inability to precisely identify specimens under damaged or processed conditions raises serious concerns about the medicinal value of the jewel orchid-derived products. Consequently, incorrect utilization will reduce effectiveness or harm the health of the patient due to the variation in medicinal compounds and application among different jewel orchid members (Du et al., 2008; Poobathy et al., 2018; Trinh et al., 2020).

Based on the rapid development of DNA sequencing technology, the application of a short standard DNA sequence, so-called DNA barcode, to identify target plant

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species has been used intensively. This method possesses several advantages in the comparison to traditional methods such as high repeatability and stability, applicability to any developmental stages of organism and ability to identify target organism being destroyed or processed (Barcaccia et al., 2016). Several barcode loci have been tried to investigate DNA barcode candidates for plant identification (Hollingsworth et al. 2011; Kress, 2017). However, two chloroplast gene namely maturase K (*matK*) and ribulose-1, 5-biphosphate carboxy lase (*rbcL*) have been proposed as preferred plant barcoding loci by consortium for the barcodes of life (CBOL, 2009). Beside *matK* and *rbcL* as standard DNA barcode, internal transcribed spacer (ITS) has also been used intensively for classification several plants recently such as *Talinum paniculatum* (Nguyen et al., 2017); *Astragalus* spp. (Zhang and Jiang, 2020); *Oryza* (Zhang et al., 2021).

In a short time, studies using these three DNA barcode loci for jewel orchid identification have been published (Chen and Shiau, 2015; Lv et al., 2015; Hu et al., 2019; Huynh et al., 2019; Sherif et al., 2020) suggesting the usefulness and potential of these three loci in plant classification. Despite the confirmed effectiveness of DNA barcode in plant identification, discrimination power and trending utilization of each barcode loci are variable. After careful survey 16 popular DNA barcode loci from 2005 to 2010, Hollingsworth and colleagues found the large difference in enthusiasm of using different locus (Hollingsworth et al., 2011). The species identification capacity of *rbcL* locus was higher than that of *matK* and ITS on mangrove plants (Wu et al., 2019). Nevertheless, another study in China at same time showed the inferior of *rbcL* to ITS and matK on *Astragalus* spp. (Zhang and Jiang, 2019). Therefore, the present study focused on evaluate the identification ability of ITS, *matK* and *rbcL* loci in jewel orchid plants by using DNA sequences available on the National Center for Biotechnology Information (NCBI). The obtained results could provide information to escalate the effectiveness in classifying and identifying different jewel orchid population at species and genus level.

MATERIAL AND METHODS

The sequences of ITS, *matK* and *rbcL* loci belonging to different jewel orchid species were downloaded from nucleotide database of NCBI (URL: http: //www.ncbi.nlm.nih.gov) by using corresponding Latin name and barcode locus as keywords. The sequences were selected for analysis based on criteria proposed by Suesatpanit and colleagues (1) sequences are not 'unverified' without a species name (2) contain <3% ambiguous base 'N' (Suesatpanit et al. 2017) and presented in Table 1.

Table 1. List of jewel orchids and number of retrieved sequences used in this study

	Sample code	Latin name	Genus	Sequence number		
				ITS	matK	rbcL
1	ARot	*Aenhenrya rotundifolia*	*Aenhenrya*	0	1	1
2	AA	*Anoectochilus albolineatus*	*Anoectochilus*	1	1	1
3	AE	*Anoectochilus elatus*		0	6	6
4	AF	*Anoectochilus formosanus*		5	1	0
5	AL	*Anoectochilus lylei*		2	2	1
6	ARox	*Anoectochilus roxburghii*		16	5	1
7	AS	*Anoectochilus setaceus*		6	0	0
8	DM	*Dossinia marmorata*	*Dossinia*	6	4	2
9	GH	*Goodyera hispida*	*Goodyera*	4	0	0
10	GP	*Goodyera pubescens*		2	6	6
11	GVir	*Goodyera viridiflora*		10	6	1
12	GVit	*Goodyera vittata*		2	1	0
13	LD	*Ludisia discolor*	*Ludisia*	8	2	6
14	MP	*Macodes petola*	*Macodes*	1	0	1

Sequences were converted into FASTA format and subjected to Multiple Sequence Alignment using Clustal W intergrated in MEGA 6 software (Tamura et al. 2013). This software was also applied to calculate the evolutionary divergence for each data set and pattern of nucleotide substitution. For phylogenetic analysis we used Neighbor-Joining tree method with 1000 bootstrap replicates and presented as circular cladograms (Ho and Nguyen, 2020). In order to estimate species resolution for a given barcode locus, the species/genus were resolved if con- specific individuals are grouped into one monophyletic branch in the phylogenetic tree with bootstrap support greater than 50% (Zhang et al., 2019). Contrarily, if separated in paraphyletic branches such species and genus are considered as identification failure (Sikdar et al. 2018).

RESULTS AND DISCUSSION

Sequence Retrieve: By using keyword "species names

+ITS/*matK*/*rbcL*" to find the sequences deposited in NCBI Gen Bank, after removal of unrealizable sequences as described by Suesatpanit and colleagues (2017), total of 124 sequences were obtained, there are 63, 35 and 26 DNA sequences of ITS, *matK* and *matK* regions, respectively (Table 1).

Table 2. Estimates of Average Evolutionary Divergence over Sequence Pairs within Group

Species	p distance of ITS	SE	p distance of matK	SE	p distance of rbcL	SE
Aenhenrya rotundifolia	NA	NA	NA	NA	NA	NA
Anoectochilus albolineatus	NA	NA	NA	NA	NA	NA
Anoectochilus elatus	NA	NA	0.000	0.000	0.000	0.000
Anoectochilus formosanus	0.002	0.002	NA	NA	NA	NA
Anoectochilus lylei	0.008	0.006	0.014	0.004	NA	NA
Anoectochilus roxburghii	0.003	0.002	0.023	0.004	NA	NA
Anoectochilus setaceus	0.044	0.008	NA	NA	NA	NA
Dossinia marmorata	0.000	0.000	0.002	0.001	0.000	0.000
Goodyera hispida	0.009	0.004	NA	NA	NA	NA
Goodyera pubescens	0.004	0.004	0.002	0.001	0.000	0.000
Goodyera viridiflora	0.009	0.003	0.012	0.003	NA	NA
Goodyera vittata	0.079	0.018	NA	NA	NA	NA
Ludisia discolor	0.003	0.003	0.000	0.000	0.004	0.002
Macodes petola	NA	NA	NA	NA	NA	NA

(The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distances. Note: SE: standard error; NA: not available).

Table 3. Estimates of Evolutionary Divergence over Sequence Pairs between Groups for ITS

	AA*	AF	AL	AR	AS	DM	GH	GP	GVir	GVit	LD	MP
AA		0.006	0.003	0.005	0.004	0.016	0.019	0.022	0.018	0.016	0.016	0.016
AF	0.010		0.003	0.002	0.006	0.014	0.018	0.021	0.016	0.015	0.015	0.014
AL	0.004	0.006		0.003	0.005	0.015	0.018	0.021	0.017	0.015	0.015	0.015
AR	0.008	0.003	0.005		0.005	0.014	0.018	0.021	0.016	0.015	0.015	0.014
AS	0.023	0.028	0.025	0.026		0.016	0.019	0.022	0.018	0.016	0.016	0.016
DM	0.062	0.055	0.058	0.055	0.079		0.018	0.021	0.017	0.016	0.015	0.013
GH	0.086	0.079	0.082	0.079	0.104	0.090		0.009	0.009	0.010	0.016	0.017
GP	0.106	0.099	0.101	0.099	0.125	0.110	0.027		0.013	0.013	0.018	0.020
GVir	0.078	0.071	0.074	0.071	0.096	0.081	0.028	0.046		0.011	0.015	0.016
GVit	0.080	0.072	0.075	0.073	0.098	0.086	0.047	0.063	0.052		0.013	0.015
LD	0.066	0.059	0.062	0.059	0.085	0.059	0.069	0.084	0.069	0.066		0.013
MP	0.058	0.051	0.054	0.051	0.076	0.049	0.077	0.096	0.067	0.073	0.042	

(*: Species names are abbreviated corresponding to Table 1, standard error of comparison is presented in italics upper diagonal).

In general, ITS locus was more intensively studied with up to 63 sequences aquired, whereas m*atK* and *rbcL* are likely unattended with lower sequence number. Considering each species, the sequence availability also is varying, in which, *Anoectochilus roxburghii, Goodyera viridiflora, Ludisia discolor* and *Goodyera pubescen* show highest sequence abundance suggesting the economic importance of these species. Since previous studies highlighted the abundance of medical compounds detected in these species. *Anoectochilus roxburghii* is rich in polysaccharides, flavonoids, glycosides, organic acids, volatile compounds, steroids, triterpenes, alkaloids, and nucleosides (Ye et al., 2017); butanolide, goodyeroside A, butanolide in *Goodyera* genus (Du amino acids and anthocyanin content in *Ludisia discolor* (Poobathy et al., 2016).

Table 4. Estimates of Evolutionary Divergence over Sequence Pairs between Groups for matK

	AA*	AF	AL	AR	DM	GP	GVIR	GVIT	LD	AE	ARot
AA		0.004	0.002	0.004	0.007	0.009	0.006	0.007	0.006	0.000	0.009
AF	0.014		0.004	0.003	0.007	0.009	0.007	0.008	0.006	0.004	0.009
AL	0.007	0.017		0.004	0.007	0.008	0.006	0.007	0.006	0.002	0.009
AR	0.012	0.007	0.013		0.007	0.009	0.007	0.007	0.005	0.004	0.009
DM	0.031	0.037	0.035	0.035		0.008	0.007	0.007	0.005	0.007	0.008
GP	0.049	0.056	0.050	0.053	0.044		0.006	0.007	0.008	0.009	0.009
GVIR	0.035	0.041	0.037	0.039	0.034	0.031		0.002	0.006	0.006	0.007
GVIT	0.033	0.040	0.035	0.038	0.032	0.030	0.007		0.006	0.007	0.008
LD	0.022	0.024	0.025	0.023	0.021	0.041	0.027	0.025		0.006	0.008
AE	0.000	0.014	0.007	0.012	0.031	0.049	0.035	0.033	0.022		0.009
ARot	0.055	0.060	0.058	0.058	0.047	0.054	0.042	0.042	0.045	0.055	

(*: Species names are abbreviated corresponding to Table 1, standard error of comparison is presented in italics upper diagonal).

Table 5. Estimates of Evolutionary Divergence over Sequence Pairs between Groups for *rbcL*

	AA*	AL	ARox	DM	GP	GVir	LD	MP	AE	ARot
AA	0.000	0.002	0.002	0.003	0.000	0.002	0.008	0.000	0.003	
AL	0.000		0.002	0.002	0.003	0.000	0.002	0.008	0.000	0.003
ARox	0.002	0.002		0.003	0.003	0.002	0.003	0.008	0.002	0.004
DM	0.002	0.002	0.004		0.003	0.002	0.003	0.007	0.002	0.004
GP	0.004	0.004	0.006	0.006		0.003	0.003	0.008	0.003	0.004
GVir	0.000	0.000	0.002	0.002	0.004		0.002	0.008	0.000	0.003
LD	0.004	0.004	0.006	0.006	0.008	0.004		0.008	0.002	0.004
MP	0.028	0.028	0.030	0.026	0.032	0.028	0.032		0.008	0.008
AE	0.000	0.000	0.002	0.002	0.004	0.000	0.004	0.028		0.003
ARot	0.006	0.006	0.008	0.008	0.010	0.006	0.009	0.035	0.006	

(*: Species names are abbreviated corresponding to Table 1, standard error of comparison is presented in italics upper diagonal)

Table 6. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution (in percentage)

	ITS				matK				rbcL			
	A	T	C	G	A	T	C	G	A	T	C	G
A	-	3.97	3.69	18.69	-	8.13	3.37	10.24	-	6.10	4.59	11.39
T	3.04	-	18.31	4.21	6.53	-	7.60	3.11	5.61	-	14.16	4.56
C	3.04	19.67	-	4.21	6.53	18.35	-	3.11	5.61	18.81	-	4.56
G	13.49	3.97	3.69	-	21.53	8.13	3.37	-	13.75	6.10	4.59	-

(Note: Each entry shows the probability of substitution from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics)

Estimation of sequence divergence: The variation within and between species based on ITS, *matK*, and *rbcL* regions were calculated and the data representing by evolutionary divergence (p distance value) are shown in Table 2. Based on p distance, it suggests that there is the large variability of three regions within a specific species

of jewel orchid. *Anoectochilus setaceus* show highest variation divergence in ITS locus (0.044), *Anoectochilus lylei* show highest at *matK* (0.014) and *Ludisia discolor* at *rbcL* (0.004).

Figure 1: Neighbor-joining tree with 1000 bootstrap replicated based on *matK* sequences (Shaded areas and dot cycles present the completely resolved species and genus, respectively).

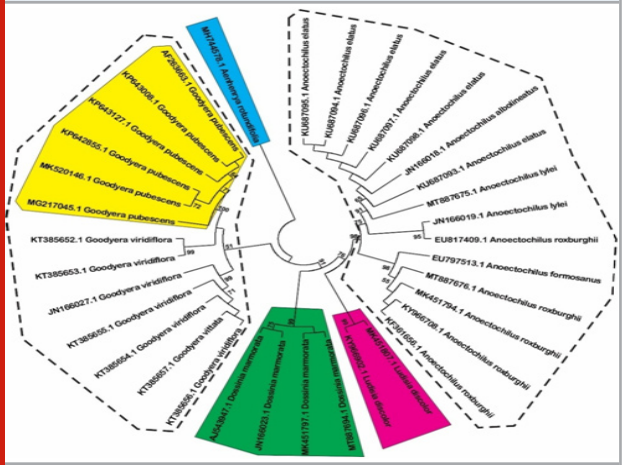
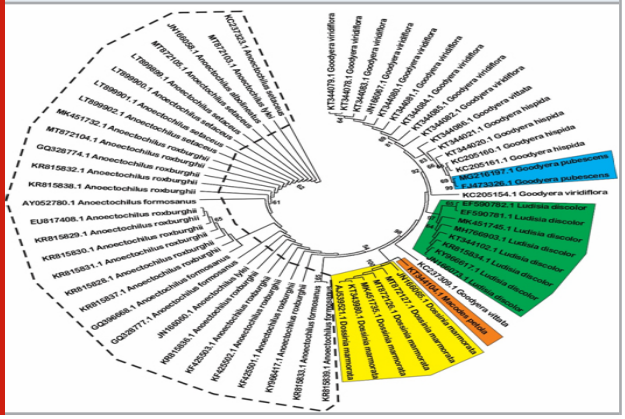


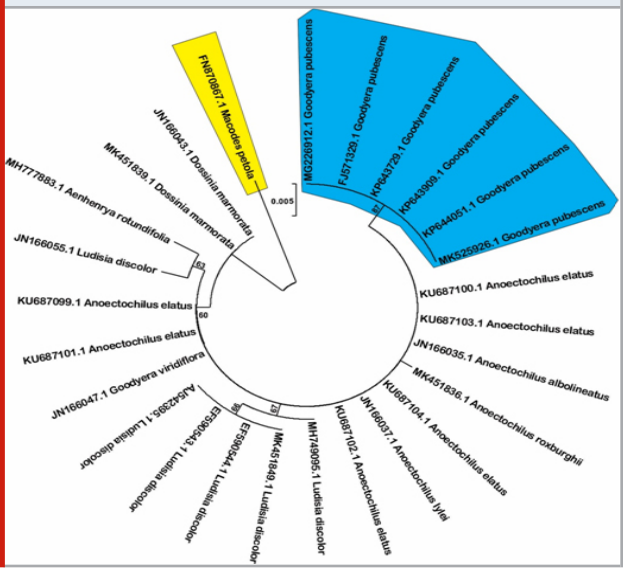
Figure 2: Neighbor-joining tree with 1000 bootstrap replicated based on ITS sequences (Shaded areas and dot cycles present the completely resolved species and genus, respectively).



The number of base substitutions per site from averaging over all sequence pairs within each group are shown in Table 3, 4 and 5. The variability among ITS, *matK* and *rbcL* regions is from 0.003-0.106, 0 - 0.06, and 0 – 0.035, respectively. Substitution bias consisting of transition and transversion at codon position for each luster could reveal the trend of evolution. In this study, the substitution of different bases in analyzed regions is evaluated on entire codon positions (1st+ 2nd + 3rd nucleotide) and shown in Table 6. In general, transitional substitution is higher than transversional substitution in all loci. Two chloroplast namely *matK* and *rbcL* show a higher transversionsal substitution than ITS.

Estimation of species resolution: Based on phylogenetic analysis, the species resolution is variable among three DNA barcode loci (Figure 1, 2 and 3). *MatK* show highest effectiveness in plant discrimination in which all of five genus are resolved (Figure 1), especially this locus is able to distinguish five per ten species consisting of *Aenhenrya rotundifolia, Goodyera pubescens, dossinia marmorata*, and *Ludisia discolor*. ITS locus could also discriminate four genus consisting of *Anoectochilus, Dossinia, Macodes* and *Lusidia*. DNA sequences from this locus could separate four species namely *Goodyera pubescens, Ludisia discolor, Macodes petola* and *Dossinia mamorata* (Figure 2). Although *rbcL* locus was reported as a good marker to differentiate species in *Prunus genus* (Singh et al. 2016), our data show that this is the poorest among three studied loci in term of discrimination power in which only *Macodes petola* and *Goodyera pubescens* are completely resolved (Figure 3). One of main reason of low discrimination power of this locus could be due tolimited number of examined sequences, Sikdar and colleagues proposed to increase sequence number to enhance the discrimination power (Sikdar et al. 2018).

Figure 3: Neighbor-joining tree with 1000 bootstrap replicated based on *rbcL* sequences (Shaded areas present the completely resolved species).



Even both *Anoectochilus* and *Goodyera* genus are rich in several medicinal compounds, the former is considered better than the later since some corresponding compounds extracted from *Goodyera* is less effective than those from *Anoectochilus* species (Du et al., 2008). Furthermore, *Anoectochilus* species are traditionally used for treating chest and abdominal pains, diabetes, nephritis, fever, hypertension, impotence, liver and spleen disorder, and pleurodynia whereas, *L. discoloris* used to reduce coughs and strengthening weak lungs (Poobathy et al., 2018). Thus, the high discrimination power of *matK* locus is highly valuable to identify, utilize and develop these two species for specific medicinal purposes.

CONCLUSION

The usefulness of three main DNA barcode loci in classify different jewel orchid plants is confirmed by *in silico* analysis. The obtained results suggest that the discrimination capacity of ITS, *matK* and *rbcL* DNA barcode loci are variable among different species of jewel orchid plants, in which *matK* and ITS loci reveal more potential for genetic classification at genus and species level of this herbal plants. In the future, higher sequence number should be included in the analysis to give more reliable result. The information from this study could be useful in conservation and development programs of jewel orchid plants.

Conflict of Interests: The author has no conflict of interest.

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