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MONOPHYLY OF FOUR SECTIONS OF GENUS *DENDROBIUM* (ORCHIDACEAE): EVIDENCE FROM NUCLEAR RIBOSOMAL DNA INTRENAL TRANSCRIBED SPACER (ITS) SEQUENCES

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Abstract: Evolutionary relationship among four sections of the genus *Dendrobium* (*Aporum, Crumenata, Strongyle* and *Bolbidium*) were inferred from nucleotide sequence variation in nuclear ribosomal DNA of Peninsular Malaysia species. The Internal transcribed spacer (ITS) of nuclear ribosomal DNA from representative of 29 species of genus *Dendrobium* and two species of genus *Bulbophyllum* as out group were analyzed by polymerase chain reaction amplification and DNA sequencing. Data analyses have been carried out using Maximum Parsimony (MP) as a character based method by different software (PAUP*4. 0 B 10 and Mega 5). Parsimony analyses of sequences resulted in a well-resolved phylogeny. The data suggested that Internal Transcribed Spacer (ITS) is a reliable marker for the phylogenetic study of *Dendrobium* compared to previous studies using chloroplast DNA with low resolution level among sections. The results demonstrated that the four sections formed a monophyletic group and that it is best to recognise only one section instead of four. Based on ICBN rules, the name *Aporum* has a priority to be used for this new classification.

Key Words: *Dendrobium*, Orchidaceae, monophyly, nuclear ribosomal DNA, Maximum Parsimony.

INTRODUCTION

With around 800-1500 species worldwide, *Dendrobium* Sw. is one of the three largest genera in family Orchidaceae[1]. This genus was first recognized by Olofswartz in 1799. The name comes from the Greek words, *Dendron* meaning tree and *bios* for life; it means that the plant which lives in trees. Most species of *Dendrobium* are epiphytes in primary forest, less often lithophytes; only very few are terrestrials[2]. Orchids are cosmopolitan in distribution, occurring in every habitat, found all over the world except in the coldest and driest regions[3]. *Dendrobium* like other orchids are generally found in wetter conditions such as the tropical regions with high annual rainfall and without too much seasonal variation throughout the year[4]. The great majority are found in the tropics, mostly in Asia, South America, and Central America. For Example, in Malaysia, the *Dendrobium* thrives well with this condition, a mean rainfall of about 2,500 mm and the lowest of about 1, 750 mm[5]. *Dendrobiums* are one of the most popular orchids due to their medicinal and commercial values[3].Some species like *D. bifarium* Lindl., *D. planibulbe*Lindl., & *D. purpureum* (Nichols) Dockr. are used in Malesia as medicinal Herbs to treat skin disorders. They have floriferous flower sprays that come in a wide variety of colors, sizes and shapes, available throughout the year and the blooms last several weeks to months[6].

Traditionally, comparative vegetative anatomy and plant systematics were two general strategies to assess the relationships among the taxa in *Dendrobium*[7]. Problems associated with variability and plant growth conditions are easy to cause confusion in the species identification. The widespread development of molecular techniques for genetic analysis in the past decade has led to the increase of knowledge of orchid genetic diversity. The common molecular data used in plant systematics comes from two sources: chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA)[8]. The nuclear ribosomal RNA (rRNA) genes involved 18S (small subunit), 28S (large subunit) and 5.8S, are separated by two internal transcribed spacer regions ITS1 and ITS2 (Figure 1). In eukaryotic genome, these genes are repeated in several hundred or perhaps thousands of copies[9]. The nuclear rDNA internal transcribed spacer (ITS) has become the most valuable region in plant molecular studies, especially in lower taxonomic level, since it was first used in phylogenetic inference by Baldwin in 1992[10]. As the nuclear gene region, it is biparentally inherited and has higher rates of base substitution. Indeed, this region can be amplified easily using standard PCR methods, because it exists in high copy number and priming sites surrounding the 18S and 26S regions are highly conserved[11]. Although usage of ITS sequence data is very common in phylogenetic studies, the characteristics of nrDNA evolution may result in a comparison of paralegals if sufficient caution is not exercised[12]. In 2003, Clements conducted a phylogenetic study on sub-tribe Dendrobiinae using ITS[13]. The genetic relationship of 36 *Dendrobium* species in China was determined using sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA by Yuan and his co-workers in 2009.

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In their study, the phylogenetic relationships made by ITS DNA analysis incompletely supported previously published morphological data. Molecular evidence from these studies recommended that the genus *Dendrobium* is polymorphic with a complex genetic background at the species level[14]. In Peninsular Malaysia, *Dendrobium* is the second largest genus of the Orchidaceae whereby it has 16 sections and 86 species3. A total of 28 sections of *Dendrobium* has been recorded from Malesia by the National Herbarium of Netherland (Source: http:// www.national herbarium. nl/pubs/orchid web/genera/*Dendrobium*). In this study, The main objectives of the present study are to determine the phylogenetic relationship between the four sections of the genus *Dendrobium* (*Aporum, Crumenata, Strongyle,* and *Bolbidium*) based on nrDNA (ITS) in Peninsular Malaysia.

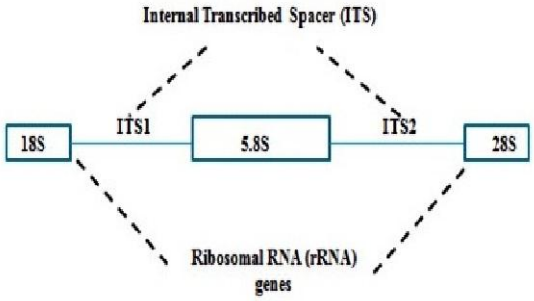

Figure 1: A sketch of the nuclear ribosomal RNA (rRNA) genes showing relative positions of rRNA genes subunit separated by the Internal Transcribed Spacer region (ITS).

Table 1: List of studied taxa

	Species	Section	Location	Gene Bank Accession Number
1	*Dendrobium aloifolium*	*Aporum*	UPM Green house, No.5	KC507775
2	*Dendrobium grande*	*Aporum*	Labuk Tapah, Selai, PM	KC507779
3	*Dendrobium leonis*	*Aporum*	UPM Green house, No.5	KC507774
4	*Dendrobium quadrilobatum*	*Aporum*	Kuala Krai, Kelantan, PM	KC507778
5	*Dendrobium rosellum*	*Aporum*	UPM Green house, No.5	KC507777
6	*Dendrobium terminale*	*Aporum*	Sungai Bertedung, Endau Rompin, PM	KC507776
7	*Dendrobium clavator*	*Crumenata*	Sungai Bertedung, Endau Rompin, PM	KC507762
8	*Dendrobium crumenatum*	*Crumenata*	Genting Highlands, PM	KC507780
9	*Dendrobium setifolium*	*Crumenata*	Sungai Bertedung, Endau Rompin, PM	KC507763
10	*Dendrobium truncatum*	*Crumenata*	Cameron Highlands, PM	KC507761
11	*Dendrobium kentrophyllum*	*Strongyle*	Fraser's Hill, PM	KC507764
12	*Dendrobium singaporense*	*Strongyle*	Cameron Highlands, PM	KC507765
13	*Dendrobium subulatum*	*Strongyle*	Gunung Nuang, PM	KC507766
14	*Dendrobium pachyphyllum*	*Bolbidium*	Fraser's Hill, PM	KC507769
15	*Dendrobium hymenanthum*	*Bolbidium*	Cameron Highlands, PM	KC507768
16	*Dendrobium striatellum*	*Bolbidium*	Kuala Krai, Kelantan, PM	KC507767
17	*Dendrobium* sp2	?	G. Kelapak buruk, PM	KC507770
18	*Dendrobium* sp3	?	UPM Green house, No.5	KC507771
19	*Dendrobium* sp4	?	UPM Green house, No.5	KC522821
20	*Dendrobium* sp5	?	UPM Green house, No.5	KC522822
21	*Dendrobium* sp6	?	UPM Green house, No.5	KC522823
22	*Dendrobium heterocarpum*	*Dendrobium*	NCBI	JF713105.1
23	*Dendrobium nobile*	*Dendrobium*	NCBI	JF713365.1
24	*Dendrobium crepidatum*	*Dendrobium*	NCBI	HM054624.1
25	*Dendrobium fimbriatum*	*Dendrobium*	NCBI	HM054636.1
26	*Dendrobium gibsonii*	*Dendrobium*	NCBI	HQ114256.1
27	*Dendrobium farmeri*	*Callista*	NCBI	HM054629.1
28	*Dendrobium thyrsiflorum*	*Callista*	NCBI	HM054759.1
29	*Dendrobium macrophyllum*	*Lautoria*	NCBI	AY239979.1
30	*Bulbophyllum inunctum*	*Sestochilus*	Cameron Highlands, PM	KC507773
31	*Bulbophyllum macranthum*	*Sestochilus*	Gunung Jerai, PM	KC507772

MATERIALS AND METHODS

Plant materials

Dendrobium samples (21 fresh samples) of the four sections (*Aporum, Crumenata, Strongyle,* and *Bolbidium*) were collected along the trials of the selected areas, UPM greenhouse and some nursery in Peninsular Malaysia (Table 1). There were five *Dendrobium* species that could not be identified because of the lack of flowers (however, their morphological characters were nearly similar to three sections *Aporum*, *Crumenata* and *Strongyle*. The fresh leaves or stems of species were used for DNA extraction. For the better comparison in the data analysis step, the sequences of the eight species of genus *Dendrobium* that belong to the three sections *Dendrobium*, *Callista* and *Latouria* retrieved from the NCBI database were used. In addition, two species of the genus *Bulbophyllum* (*B. macranthum and B. inunctum*) were used as outgroup.

Genomic DNA extraction, amplification, and sequencing

Genomic DNA was extracted from leaf samples using a cetyltrimethyl ammonium bromide (CTAB) method as described previously by Wang *et al* 2004 with minor modification[15]. The concentration of genomic DNA samples was determined by Nano drop machine and necessary dilutions were done, followed by verification with 0.8% agarose gel electrophoresis. The PCR conditions were 95°C 5 min; 35 cycles: 95°C 1 min; 50/55°C 2 min; 72°C 2 min; 72°C 7 min. The PCR products were separated by1% agarose gel electrophoresis and purified by Wizard_ SV Gel and PCR Clean-Up System (Promega, USA). After that, all of purified PCR products were sent to the First Base Company for sequencing. Finally the sequences were submitted to the NCBI database (Table 1).

DNA sequence data analysis

DNA sequences obtained from ITS region were aligned by BioEdit ver.7.0.2.[16]. The Maximum Parsimony (MP) method was selected for the construction of phylogenetic trees. The output data were processed using PAUP* 4.0B10[17] and MEGA5[18] to draw the phylogenetic trees. A total of 1000 bootstrap replicates was calculated for all methods.

RESULTS AND DISCUSSION

Complete ITS sequences were analysed for 29 *Dendrobium* species plus *Bulbophyllum inunctum* and *Bulbophyllum macranthum* as outgroup. The aligned sequences consisted of 490 nucleotide characters. 170 characters were conserved among all taxa, 291 were variable, and 180 were parsimony informative. The analysis methods, Maximum Parsimony (MP) were used to infer the evolutionary history. Unweighted parsimony analysis resulted in 12 most parsimonious trees [tree length = 589, consistency index (CI) = 0.58, retention index (RI) = 0.71 and homoplasy index (HI) = 0.33]; One of the most parsimonious trees was shown in Figure.2.

One of the loci that is commonly used for phylogenetic inference is ITS with a high copy number and relative range of phylogenetic utility[19]. The use of nrDNA regions has become popular and is used in different level of taxa, especially the low level taxa, such as infrageneric species[20]. The utility of this region in the molecular study of family Orchidaceae is very widespread. It is often used in phylogenetic studies at various taxonomic levels in most groups of the family Orchidaceae[21]; Salazar *et al.,* 2003[22]; Van den Berg *et al.*, 2005[23]; whitten *et al.*, 2007[24]). Andre Schuiteman (2011) has used ITS to do phylogeny of genus *Dendrobium.* Actually, the obtained results from ITS marker in this study confirmed and extended his hypothesis, which indicated that the four sections (*Aporum, Crumenata, Strongyle* and *Bolbidium*) can be considered as one section instead of four. The original studies by Yukawa and co-workers (1993[25], 1996[26], 2000[27], 2001[28]) using DNA markers (*mat*K and ITS) have made available a number of insights concerning the phylogeny of genus *Dendrobium* and subtribe Dendrobiinae, which were confirmed and improved by later studies (Clements 2003[13], 2006[29]).

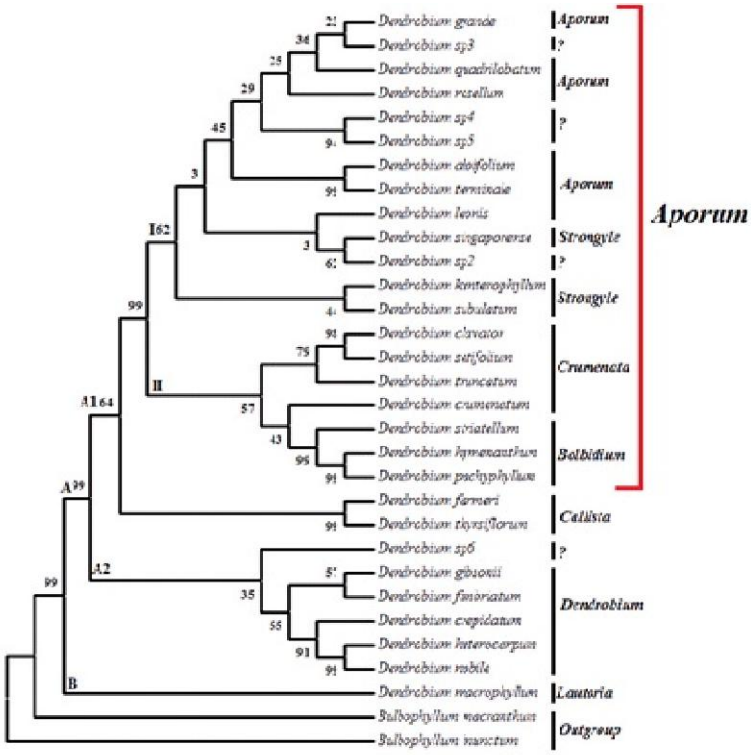

Figure 2: One of most parsimonious trees of selected *Dendrobium* sections based on nrITS sequence data. A (1-2) and B are mainclades and include the different sections of genus *Dendrobium.* A1 (I and II) denote the four sections *Aporum, Crumenata, Strongyle* and *Bolbidium* form a monophyletic group and can be considered as one section named *Aporum.*

In this study, several attempts were made to amplify ITS from a number of *Dendrobium* species. The lower level of homoplasy and around 37% PIC was established in this region to show a high level of branch support in data analysis. Furthermore, it confirmed that sections *Crumenata, Bolbodium, Aporum* and *Strongyle* formed a well-supported monophyletic group. That it is best to recognise only one section instead of four. Based on ICBN rules, the name *Aporum* has a priority to be used for this new classification. It also suggested that each three sections *Aporum, Crumenata and Strongyle* were not monophyletic, whereas section *Bolbidium* was monophyletic with strong support is around 99%. Furthermore, the results showed the unidentified *Dendrobium* excluding *D.* sp6 were nested with species of two sections *Aporum* and *Strongyle*; therefore they may be belonged to these two sections.

The species *D.* sp2 was close to *D. singaporense* (*Strongyle*) with bootstrap percentage of more than 50%. The two species *D.* sp4 and *D.* sp5 were close together with strong support (BP of more than 90%). Also, based on the results *D.* sp3 was related to the section *Aporum.* This species was close to *Dendrobium grande* but with not strong bootstrap. Although *D.* sp6 was similar to section Crumenata species, according to the morphological characters, but the result showed that this specie was close to the section *Dendrobium* based on Molecular evidence.

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