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PHYLOGENETIC ANALYSIS AMONG FOUR SECTIONS OF GENUS DENDROBIUM SW. (ORCHIDACEAE) IN PENINSULAR MALAYSIA USING RBCL SEQUENCE DATA

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Abstract: Phylogenetic analysis using chloroplast DNA, the ribulose-bisphosphate carboxylase gene (*rbc*L), was conducted to examine relationship among four sections of the genus *Dendrobium* (Orchidaceae): *Aporum, Crumenata, Strongyle,* and *Bolbidium* in Peninsular Malaysia. Classifications based on morphological characters have not been able to clearly divide these four sections, therefore deeper and detailed analyses are required to ascertain their status. In this study, the phylogenetic relationships among species of the four sections were investigated to clarify their relations either to lump them into one section or reduce them into two.

Keyword: Dendrobium, Phylogeny, rbcL, Orchidaceae.

INTRODUCTION

The genus *Dendrobium* Sw. is one of the largest genera in Orchidaceae with 800-1400 species in the world[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 on 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 is 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 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[4]. Some species like *D. bifarium* Lindl., *D. planibulbe*Lindl., & *D. purpureum* (Nichols) Dockr. are used in Malesia as medicinal herbs to treat skin disorders[6]. They have floriferous flower sprays that come in a wide variety of colours, sizes and shapes, available throughout the year and the blooms last several weeks to months[7]. More than 8000 novel *Dendrobium* hybrids and cultivars have been bred in horticulture through inter specific hybridization for novel flower morphological characteristics since 18th century[6].

In scientific classification of Orchidaceae family, *Dendrobium* belongs to the Subfamily Epidendroideae, Tribe: Epidendreae, and Sub tribe: Dendrobiinae[8]. The new classification system proposed by Chase *et al*., (2003) was based on DNA studies, which divided Orchidaceae into 5 subfamilies, 21 tribes, and 43 sub tribes[9]. *Dendrobiums* are placed in the sub family Epidendroideae, tribe Epidendreae, sub tribe Dendrobiinae. The sub-tribe Dendrobiinae Lindl. comprises a diverse group of epiphytic, lithophytic and terrestrial herbs classified into six genera[8]. Species of this sub tribe occurred mainly throughout the paleotropical regions with extensions to temperate Asia[3], the Philippines, the Malay Archipelago, New Guinea, Australia, New Caledonia, Southwest pacific islands, and New Zealand[10]. In Peninsular Malaysia, *Dendrobium* is the second largest genus of the Orchidaceae whereby it has 16 sections and 86 species[3]. A total of 28 sections of *Dendrobium* has been recorded from Malesia by the National Herbarium of Netherland (Source: http:// www.nationaal herbarium.nl/pubs/orchid web/genera/*Dendrobium*).

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Appendix A: Characteristics of four *Dendrobium* sections

Sections	Characteristics
Aporum (Blume) Lindl.	This section has about 45 species taking place from India to New Guinea with the centre of distribution being South-East Asia and the Indonesia Islands. Myanmar includes about 25 species and Borneo has 24. They are often small epiphytes of lowlands but some of them are found at altitudes up to 1600 m. They are small plants that initially are erect, then pendulous, with short to moderately long leafy stems with flattened, usually sharp-pointed, fleshy leaves in 2 ranks. The leaves are usually closely spaced or overlapping at their bases. The flowers are borne laterally, usually singly from a cluster of chaffy bracts. They are small and last a few days. Some species have an elongated terminal extension of the stem that lacks leaves and bears the flowers (Lavark *et al.*, 2000).
Crumenata Pfitzer. Synonyme: *Rhopalanthe* Schltr.	The species of this section sometimes occurred in *Aporum*. This section has been seen from Myanmar through Indonesia and New Guinea to Samoa. There are 45 species centring in Borneo with 25 species. They are found in low land areas below 500 m often growing in coastal situations, but some are at higher altitudes. The stems are long and slender, but with the basal few nodes swollen, this characteristic can help to it to be separated from *Aporum*. The leaves are fleshy and overlapping. The flowers are produced along the stems, usually singly, and are short – lived. The lip is 3-lobed (Lavark *et al.*, 2000).
Stongyle Lindl.	There are about 20 species in this section that occurred from Myanmar to New Guinea. Also the centre of this section could be defined as the South-East-Asian mainland and the Indonesian islands. These have been found in the low lands below 500 m. This section is closely related to *Aporum* and *Crumenata*, actually it can be a link between the two sections, some taxonomists prefer to absorb this section in *Aporum*, but most kept them separate. The plants grow in small clumps. The stems are erect or pendulous, leafy throughout their length. The leaves are fleshy, not overlapping at the base; often terete, 1 or 2 species have flattened leaves. When the flowers are borne, usually there is a leafless terminal part to the stem. The flowers are singly, or rarely 2 at the time, they are small and not long-lasting (Lavark *et al.*, 2000).
Bolbidium Lindl.	*Bolbidium* is small section with 6 or 7 species that have been seen from India to Borneo. There is one doubtful record from New Guinea. Peninsular Malaysia with 5 species is the centre of this section. This section is related to section Dendrobium. They are epiphytes of low to moderate altitudes in areas with seasonal climate. The pseudobulbs are small and crowded close together. There are 2 leaves without leaf sheaths, opposite each other at the apex. The flowers are produced singly from a group of bracts between the leaves. The lip is entire and there is a long mentum (Lavark *et al.*, 2000).

In this study, four sections of this genus (*Aporum, Crumenata, Strongyle,* and *Bolbidium*) were studied. The morphological characteristics for each section were shown in Appendix A. According to traditional classification, these four sections occurred in four clades separately. Therefore, analysis using more robust characters than morphological characters is needed to better classify them as a single or two sections, even though there are considerable differences among the species, especially in the vegetative characters.

Traditionally, comparative vegetative anatomy and plant systematics were two general strategies to assess the relationships among the taxa in *Dendrobium*[11]. 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)[12]. Chloroplast DNA has been the most extensively used source of data in plant phylogenetic analysis[13, 14]. In 2010, Asahina *et al.* worked on phylogenetic analysis of medicinal *Dendrobium* species by using *matK* and *rbcL*. In plants, *rbc*L gene is the most common gene that has been used for molecular phylogenetic analysis[15]. This gene can act as a code for the large subunit of ribulose 1, 5 bisphosphate carboxylase / oxygenase (Rubisco). This gene has been used to examine phylogenetic relationships since it exists in a single copy per genome, is large enough to provide sufficient number of characters for phylogenetic purposes, has few insertions and deletions that are known, and is appropriate for phylogenetic studies especially at higher levels[14].

The main objectives of the present study are to determine the phylogenetic relationship among the four sections of genus *Dendrobium* (*Aporum, Crumenata, Strongyle,* and *Bolbidium*) based on cpDNAin Peninsular Malaysia and to compare the traditional classification with molecular classification.

MATERIALS AND METHODS

Sample Collection:

In this study, novel *rbc*L gene sequences were determined for eight species of *Dendrobium*, including two out group taxa (*Bulbophyllum macranthum and Bulbophyllum inunctum*). *Dendrobium* species of the four sections (Aporum, Crumenata, Strongyle and Bolbidium) and Bulbophyllum *species* were collected from areas in Peninsular Malaysia. In addition, sequences of two species of genus *Dendrobium* (*D. heterocarpum* and *D. nobile*) from section *Dendrobium* retrieved from NCBI database were used. For data analysis, the list of samples is summarised in Table 1.

Genomic DNA extraction, amplification, and sequencing:

Genomic DNA was extracted from leaf samples using the cetyltrimethylammonium bromide (CTAB) method as described previously by Wang *et al.* (2004) with minor modifications[16]. The concentration of genomic DNA samples was determined by verification on 0.8% (w/v) agarose gel. The primers set used for amplification of *rbc*L gene were as follows: *rbc*La_f

(5'ATGTCACCACAAACAGAGACTAAAGC3') and *rbc*La_rev (5'- GTAAAATCAAGTCCACCRCG -3'). The PCR reaction mixtures contained approximately 5 ng of DNA template, 2.5 µL of 10× reaction buffer, 1µL dNTPs (each 2.5mM), 1.0U Taq polymerase, and 1 µL of each oligonucleotide primer (each at 10 µM concentration) in a final volume of 25 µL. The chloroplast *rbc*L coding region was amplified with the following thermocycling conditions: an initial denaturation at 95°C for 4 min;[4 cycles : 30 sec denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C then followed by [29 cycles: 30 sec denaturation at 94°C, 1 min annealing at 54°C and 1 min extension at 72°C] and 5 min final extension at 72°C.The PCR products were separated on 1% (w/v) agarose gel and purified by Wizard_ SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR products were sent for sequencing to First BASE Laboratories Sdn. Bhd., Malaysia. Sequencing was carried out by ABI Big dye version 3.1 (USA) and 3730xl DNA Analyzer (USA) (Applied Biosystems) using pGEM as control and applying Biosystems Sequencing Analysis software v5.2.0 for analyzing data from the machine. All DNA sequences produced were submitted to NCBI GenBank and their accession numbers were as listed in Table 1.

Table.1: Taxa used in cpDNA study of four sections of genus *Dendrobium*

Section	Taxon	Location	Voucher	Accession No
Aporum	*D. quadrilobatum*	Kuala Kari, Kelantan	RG 2970	KC618534
Aporum	*D. rosellum*	UPM, Green house, No 5 5number5	D001	KC618533
Crumenata	*D. crumenatum*	Genting Highlands	D008/M.M.1	KC660968
Crumenata	*D. truncatum*	Cameron Highlands	RG 2625	KC660969
Strongyle	*D. kentrophyllum*	Fraser's Hill	FAN.FH.162	KC660974
Strongyle	*D. singaporensis*	Cameron Highlands	RG 2635	KC660975
Bolbidium	*D. pachyphyllum*	Fraser's Hill	FAN.FH.392	KC660979
Bolbidium	*D. hymenantum*	Cameron Highlands	RG 2154	KC660978
Dendrobium	*D.heterocarpum*	Sequence from NCBI	SBB-1008	JF713182.1
Dendrobium	*D. nobile*	Sequence from NCBI	SBB-0541	HM055129.1
Sestochilus	*B. macranthum*	Cameron Highlands number5	FAN.FH.462	KC618531
Sestochilus	*B. inunctum*	Gunung Jerai	B002/ SH.K-109	KC618532

DNA sequence data analysis:

All sequences obtained were assembled for both forward and reverse sequences to produce contig file using BioEdit ver.7.0.2[17]. Multiple alignments of all of the sequences obtained in this study and from NCBI were performed using CLUSTAL X[18]. Data for each gene region were aligned manually using PAUP* 4.0B10[19]. Two methods were used in reconstructing the phylogenetic tree: Distance-based method (UPGMA and Neighbor-Joining) and Characters-based (Maximum Parsimony, Maximum Likelihood and Bayesian analysis). The Parsimony and Likelihood analyses were run separately for *rbc*L region using PAUP* 4.0B10[19]. The different bootstrap replicate during analysis can caused different bootstrap value. For maximum parsimony, 1000 bootstrap replicate were used but for maximum likelihood Levels of support were estimated with 500 bootstrap replicates. All trees produced from the Parsimony and Likelihood was visualized using Tree View ver1.6.6[20].

RESULTS

The parsimonious trees were determined by a heuristic search algorithm with 1000 replicates of random taxon entry, TBR branch swapping. The parsimony analysis resulted in 3480 equally parsimonious trees as shown in Figure 1. [Tree length= 247, consistency index (CI) =0.89, retention index (RI) =0.67, homoplasy index (HI) = 0.1093]. Branches corresponding to partitions reproduced in less than 50% trees are collapsed.

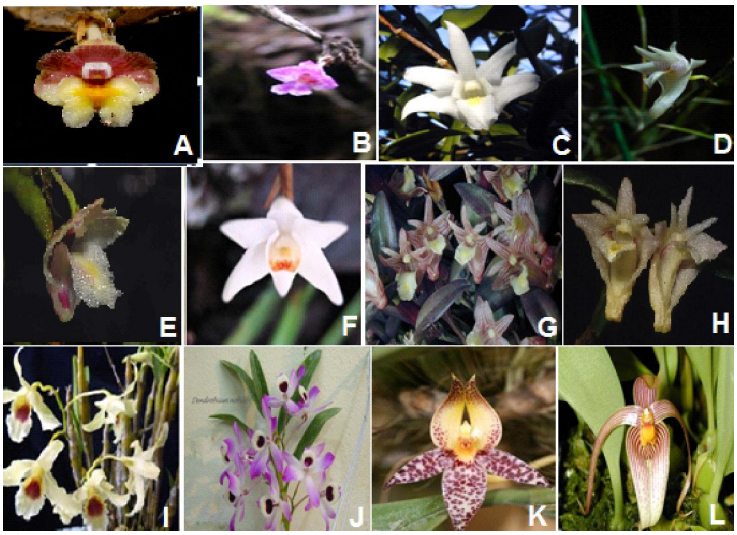

Figure.1: *Dendrobium* species and 2 species of genus *Bulbophyllum*. A. *D. quadrilobatum*; B. *D. rosellum*; C. *D. crumenatum*; D. *D. truncatum*; E. *D. kentrophyllum*; F. *D. singaporensis*; G. *D. pachyphyllum*; H. *D. hymenanthum;* I. *D. heterocarpum*; J. *D. nobile*; K. *B. macranthum*; L. *B. inunctum* (A,C,D,E,G,H,J,K,L Source: http://www.orchidspecies.com/indexde.htm)

In the MP tree, the results showed that the tree formed one main clade that included *Dendrobium* species whereas the other two clades consisted of two species of genus *Bulbophyllum*as out group. Based on MP tree results, these two species were not monophyletic and occurred in two separate clades.

The main clade was divided into two sub-clades (I-II). Clade I included the four sections (*Aporum, Crumenata, Strongyle* and *Bolbidium*) (BP83) whereas clade II consisted of section *Dendrobium* (BP70) that is monophyletic. Sub-clade I consisted of three clades (III-V). According to the results, two sections *Aporum* and *Strongyle* were nested together (BP61) and formed clade III. On the other hands, each of the other two sections (*Crumenata* and *Bolbidium*) formed a separate clade by itself. Based on the MP tree, two sections *Crumenata* and *Bolbidium* were monophyletic. In contrast, two sections *Aporum* and *Strongyle* were polyphyletic. However, the four sections (*Aporum, Crumenata, Strongyle* and *Bolbidium*) formed a monophyletic group compared to section *Dendrobium* and the outgroup (Fig.2).

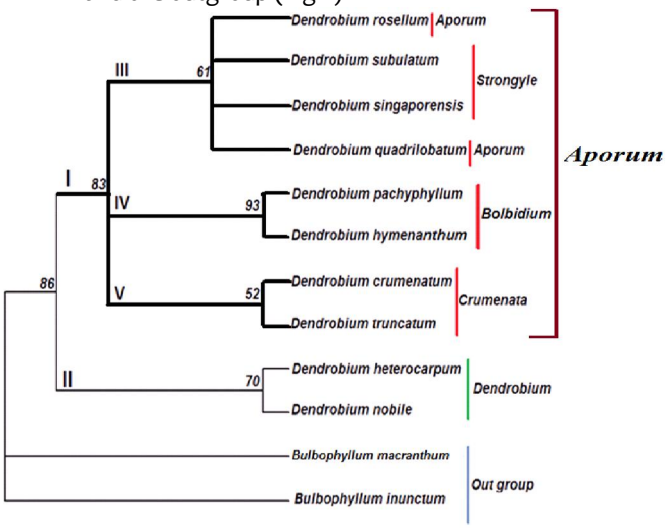

Figure.2: Strict consensus tree resulted from 3480 most parsimonious trees is shown for *rbc*L region. Bootstrap percentage > 50 are indicated above the nodes. Section names are shown.

For maximum likelihood (ML) analysis, Model test 3.7 was used to determine the optimal model of nucleotide evolution[21]. The F81+I substitution model [Lset Base= (0.3079 0.2237 0.2117) Nst=1 Rates=equal Pinvar=0] was selected using Model test. The ML method was then performed to find the optimal ML tree with a heuristic search as implemented in PAUP* 4.0b10[19], with TBR branch-swapping and 10 random sequence additions. The ML tree (Fig. 3) was the same to the MP, with the following exceptions: (1) section *Crumenata* was not monophyletic, the two species of this section formed two separate clades, (2) two species of *Bulbophyllum* as out group were monophyletic with strong support (BP100), and (3) different bootstraps support for almost all branches compared to the MP tree.

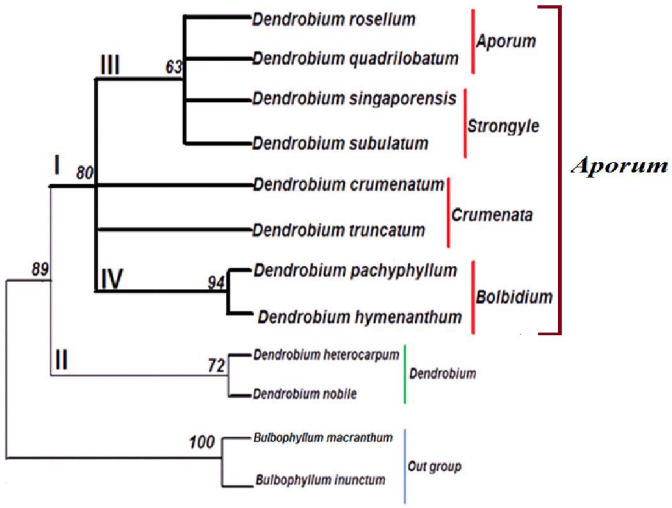

Figure.3: Bootstrap 50% majority-rule consensustreeis resulting from maximum likelihood analysis of the *rbc*L gene dataset. Numbers at nodes represent bootstrap (500 replicates). Section names have been shown.

DISCUSSION

The *rbc*L region was one of the markers that has been widely used for phylogenetic studies. It has been sequenced in over 5000 plant species[22]. This gene is located in the large single copy region of the chloroplast genome and encodes the large subunit of ribulose 1, 5-biphosphate carboxylase/oxygenase (RUBISCO; a critical photosynthetic enzyme[23]). To infer the relationships at the family level and above, *rbc*L is still the first choice, but there is a lower limit of its applicability at the genus or species level. This gene seems suitable for phylogenetic studies in Orchidaceae. It was used by Cameron in 1999 for phylogenetic analysis of the Orchidaceae. It was powerful in assessing monophyly of clades within the family, but failed to provide strong support for the interrelationships of the subfamilies[24]. In 1996, Yukawa and his co-workers showed that the character support for the *rbc*L tree is weak as indicated by the short branch length. The results showed that the four sections (*Aporum, Crumenata, Strongyle* and *Bolbidium*) formed a monophyletic group compared to the other section of genus *Dendrobium* (section *Dendrobium*) and genus *Bulbophyllum* as an out group. The results confirmed that two sections, *Aporum* and *Strongyle*, were polyphyletic, whereas the other two sections, *Crumenata* and *Bolbidium*, were monophyletic.

Traditional morphological assessment and histological method cannot help in species identification, as some species from different areas are similar in their morphologies and anatomical characteristics[25]. In addition, it is difficult to distinguish *Dendrobium* species due to the lack of research conducted on components concerning the nature of *Dendrobium*[26]. The extensive advances in molecular

techniques utilizing genetic analysis in the past decade have causedan increase in the information concerning orchid genetic diversity. Molecular techniques have been used in studying DNA sequence variation within and among orchid species and cultivars[4].

Based on the traditional classification; the sections *Aporum, Crumenata, Strongyle,* and *Bolbidium* have been considered as four separate sections. For the first time, the similarities among species of two sections, *Aporum* and *Strongyle*, had been noted by Schlechter in 1912[10]. Seidenfaden and Wood (1992) maintained that section *Strongyle* is thelink between sections *Crumenata* (synonym: *Rhophalanthe*) and *Aporum*[3]. In addition, they demonstrated that two sections, *Aporum* and *Crumenata*, are the same in some morphological characters, so some species of section *Crumenata* may belong to section *Aporum*[3]. According to morphological characters, section *Aporum* is closely related to the sections *Crumenata*, *Oxystophyllum* and *Strongyle*. *Aporum* lacks the swollen basal nodes of *Crumenata*, and also the small projection under the apex of the lip, which is present in *Oxytophyllum*, while the leaves of *Strongyle* are terete compared to the flattened leaves in *Aporum*. In addition, *Strongyle* is closely related to *Aporum* and *Crumenata*, some taxonomists prefer to absorb section *Strongyle* into *Aporum*, but most kept them separate (Lavarack *et al*., 2000). The study done by Yukawa (1996) on subtribe Dendrobiinae and genus *Dendrobium* using molecular markers showed that two sections, *Aporum* and *Crumenata* (*Rhophalanthe*), formed the same clade[27]. Other study by Yukawa on molecular phylogeny of *Dendrobium* using *mat*K and ITS (Internal Transcribed Spacer region) revealed that three sections, Aporum, *Crumenata,* and *Bolbidium*, were close together with bootstrap value of 100% and formed a well-supported monophyletic group[28]. In 2011, Schuiteman using ITS showed three sections, *Aporum*, *Crumenata*, and *Bolbidium*, occurred in one clade forming a monophyletic group[2].

In this study, the most significant result based on phylogenetic relationships of the four sections of genus *Dendrobium* is that sections *Crumenata, Bolbodium, Aporum* and *Strongyle* formed a well-supported monophyletic group. It also suggested that these four sections were not all monophyletic. Based on MP and ML analyses, sections *Aporum* and *Strongyle* were nested together with bootstrap value more than 80%, so it confirmed that section *Strongyle* can be absorbed into section *Aporum*.

CONCLUSION

This study highlighted that the four sections of genus *Dendrobium* Sw., *Aporum, Crumenata, Strongyle* and *Bolbidium* are probably best considered as a single section instead of four. Based on ICBN rules, the name *Aporum* has the priority to be used for this new classification. To obtain better or more accurate results, we are currently analyzing more samples in terms of species and performing detailed analysis using other more robust molecular markers.

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