INTERNATIONAL JOURNAL OF BIOASSAYS
ISSN: 2278-778X
CODEN: IJBNHY
ORIGINAL RESEARCH ARTICLE
OPEN ACCESS

The comparison between nuclear ribosomal DNA and chloroplast DNA in molecular systematic study of four sections of genus *Dendrobium* sw. (Orchidaceae)

Maryam Moudi[1]* and Rusea Go[2,3]
[1]Department of Biology, Faculty of Science, University of Birjand, Birjand, South Khorasan, Iran.
[2]Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.
[3]Institute of Tropical Forestry & Forest Products, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul Ehsan, Malaysia.

Received for publication: December 27, 2015; **Accepted:** January 14, 2016

Abstract: Phylogenetic study of the four sections (*Aporum, Crumenata, Strongyle, and Bolbidium*) of genus *Dendrobium* (family Orchidaceae) was conducted using molecular data. Classifications based on morphological characters have not being able to clearly divide these four sections neither do they supported their monophyly origin. Therefore, deeper and detailed analysis especially using molecular data is required to ascertain their status. Molecular evidences were used to clarify their relations either to lump them into one section or reduce them into two. The study has been carried out for the 34 species of *Dendrobium* using Maximum Parsimony (MP). Three nucleotide sequences data sets from two distinct genomes chloroplast DNA genes (*rbc*L and *mat*K) and nuclear ribosomal DNA (ITS) were used to construct cladograms. The results that obtained from the Internal Transcribed Spacer (ITS) gene showed that the nuclear genes are reliable marker for the phylogenetic study of *Dendrobium* compared to chloroplast DNA with low resolution level among sections.

Key words: *Dendrobium*, phylogeny, ITS, *rbc*L, *mat*K

Introduction

The widespread development of molecular techniques for genetic analysis in the past decade has led to the increase of the knowledge of orchid genetic diversity. The common molecular data used in plant systematics comes from two sources: chloroplast DNA (cpDNA) and nuclear ribosomal DNA[1]. Chloroplast DNA has been the most extensively used source of data in the plant phylogenetic analysis. The use of cpDNA has been reviewed widely[2]. Some of the chloroplast genes commonly used in phylogenetic studies are ribulose-bisphosphate carboxylase gene (*rbc*L) and MaturaseK gene (*mat*K). The other widely DNA marker used is the ribosomal DNA [e.g. Internal Transcribed Spacer (ITS)] that could be used to complement data based on plastid genes[3].

The genus *Dendrobium* Sw. is one of the largest genera in Orchidaceae which 250 and 86 species are found in Malaysia and Peninsular Malaysia respectively[4,5]. This genus was recognized by Olofswartz in 1799 for the first time, as cited in Seidenfaden and Wood (1992[4]). *Dendrobiums* are distributed in the tropical and subtropical regions in South, East and Southeast of Asia, north of Australia, New Zealand and New Guinea[6]. They are one of the most popular orchids because of their medicinal and commercial values[7]. In addition, genus *Dendrobium* is also famous due to their floriferous flower sprays, wide variety of colors, sizes and shapes, year-round accessibility, and long flowering life of several weeks to months[8]. However, it is known that many *Dendrobium* plants are morphologically similar. Thus, making their identification based on morphological characters difficult, except during flowering, when they can be easily classified based on their flower morphologies[7]. Yukawa and his co-workers (1996[9]) showed that the problems in variability and plant growth conditions caused confusion in the species identification. Many problems remain in the classification of this large genus based on morphological characters, so advanced studies using molecular methods are needed. In the traditional classification of genus *Dendrobium*, the four sections (*Aporum, Crumenata, Stongyle* and *Bolbidium)* remain separate[4].

Based on the limited studies done so far, especially in Malaysia, more detailed analyses are needed. Perhaps it is better to classify them as a single, two or three sections, even though there are considerable differences among the species, especially in the vegetative characters. For example, many species have laterally flattened leaves, some have terete leaves, and again many have 'normal' (dorso-ventrally flattened) leaves. In addition, some have stems with swollen internodes, while others have wiry stems. Many have thin-textured flowers that last only a single day, others (in section *Aporum*) have fleshier flowers that last longer for more than two or three weeks. Therefore, the main problems are difficulty in identifying species within the complex of the four sections in the genus *Dendrobium* (*Aporum, Strongyle, Crumenata* and *Bolbidium*) through morphological characters. It is important to determine whether the molecular study supports

***Corresponding Author:**
Maryam Moudi
Department of Biology,
Faculty of Science, University of Birjand,
Birjand, South Khorasan, Iran.

the morphological approach of dividing the sections or not. Previous classification using morphological characters separated these four sections into different clades. Therefore, an analysis using more robust characters than morphological data is needed. In this study, four sections of *Dendrobium* (*Aporum, Crumenata, Strongyle*, and *Bolbidium*) have been studied. Phylogenetic trees for chloroplast markers (*rbc*L and *mat*K) and nuclear ribosomal DNA (ITS) were constructed based on the phylogenetic analysis method, Maximum Parsimony (MP) using different software (PAUP*4.0 B 10, and Mega 5). In addition, some species from another section of *Dendrobium* (*Dendrobium*, *Callista* and *Latouria*) were also considered for better comparison of the relationship among the sections and two species of genus *Bulbophyllum* Thouars. was used as the outgroup. The main goals of the present study were: 1. To determine the monophylyof four sections of genus *Dendrobium* SW. 2. To evaluate which molecular markers (cpDNA, nrDNA genes) are appropriate for molecular study of *Dendrobium* species.

Materials and Methods

Plant materials: *Dendrobium* samples (23 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 six *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[10]) with minor modification. 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. For the amplification of these selected gene regions; Chloroplast (*rbc*L and *mat*K), nuclear ribosomal (ITS) regions were amplified in 20µl total reaction volume. The PCR products were separated by 1% agarose gel electrophoresis and purified by Wizard_ SV Gel and PCR Clean-Up System (Promega, USA). The purified PCR products were sent to First BASE Laboratories Sdn. Bhd., Malaysia, for sequencing. 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 the DNA sequences produced for this study were checked for stop codones and then submitted to the NCBI GenBank; their accession numbers are listed in (Table 1).

DNA sequence data analysis

DNA sequences obtained from ITS region were aligned by BioEdit ver.7.0.2.[11]. The Maximum Parsimony (MP) method was selected for the construction of phylogenetic trees. The output data were processed using PAUP* 4.0B10[12], MEGA5[13] to draw the phylogenetic trees. The ILD test (Incongruence Length Difference) or Partition homogeneity (PH) test was conducted using PAUP* 4.0B10[12] to determine whether or not the gene regions can be combined. To find the most parsimonious trees, maximum parsimony (MP) analyses were run using a heuristic strategy of branch-swapping by tree bisection-reconnection (TBR) step wise addition with 1000 randomly-addition replicates and holding 10 trees in each step, levels of support were estimated with 1000 bootstrap replicate (BP), using the TBR algorithm of branch swapping for 10 random-addition replicates per bootstrap replicate. Parsimony analyses were run separately for each gene region and combination of gene regions.

Table 1: List of studied taxa

Species	Section	Location	Gene bank accession number		
			*rbc*L	*mat*K	ITS
Dendrobium aloifolium	*Aporum*	UPM Green house, No.5	KC660972	KC682481	KC507775
Dendrobium grande	*Aporum*	Labuk Tapah, Selai, PM	KC618535	KC682482	KC507779
Dendrobium leonis	*Aporum*	UPM Green house, No.5	KC559780	KC682484	KC507774
Dendrobium quadrilobatum	*Aporum*	Kuala Krai, Kelantan, PM	KC618534	KC682493	KC507778
Dendrobium rosellum	*Aporum*	UPM Green house, No.5	KC618533	KC663439	KC507777
Dendrobium terminale	*Aporum*	Sungai Bertedung, Endau Rompin, PM	KC660970	-	KC507776
Dendrobium clavator	*Crumenata*	Sungai Bertedung, Endau Rompin, PM	KC660971	KC682490	KC507762
Dendrobium crumenatum	*Crumenata*	Genting Highlands, PM	KC660968	KC682479	KC507780
Dendrobium setifolium	*Crumenata*	Sungai Bertedung, Endau Rompin, PM	KC660973	KC663438	KC507763
Dendrobium truncatum	*Crumenata*	Cameron Highlands, PM	KC660969	KC682483	KC507761
Dendrobium acerosum	*Strongyle*	Biology Department Herbarium, UPM	KC660976	-	-
Dendrobium keentrophyllum	*Strongyle*	Fraser's Hill, PM	KC660974	KC682486	KC507764

Dendrobium singaporense	*Strongyle*	Cameron Highlands, PM	KC660975	KC682488	KC507765
Dendrobium subulatum	*Strongyle*	Gunung Nuang, PM	KC559781	KC682480	KC507766
Dendrobium pachyphyllum	*Bolbidium*	Fraser's Hill, PM	KC660979	KC682485	KC507769
Dendrobium hymenanthum	*Bolbidium*	Cameron Highlands, PM	KC660978	KC682487	KC507768
Dendrobium striatellum	*Bolbidium*	Kuala Krai, Kelantan, PM	KC660977	KC682489	KC507767
Dendrobium sp1	?	Fraser's Hill, PM	KC660980	-	-
Dendrobium sp2	?	G. Kelapak buruk, PM	KC660981	-	KC507770
Dendrobium sp3	?	UPM Green house, No.5	KC660982	KC682491	KC507771
Dendrobium sp4	?	UPM Green house, No.5	KC660983	-	KC522821
Dendrobium sp5	?	UPM Green house, No.5	KC660984	-	KC522822
Dendrobium sp6	?	UPM Green house, No.5	KC660985	-	KC522823
Dendrobium heterocarpum	*Dendrobium*	NCBI	JF713182.1	JF713415.1	JF713105.1
Dendrobium nobile	*Dendrobium*	NCBI	HM055129.1	HM055320.1	JF713365.1
Dendrobium crepidatum	*Dendrobium*	NCBI	**JF713162.1**	HM055232.1	HM054624.1
Dendrobium fimbriatum	*Dendrobium*	NCBI	JF713178.1	JF713410.1	HM054636.1
Dendrobium gibsonii	*Dendrobium*	NCBI	FJ216637.1	-	HQ114256.1
Dendrobium farmeri	*Callista*	NCBI	**HM055099.1**	HM055240.1	HM054629.1
Dendrobium thyrsiflorum	*Callista*	NCBI	HM055145.1	HM055360.1	HM054759.1
Dendrobium macrophyllum	*Lautoria*	NCBI	-	-	AY239979.1
Bulbophyllum inunctum	*Sestochilus*	Cameron Highlands, PM	KC618532	KC682494	KC507773
Bulbophyllum macranthum	*Sestochilus*	Gunung Jerai, PM	KC618531	KC682492	KC507772

Results

Phylogenetic Analysis Based on *rbc*L Region: It was obvious that the amplifications of *rbc*L regions for some species were not successful. This problem was mainly related to the quality of DNA, especially for the herbarium specimen. The *rbc*L gene sequences were obtained from 30 *Dendrobium* species and two species of genus *Bulbophyllum, B. inunctum* and *B. macranthum* as outgroup. The aligned sequences consisted of 533 nucleotide characters; 374 characters were conserved among all taxa, 129 characters were variable, and 32 were parsimony informative. The means base composition was found to be fairly uniform among all taxa analyzed (30% A, 22% C, 21.2% G and 26.8% T). The estimated Transition/ Transversion bias (*R*) was 0.57. The nucleotide frequencies were A = 32.38%, T/U = 22.75%, C = 23.07%, and G = 21.80%.

The evolutionary history was inferred using the Maximum Parsimony (MP) method. In MP, the consensus tree which was inferred from 227 most parsimonious trees is shown (Figure 1). Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The tree length = 149, consistency index (CI) = 0.65, retention index (RI) = 0.82 and homoplasy index (HI) = 0.098. The nucleotide sequences of *the rbcL region* in these *Dendrobium* sections comprised mostly of conserved characters, therefore differentiation among sections was not successful.

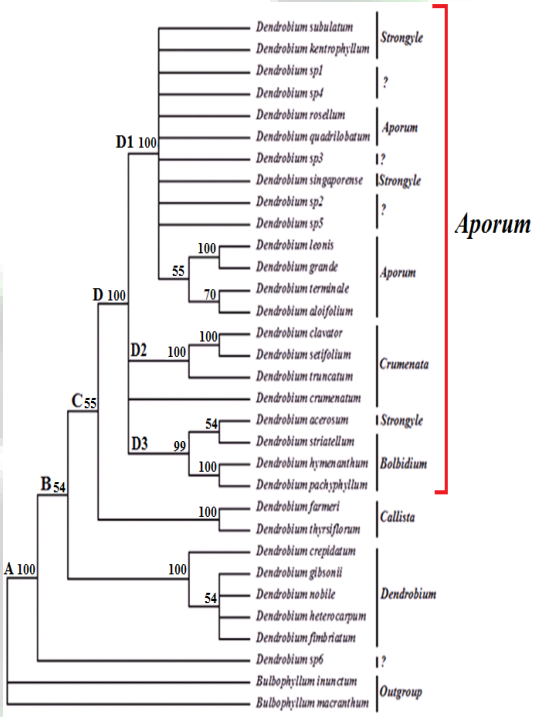

Figure 1: The consensus tree inferred from most parsimonious trees is shown for *rbc*L region. Bootstrap percentage ≥ 50 are indicated above the nodes. A, B, C, D, D (1-3) denotes the label of clades. D (1-3) indicate the four sections *Aporum, Crumenata, Strongyle* and *Bolbidium* that formed a monophyletic group and can be considered as one section named *Aporum*.

Phylogenetic Analysis based on *mat*K Region

The *mat*K gene sequences were achieved from 23 *Dendrobium* species and two species of genus *Bulbophyllum; B. inunctum* and *B. macranthum* as outgroup. The aligned sequences consisted of 727 nucleotide characters in which 610 characters were conserved, 109 were variable and 46 were parsimony informative among all taxa. The means base composition was found to be fairly uniform among all taxa analyzed (30.2% A, 16.8% C, 14.9% G, and 38.2% T). The estimated Transition/ Transversion bias (*R*) was 0.44. The nucleotide frequencies were A = 31.55%, T/U = 35.29%, C

= 17.24%, and G = 15.92%. To infer the evolutionary history, maximum parsimony (MP) was used. The strict consensus tree (Figure 2) was constructed from 121 most parsimonious trees as shown. The tree length = 122, consistency index (CI) = 0.85, retention index (RI) = 0.91 and homoplasy index (HI) = 0.087.

Molecular data analysis of matK region showed the close relatedness of the four sections (Aporum, Crumenata, Strongyle and Bolbidium) compared to the other two sections of Dendrobium and Callista with bootstrap value more than 80%. Clade A in MP was divided in to three sub-clades (A1-A3). The first sub-clade A1 consisted of taxa assigned to section Aporum (BP50) that is monophyletic. The second sub-clade A2, involved two species of section Strongyle with strong support (BP100). However, section Strongyle and the D. sp3 were polyphyletic. The results showed that the D. sp3 could belong to one of the four sections (Aporum, Crumenata, Strongyle and Bolbidium); although the morphological characters especially its leaf shape was close to section Aporum. The third sub-clade A3 included two sections Crumenata and Bolbidium with a weak bootstrap percentage (BP57) meaning that both of them were polyphyletic. The other two main clades, clade B consisted of section Dendrobium (BP82), and clade C included section Callista (BP71) meaning that both of them were monophyletic.

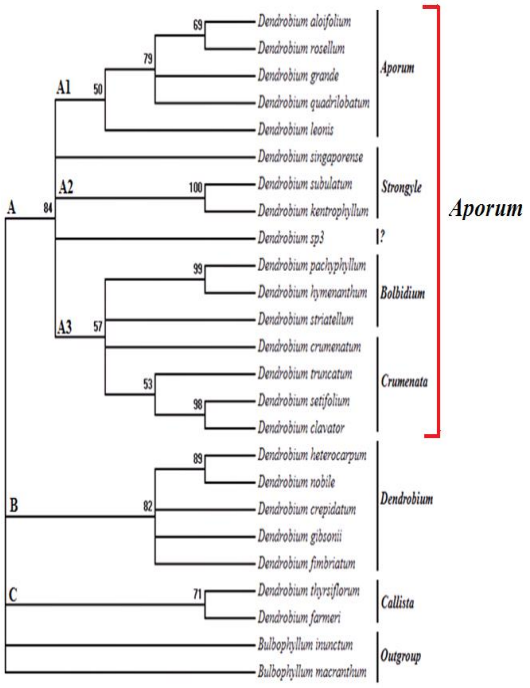

Figure 2: Consensus tree inferred from most parsimonious trees is shown for *mat*K region. Bootstrap percentage ≥ 50 are indicated above the nodes. A, B and C show the different sections used in this study. A (1-3) denote the four sections *Aporum, Crumenata, Strongyle* and *Bolbidium* that form a monophyletic group and can be considered as one section named *Aporum*.

Analysis of Chloroplast Sequence Data

Combined data analysis provided better insight into section separation rather than a separate analysis of the sequence data. Sequence analysis of *mat*K was much better. Polyphyletic clades were still being observed. Therefore, analysis of a combination of data is the best way to solve this kind of problems. However, polyphyletic clades were still observed in this analysis, the result is better than analyses with each plastid marker individually. To find the congruency among trees from each region, the test described as the Incongruence-Length Differences (ILD) test by Farris *et al.,* (1995[14]) was implemented. Each partition homogeneity test was performed on 100 replicates using PAUP*4.0 B 10[12]. The PH test between chloroplast dataset, gave a p-value of 0.01 which showed significant heterogeneity. However, given the oversensitivity of this statistical test seen in other studies; it seems likely that the partition homogeneity test would be overly sensitive comparing nuclear and chloroplast datasets. The much better resolved tree of the combined analysis compared to the separate analysis of the datasets supports our assumption that the partition homogeneity test sometimes reveals unreliable results especially for large datasets. It is obvious that these incongruencies are due to technical issues such as phylogenetic signal and homoplasy[15]. Therefore, the datasets were combined to give more robust phylogeny analysis.

Two gene regions (*rbc*L and *mat*K) were treated as separate partition. The combined chloroplast matrix included 20 in-group species and 2 outgroup species. The combined alignment of all chloroplast markers consisted of 1809 positions, where 1426 were conserved characters, 331 were variable and 113 were parsimony informative characters. Means base composition was found to be fairly uniform among all taxa analyzed (32.5% A, 18.0% C, 16.4% G, and 33.1% T). The estimated Transition/Transversion bias (*R*) was 0.52. The nucleotide frequencies are A = 32.91%, T/U = 31.31%, C = 18.79%, and G = 16.99%. The evolutionary history was inferred using the Maximum Parsimony (MP. Unweighted parsimony analysis resulted in 58 most parsimonious trees [tree length = 329 consistency index (CI) = 0.74, retention index (RI) = 0.82 and homoplasy index (HI) = 0.12]; the strict consensus tree was shown (Figure 3). The results showed that the tree had two main clades (A-B) (BP98) with outgroup clades. The clade A consisted of five sections of *Dendrobium*, whereas section *Callista* formed clade B by itself. Clade A included two sub-clades (I-II). Sub-clade I consisted of four sections (*Aporum, Crumenata, Strongyle* and *Bolbidium*) with strong support (BP 99). The results showed that these four sections formed a monophyletic group compared to the other two sections *Dendrobium* and *Callista*. The sections *Aporum, Crumenata* and

Strongyle were polyphyletic, whereas section *Bolbidium* was monophyletic with strong BP.

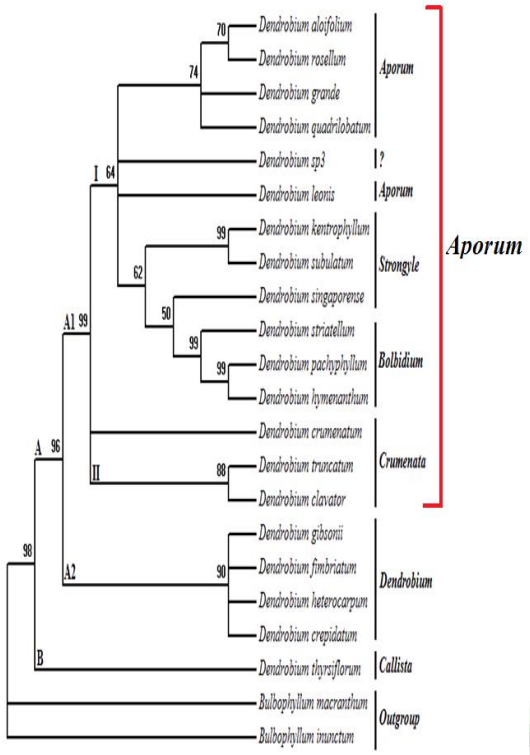

Figure 3: Consensus tree inferred from most parsimonious trees is shown for chloroplast sequence data. Bootstrap percentage ≥ 50 are indicated above the nodes. A (1-2) and B are mainclades and include the different sections of genus *Dendrobium*. A1 (I and II) denote the four sections *Aporum, Crumenata, Strongyle* and *Bolbidium* form a monophyletic group and can be considered as one section named *Aporum*.

Phylogenetic Analysis based on nrITS

Complete ITS sequences were analyzed for 29 *Dendrobium* 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 means base composition was found to be fairly uniform among all taxa analyzed (21.4%A, 30.8%C, 24.7%G, 23.2% T). The estimated Transition/ Transversion bias (*R*) was 1.08. The nucleotide frequencies were A = 28.27%, T/U = 24.22%, C = 27.35%, and G = 20.21%.

Maximum Parsimony (MP) analysis was 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]; the strict consensus tree is shown (Figure 4). The results showed that there were two main clades (A-B) with strong bootstrap value (BP99). Clade A included sections *Aporum, Crumenata, Strongyle, Bolbidium, Dendrobium* and *Callista*. In contrast, section *Lautoria* formed by itself the other main clade, clade B. Clade A was divided into three sub-clades (A1-A3) (BP99). A1 formed two sub-clades (A1.1 and A1.2) with bootstrap percentage more than 60% that involved five sections of *Dendrobium* (*Aporum, Crumenata, Strongyle, Bolbidium* and *Callista*). D. sp6 formed sub-clade A2 by itself; therefore, it showed that this species could not be close to the four sections (*Aporum, Crumenata, Strongyle* and *Bolbidium*). The sub-clade A3 consisted of section *Dendrobium* with weak support (BP53). Clade A1 was divided in to two sub-clades with strong support (BP of more than 90%). The first sub-clade consisted of two clades I and II. Clade I included two sections *Aporum* and *Strongyle* (BP of more than 50%) whereas clade II included sections *Crumenata* and *Bolbidium*. Section *Bolbidium* was monophyletic with bootstrap value of more than 90%. Overall, molecular Data analysis based on ITS indicated that the four sections (*Aporum*, *Crumenata, Strongyle, and Bolbidium*) formed a monophyletic group compared to the other three sections (*Dendrobium, Callista* and *Lautoria*). The results showed that sections *Aporum* and *Strongyle* were nested in one clade, whereas sections *Crumenata* and *Bolbidium* were close together.

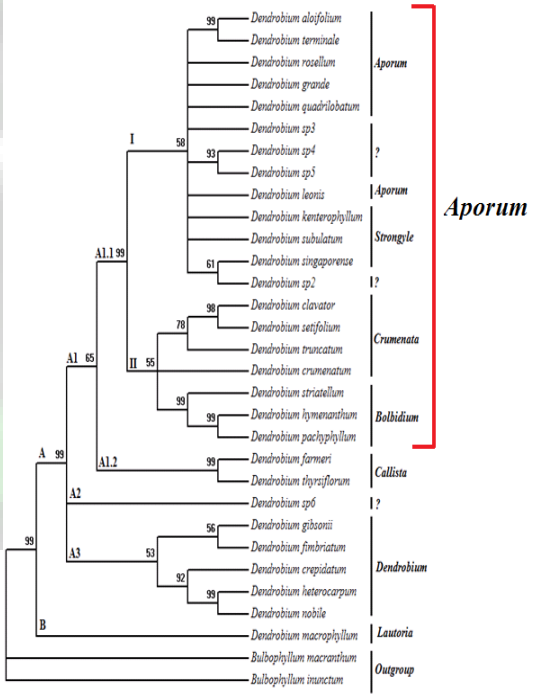

Figure 4: Consensus tree inferred from most parsimonious trees is shown for nrITS region. Bootstrap percentage ≥ 50 are indicated above the nodes. A (1-3) and B are mainclades and include the different sections of genus *Dendrobium*. A1.1 (I and II) denote the four sections *Aporum, Crumenata, Strongyle* and *Bolbidium* form a monophyletic group and can be considered as one section named *Aporum*

Discussion

In this study, the markers from different regions of the chloroplast and nuclear ribosomal DNA were used. One of the genes used in this study was *rbc*L. The *rbc*L region was one of the markers that have been widely used for phylogenetic studies. This gene has been sequenced in over 5000 plant species[16]. 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[17]). 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. *rbc*L was used by Cameron (1999[18]) for phylogenetic analysis of the family Orchidaceae. It was powerful in assessing monophyly of clades within the family, whereas it failed to provide strong support for the interrelationships of the subfamilies. Yukawa and his co-workers (1996[9]) showed that character support for the *rbc*L tree is weak as indicated by the short branch length. Actually, they indicated that *rbc*L sequences have limited utility to construct phylogenetic relationships in family Orchidaceae. In this study, *rbc*L region was used for phylogenetic analysis, and when used individually, it produced a tree with an internal polytomy. Therefore, the results showed that this gene is less suitable to clarify the relationships among the four sections, although this gene showed that the four sections formed a monophyletic group but it confirmed that individual section they were not monophyletic. Based on results from MP, the four sections *Aporum, Crumenata, Strongyle* and *Bolbidium* were polyphyletic.

The *mat*K region contained 6.3% PIC (Parsimony Informative Characters) in this study. This gene encodes a protein (maturase) involved in splicing type II introns from RNA transcripts[19]. This region is located in a large single-copy region of the chloroplast genome. It can be easily amplified due to the highly conserved region. Partial *mat*K sequences are able to produce a phylogenetic tree that is comparable in resolution and support to the trees obtained from *rbc*L, *atp*B and 18S[20,21]. Asahina and his co-workers (2010[7]) conducted a study concerning the identification of medicinal *Dendrobium* species by phylogenetic analyses using *mat*K and *rbc*L sequences. They demonstrated that *mat*K rather than *rbc*L offered a higher resolution and is better suited in identifying medicinal *Dendrobium* species. They confirmed that the utility of the *mat*K sequences as barcodes for the first identification process was so efficient. In this study, *mat*K contain PIC more than *rbc*L and the results showed that there was almost good separation among sections with a bootstrap value around of 50% support compared to the trees inferred from *rbc*L. The nucleotides of the *rbc*L region into *Dendrobium* sections comprised mostly of conserved characters, therefore differentiation among sections was not successful.

One of the loci that is commonly used for phylogenetic inference is ITS with a high copy number and relative range of phylogenetic utility. The Internal Transcribed Spacers (ITS) regions of nuclear ribosomal DNA (nrDNA) are very heterogeneous both in size and nucleotide sequences among various angiosperms22. The use of nrDNA regions has become popular and is used in different level of taxa, especially the low level taxa, such as infrageneric species23,24,25,26. 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 Orchidaceae27,28,29,30. Clements (200331) conducted a phylogenetic study on sub-tribe Dendrobiinae using ITS. The overall results achieved in these ITS sequence analyses coupled with data on the morphology of the study species, provides a basis for a clearer understanding of the phylogeny of a major part of the Dendrobiinae. At the broadest level, the present ITS results link strongly with those produced from analyses of rbcL and matK chloroplast DNA sequences and chloroplast DNA restriction sites7,32,33. In addition, Andre Schuiteman (201134) 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 (199332, 19967, 200033, 200135) using DNA markers (matK 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 by Clements22. In this study, several attempts were made to amplify ITS from a number of Dendrobium species, which resulted with some success but nevertheless unsuccessful in some for example *Dendrobium acerosum* and Dendrobium sp1. The low level of homoplasy and around 37% PIC were 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. It also suggested that three sections Aporum, Crumenata, Strongyle, were not all monophyletic, whereas section Bolbidium was monophyletic with strong support is around 99%. Based on the analyses of both separately and combined data, the most important result regarding the phylogenetic relationships of four sections Aporum, Crumenata, Strongyle and

Bolbidium, was the demonstration that although they were not all monophyletic they formed a well-supported monophyletic group. All of the four sections described by Seidenfaden and Wood, (19924) and Lavarack *et al.,* (200036), strongly supported monophyletic clade compared to the other sections of genus Dendrobium, in molecular data analysis. Actually, the results especially from nuclear data set showed that two sections Crumenata and Bolbidium were close together, although in some trees these two sections formed separate clades. In contrast, the other two sections were mostly nested together in one clade. Sections Aporum, Crumenata and Strongyle were often polyphyletic, whereas section Bolbidium was usually monophyletic with strong support (BP100).

In conclusion, phylogenetic relationships among the four sections of the genus Dendrobium were shown based on molecular markers separately and in combination using MP. In conclusion, sections *Aporum, Crumenata, Strongyle* and *Bolbidium* formed a monophyletic group. The results showed that sections *Aporum* and *Strongyle* were genetically closely related, whereas sections *Crumenata* and *Bolbidium* were nested together in one clade indicating the high genetic similarity. Therefore, based on the results it can be concluded that the four sections *Aporum, Crumenata, Strongyle* and *Bolbidium* can be considered as one section, named *Aporum* according to the ICBN rules (Figure 5). However, there is another division that can be proposed for these four sections (*Aporum, Crumenata, Strongyle* and *Bolbidium*). They can be divided into two clades, one having stems with a few swollen internodes (*Crumenata* and *Bolbidium*), the other having stems without any swollen internodes (*Aporum* and *Strongyle*).

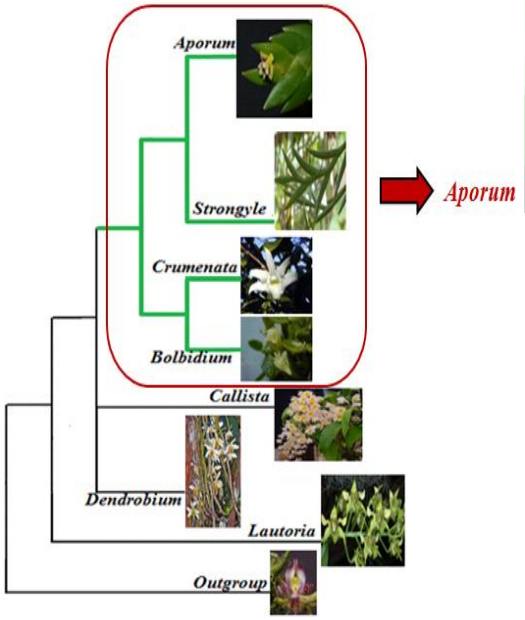

Figure 5: Sectional relationship among four sections of the genus *Dendrobium* (*Aporum, Crumenata, Strongyle* and *Bolbidium*) compared to the other sections of this genus and genus *Bulbophyllum* as outgroup from phylogenetic analysis. The four sections (*Aporum, Crumenata, Strongyle* and *Bolbidium*) Form a monophyletic group and can be considered as one section named *Aporum*.

Acknowledgement

The authors would like to appreciate Andre Schuiteman (Senior Researcher, Orchidaceae, Herbarium, Library, Art and Archives, Royal Botanic Gardens, Kew) for his helpful comments throughout this research

References

1. Small RL, Cronn RC and Wendel JF, LAS. JOHNSON REVIEW No. 2. Use of nuclear genes for phylogeny reconstruction in plants, Australian systematic Botany, 2004, 17, 145- 170.

2. Olmstead RG, Palmer JD. Chloroplast DNA systematics: a review of methods and data analysis. American Journal of Botany, 1994, 81, 205–1224.

3. Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP, Haidar N. Land plants and DNA barcods: short-term and long-term goals. Philosophical Transactions of the Royal Society B, 2005, 360, 1889-1895.

4. Seidenfaden G, Wood JJ. The Orchids of Peninsular Malaysia and Singapore. Fredensborg: Olsen and Olsen, 1992.

5. Turner IM. A catalogue of the vascular plants of Malaya. The Gardens' Bulletin Singapore, 1995, 47 (1): 559-620.

6. Wang HZ, Fenga SG, Lua JJ, Shia NN, Liub JJ. Phylogenetic study and molecular identification of 31 *Dendrobium* species using inter-simple sequence repeat (ISSR) markers. Scientia Horticulture, 2009, 122, 440-447.

7. Asahina H, Shinozaki J, Masuda K, Morimitsu Y, Satake M. Identification of medicinal *Dendrobium* species by phylogenetic analyses using *matK* and *rbcL* sequences. Journal of Natural Medicines, 2010, 64, 133-138.

8. Kuehnle AR. Chapter 20: Orchids, *Dendrobium*. In: N.O Anderson (ed.), Flower Breeding and Genetics. Springer, 2007, 539-560.

9. Yukawa T, Ohba H, Cameron, KM, Chase MW. Chloroplast DNA phylogeny of subtribe Dendrobiinae (Orchidaceae): insights from a combined analysis based on *rbcL* sequences and restriction site variation. Journal Plant Research, 1996, 109 (2), 169–176.

10. Wang HZ, Wang YD, Zhou XY, Ying QC, Zheng KL. Analysis of genetic diversity of 14 species of *Cymbidium* based on RAPDs and AFLPs. Acta Biologiae Exprimetalis Sin, 2004, 37 (6), 482–486.

11. Hall AT. Bio Edit: a user friendly biological sequence alignment editor and analysis performer windows 95/98/NT. Nucleic Acid Symposium series, Axford Journals, 1999, 41, 95-98.

12. Swofford DL. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4b10. Sinauer, Sunderland, Massachusetts, USA, 2002.

13. Tamura K, Peterson D, Peterson N. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution, Advance access: 10.1093/molbev/msr121, 2011.

14. Farris JS, Källersjö M, Kluge AG, Bult C. Constructing a significance test for incongruence. Systematic Biology, 1995, 44(4), 570-572.

15. Wendel JF, Schnabel A, Seelanan T. Bi-directional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proceeding of the Naionatl Academy Sciences of the USA, 1995, 92, 280-284.

16. Sanderson MJ, Driskell AC, Ree RH, Eulenstein O, Langley S. Obtaining maximal concatenated phylogenetic datasets from large sequence databaces. Molecular Biology and Evolution, 2003, 20(7), 1036-1042.

17. Soltis DE, Soltis PS. Molecular systematics of plants II: DNA sequencing. Boston: Kluwer Academic Publishers, 1998.

18. Cameron KM, Chase MW, Whitten WM, Korse PJ, Jarrell DC, Albert VA. A phylogenetic analysis of the Orchidaceae: Evidence from rbcL nucleotide sequences. American Journal of Botany, 1990, 86(2), 208-224.

19. Wolf KH, Morden CW, Palmer JD. Function and Evolution of a minimal plastid genome from nonphotosynthetic parasitic plant. Proceeding of the National Academy of Sciences USA, 1992, 89(22), 10648-10652.

20. Soltis DE, Soltis PS, Chase MW, Mort ME, Albach DC, Zanis M. Angiosperm phylogeny inferred from 18S rDNA, *rbc*L, and *atp*B sequences. Botanical Journal of the Linnean Society, 2000, 133(4), 381-461.

21. Hilu KW, Borsch T, Müller K, Soltis DE, Soltis PS, Savolainen V. Angiosperm phylogeny based on *mat*K sequence information. American Journal of Botany, 2003, 90, 1758-1776.

22. Moudi M, Go R. Monophyly of four sections of genus *Dendrobium* (ORCHIDACEAE): Eidence from nuclear ribosomal DNA Internal Transcribed Spacer (ITS) sequencs. Internatinal journal of Bioassays, 2015, 4(01), 3622-3626.

23. Kim KJ, Jansen RK. Comparisons of phylogenetic hypotheses among different datasets in dwarf dandelions (Kriga, Asteraceae): Additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. Plant Systematic and Evolution, 1994, 190(3-4), 157-185.

24. Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ. The ITS region of nuclear ribosomal DNA — a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri Botanical Garden, 1995, 82, 247–277.

25. Choi BH, Kim JH. ITS sequences and speciation on far Eastern *Indigofera* (Leguminosae). Plant Research, 1997, 110, 339-346.

26. Choi HK, Kim CK, Shin HC. Molecular reexamination of Korean Umbelliferae based on internal transcribed spacer sequences of rDNA: *Ligusticum tenuissimum* (Nakai) Kitagawa. and *Libanotis coreana* (Wolf)vKitagawa. Plant Biology, 2000, 43, 136-142.

27. Koehler K, Stechele M, Hetzel U, Domingo M, Schönian G, Zahner H. Cutaneous leishaniosis in a horse in Southern Germany caused by *Leishmania infantum*. Veterinary Parasitology, 2002, 109(1-2), 9-17.

28. Salazar GA, Chase MW, Soto Arenas MA, Ingrouille M. Phylogenetics of Cranichideae with emphasis on Spiranthinae (Orchidaceae: Orchidoideae): Evidence from plastid and nuclear DNA sequences. American Journal of Botany, 2003, 90(5), 777-795.

29. Van Den Berg C, Goldman DH, Frudenstein JV, Pridgeon AM, Cameron KM, Chase MW. An overview of the phylogenetic relationships within Epidendroideae inferred from multiple DNA regions and recircumscription of Epidendreae and Arethuseae (Orchidaceae). American Journal of Botany, 2005, 92(4), 613-624.

30. Whitten WM, Blanco MA, Williams NH, Koehler S, Carnevali G, Singer RB. Molecular phylogenetics of Maxillaria and related genera (Orchidaceae: Cymbidieae) based on combined molecular datasets. American Journal of Botany, 2007, 94(11), 1860-1889.

31. Clements MA. Molecular phylogenetic systematics in the Dendrobiinae (Orchidaceae), with emphasis on *Dendrobium* section *Pedilonum*. Telopea, 2003, 10(1), 247-272.

32. Yukawa T, Kurita S, Nishida M, Hasebe M. Phylogenetic implications of chloroplast DNA restriction site variation in subtribe Dendrobiinae (Orchidacaeae). Lindleyana, 1993, 8, 211–221.

33. Yukawa T, Kita K, Handa T. DNA phylogeny and morphological diversification of Australian *Dendrobium* (Orchidaceae). In: Wilson, K.L. and Mossison, D.A. (eds) Monocots: Systematics and Evolution. Melbourne: CSIRO Publishing, 2000, 465-471.

34. Schuietman A. *Dendrobium* (*Orchidaceae*): to split or not split?. Gardens Bulletin Singapore 1and, 2011, 2, 245-257.

35. Yukawa T. Molecular phylogeny of *Dendrobium*. In: Nagata, H. and Ichihashi, S. (eds) Proceedings of the seventh Asia Pacific orchid conference (APOC7), 2001.

36. Lavarack PS, Harris W, Stocker G. *Dendrobium* and its relatives. Oregon, Portland: Timber Press, 2000.

Cite this article as:
Maryam Moudi and Rusea Go. The comparison between nuclear ribosomal DNA and chloroplast DNA in molecular systematic study of four sections of genus *Dendrobium* sw. (orchidaceae). *International Journal of Bioassays* 5.3 (2016): 4944-4952.

Source of support: Nil
Conflict of interest: None Declared