PHYLOGENETIC ANALYSIS AMONG FOUR SECTIONS OF THE GENUS DENDROBIUM SW. (ORCHIDACEAE) BASED ON LOW COPY NUCLEAR GENE (XDH) SEQUENCES IN PENINSULAR MALAYSIA

M. Moudi & R. Go

Received 2015.09.06; accepted for publication 2015. 12. 12

Moudi, M., Go, R. 2015. 12. 31: Phylogenetic analysis among four sections of the genus *Dendrobium* SW. (Orchidaceae) based on low copy nuclear gene (*xdh*) sequences in Peninsular Malaysia.- *Iran. J. Bot. 21 (2): 169-17*8. Tehran.

The present study focused on the molecular phylogeny of the four sections (*Aporum, Crumenata*, *Strongyle*, and *Bolbidium*) of the genus *Dendrobium* SW. (Orchidaceae) based upon low copy nuclear gene, Xanthine Dehydrogenase (*Xdh*) sequences using maximum parsimony (MP), maximum likelihood (ML) and Bayesian (BI) methods. Single and low copy nuclear genes have been increasingly used in phylogenetic reconstruction because they contain large amounts of genetic information and are biparentally inherited. Classifications based on morphological characters have not been able to clearly divide these four sections and neither do they support their monophyletic origins. Therefore, detailed analysis using molecular data is required to ascertain their status. This study includes 20 species of genus *Dendrobium* and 2 species from genus *Bulbophyllum* (section *Sestochilus*) as outgroup taxa. According to the results, the aligned sequences consisted of 733 nucleotide characters of which 131characters were parsimony informative. The analysis revealed that the nuclear genes can be reliable marker for the phylogenetic study of genus *Dendrobium*. The results suggested that the four sections are probably best considered as one section instead of four and based on the International Code of Botanical Nomenclature (ICBN) rules can be named as section *Aporum* for this new classification.

Maryam Moudi (correspondence < maryammoudi@birjand.ac.ir>), Faculty of Science, University of Birjand, Birjand, South Khorasan, Iran.- Rusea Go, Department of Biology, Faculty of Science, University Putra Malaysia & Institute of Tropical Forestry & Forest Products, University Putra Malaysia

Key Words: *Dendrobium*; Orchidaceae; low copy nuclear gene; *Xdh;* monophyly; phylogeney

فیلوژنی چهار بخشه از جنس.Dendrobium SW (Orchidaceae) بر اساس ترکیب داده های توالی ژن هسته ایXdh در شبه جزیره مالزی

مریم مودی، دانشکده علوم دانشگاه بیرجند، خراسان جنوبی

روسه آ گو، دپارتمان بیولوژی، دانشکده علوم و دانشکده جنگلداری گرمسیری و محصولات جنگلی، دانشگاه پوترا ، مالزی

در مطالعه حاضر با استفاده ازتوالی ژن هسته ای Xdh بازسازی فیلوژنی چهار بخشه از جنس Dendrobium متعلق به تیره گیاهی Orchidaceae با روشهای بیشینه صرفه جویی، درست نمایی حداکثر و بیسین صورت گرفته است. ژن های هسته ای با نسخه کم برای مطالعات فیلوژنتیکی مورد استفاده قرار می گیرند. این ژن ها دارای مقادیر زیادی از اطلاعات ژنتیکی و توارثی از هر دو والد می باشند. مطالعات مورفولوژیکی در ارتباط با مونوفیلی چهار بخشه (Aporum, Crumenata, Strongyle and Bolbidium) قابل استناد نمی باشد. لذا برای دستیابی به اطلاعات دقیقتر نیاز به مطالعات مولکولی می باشد. در مطالعه حاضر ۲۰گونه گیاهی متعلق به جنس Dendrobium به همراه دو گونه گیاهی از جنس Bulbophyllum (section Sestochilus) به عنوان برون گروه مورد بررسی قرار گرفته است .بر پایه نتایج ، توالی نوکلئوتیدی همردیف سازی شده دارای ۷۳۳ جایگاه نوکلئوتیدی می باشد که از این تعداد ۱۳۳جفت باز به لحاظ پارسیمونی اطلاعاتی است. آنالیز داده ها

نشان می دهد که ژن های هسته ای می توانند به عنوان یک نشانگر معتبر مولکولی برای مطالعات فیلوژنتیکی خصوصا در ارتباط با این جنس گیاهی مورد استفاده قرار گیرند. با توجه به نتایج به دست آمده ، پیشنهاد می شود که بهتر است چهار بخشه به صورت یک بخشه در نظر گرفته شده و بر اساس قوانین بین المللی نامگذاری گیاهان به نام بخشه Aporum نامیده شود.

INTRODUCTION

Family Orchidaceae is one of the two largest families in flowering plants and is perhaps second only to Asteraceae (Chase et al., 2015). It is one of the most well-known by reason of their diversity and specialty in floral structure (Dressler and Dodson, 1960; Pridgeon, 2003). Most of classifications in this family are based on morphological characters. Apart from taxonomic study, morphological characters have been used in numerous studies like population study, agriculture, botany and plant breeding (Storfer, 1996). Although, there is an updated classification of Orchidaceae has been done by Chase and his co-workers in 2015. This classification is not based on phylogenetic analysis but rather it is summary of the published literatures recently. The genus *Dendrobium* Sw. is one of the three largest genera in Orchidaceae with around 800-1500 species and are found in tropical Asia, Australasia, Australia with a few species extending in to the temperate Asian regions and New Zealand (Xiang et al., 2013). This genus was recognized by Olof Swartz in 1799 for the first time, as cited in Seidenfaden and Wood in 1992. *Dendrobiums* are distributed in the tropical and subtropical regions in South, East and Southeast of Asia, north of Australia, New Zealand and New Guinea (Wang et al., 2009). They are one of the most popular orchids because of their medicinal and commercial values (Asahina et al., 2010). In addition, the 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 (Kuehnle, 2007). 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 (Asahina et al., 2010). Yukawa and his co-workers in1996 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. 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 systematic comes from two sources: chloroplast DNA (cpDNA) and nuclear ribosomal DNA (Small et al., 2004). Chloroplast DNA has been the most extensively used source of data in the plant phylogenetic analysis. The use of cpDNA has been reviewed widely (Olmsted and Palmer 1994). 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 (Chase et al., 2005). However, there is another rich source of phylogenetic information on plants, which are the low-copy nuclear genes [e.g. Xanthine Dehydrogenase (*Xdh*), Alcohol dehydrogenase (*Adh*)]. Low copy nuclear genes have great potentials to be used as robust characters of phylogenetic reconstruction at all taxonomic levels, especially where universal markers, such as cpDNA and nrDNA cannot reveal the strong phylogenetic hypotheses (Sang, 2002). Single and low copy nuclear genes include a large amount of genetic information and are biparentally inherited, therefore they have been increasingly used in phylogenetic studies, albeit with many difficulties (e.g., paralogy and copy number) (Li, 2008). Low copy nuclear genes are underused in plant phylogenetic studies due to the practical and theoretical problems in separating the evolutionary dynamics of nuclear gene families. However, it has recently become clear that low-copy nuclear genes are mainly useful in resolving close interspecific relationships and in reconstructing allopolyploidization in plants. Nonetheless, applying low copy nuclear genes in molecular studies usually needs more lab work, such as designing PCR primers, PCR-cloning, and/or Southern blots, rapid accumulation of gene sequences in the databases and advances in cloning techniques. Phylogenetic and molecular evolutionary analyses of developmentally important genes have inserted a new aspect to systematic and evolutionary studies of plant diversity (Sang, 2002; Small et al., 2004). One of the most studied gene-enzyme systems is the *Xdh* locus and its encoded protein, xanthine dehydrogenase (XDH; EC 1.1.1.204) (Rodríguez-Trelles et al., 2001). Xanthine dehydrogenase is a member of the molybdenum hydroxylase family of enzymes, which have important metabolic roles in purine metabolism and hormone biosynthesis (Morton, 2011). Molybdenum (Mo) is necessary for almost all organisms and functions in

more than 40 enzymes through catalyzing diverse redox reactions. Only four of these enzymes are found in plants: nitrate reductase; aldehyde oxidase (s); Xanthine Dehydrogenase, which is involved in purine catabolism, stress reactions and sulphite oxidase (Mendel and Hänsch, 2002). Sequences of *Xdh* have been used to estimate angiosperm phylogeny (Morton and Mazie, 2007). They sequenced over 200 genera, including several genera of gymnosperms and finally, applied for studying phylogenetic relationships within Orchidaceae (Gorniak et al., 2010). There is some molecular studies done on the genus *Dendrobium* using plastid DNA and nuclear ribosomal DNA (Yukawa et al., 1996, 2000, 2001, Clements 2003, Wang et al., 2009, Asahina et al., 2010, Schuiteman 2011, Moudi et al., 2013, Xiang et al., 2013, Moudi and Go, 2015). In this article, the low copy nuclear gene *Xdh* was used to study and discuss the monophyly among four sections (*Aporum*, *Crumenata*, *Strongyle*, and *Bolbidium*) of genus *Dendrobium*. The main objectives of the present study are to (1) Study on *Xdh* sequences as low copy nuclear gene of the *Dendrobium* species of four sections; (2) Determine the phylogenetic of four sections of the genus *Dendrobium* in Peninsular Malaysia.

MATERIALS AND METHODS

Sample collection: *Dendrobium* species of sections *Aporum, Crumenata, Strongyle and Bolbidium* were collected from different areas in Peninsular Malaysia (table 1). All observations concerning the nature of the habitat and findings were recorded for the fresh samples. Field collections were executed during orchids' flowering season, mainly at the end of the year (November - December) and middle of the year (May - June). All the samples were identified based on morphological characters. Several reference books were used to facilitate the identification process. For this study, the *Xdh* gene sequences were determined for 20 species of *Dendrobium*, including 2 outgroup taxa (*Bulbophyllum inunctum* and *Bulbophyllum macranthum* from section *Sestochilus*). Fourteen species are representing 4 sections of *Dendrobium* from Peninsular Malaysia as described by Seidenfaden and Wood (1992). Two samples of *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*). Voucher specimens for all accessions have been deposited in herbarium of biology department, University Putra Malaysia (UPM). For better comparison in the phylogenetic analysis, the sequences of one species of genus *Dendrobium* that belong to the section *Latouria* and also the other sequences of three species: *Flickingeria insularis* Seidenf., *Diplocaulobium validicolle* (J.J.Sm) Kranesl. and *Cadetia hispida* (A. Rich.) Schltr., retrieved from the NCBI (National Center for Biotechnology Information) database were included. In a recent research that was done by Schuiteman in 2011, it was argued that the genus *Dendrobium* includes genera like *Cadetia* Gaudich., *Flickingeria* A. D. Hawkes and *Epigeneium* Gagnep. Gorniak and his co-workers in 2010 demonstrated that these species (*Cadetia*, *Flickingeria* and *Epigeneium*) are genetically closely related to *Dendrobium macrophyllum*. The Basionym of three species that were used in this study are: *Flickingeria insularis* Seidenf. =*Dendrobium phuketense* Schuit. & Peter B. Adams. [Section: *crinifera*] *Diplocaulobium validicolle* (J.J.Sm) = *Dendrobium validocolle* J.J.Sm. [Section: *Diplocaulobium*], and *Cadetia hispida* (A. Rich.) Schltr. = *Dendrobium hispidum* A. Rich. [Section: *Cadetia*] (Muelleria 29 (1): 67. 2011).

DNA extraction, PCR amplification and sequencing: DNA was extracted from fresh material using Cetyl Trimethyl Ammonium Bromide (CTAB) method, as described previously by Wang et al., (2004) with minor modification. During extraction, the DNA pellets for some species were of good quality and quantity, whereas for some of them the pellets were mostly brownish. Therefore, DNA purification (polysaccharides and protein purification) using manual methods were performed. DNA quantity estimation was done using electrophoresis on 0.8% (w/v) agarose gel. After running the gel at 70 voltages and staining the gel in ethidium bromide for 30- 45 minutes (according to the concentration of ethidium bromide), DNA were visualized using UV transluminator and quantified in comparison to the standard ladder. Estimation of the quality of DNA can also be checked by agarose gel electrophoresis. A clear and sharp DNA band without any smearing is a reliable sign of obtaining DNA with good quality. The *Xdh* gene was amplified from total DNA extracts using the polymerase chain reaction (PCR). *Xdh* was amplified using two sets of primers: X502F and X1599R or X551F and X1591R (Gorniak et al., 2010). This region includes *Xdh* gene and is 1000 bp long. For amplification of *Xdh*, the reaction mixtures contained approximately 10-50 ng of DNA template, 5 µL of 10× reactions buffer, 2µL dNTPs (each 2.5mM), 2.0 U Taq polymerase and 1µL of each oligonucleotide primer, each at 10 µM concentration, in a final volume 50µL. A touchdown protocol was used for PCR amplification: the initial denaturation step (94 °C for 1min) was followed by six cycles of 94°C for 45 s; the annealing temperature 53°C-47°C reducing 1°C per cycle for 45

s, and 72°C for 90 s. The next 28 cycles used 94°C for 45 s, an annealing of 47°C for 45 s, and 72°C for 90 s. The final extension step used 72°C for 5 min.

Table1. List of studied taxa and their gene bank accession numbers.

	Species	Section	Location	Gene bank accession number
1	*Dendrobium aloifolium* (Blume) Rchb.f.	*Aporum*	UPM Green house, No.5	**KC709960**
2	*Dendrobium leonis* (Lindl.) Rchb.f.	*Aporum*	UPM Green house, No.5	**KC709959**
3	*Dendrobium quadrilobatum* Carr.	*Aporum*	Kuala Krai**,** Kelantan, PM	**KC709963**
4	*Dendrobium rosellum* Ridl.	*Aporum*	UPM Green house, No.5	**KC709961**
5	*Dendrobium terminale* Parish & Rchb.f	*Aporum*	Sungai Bertedung, Endau Rompin, PM	**KC709962**
6	*Dendrobium clavator* Ridl.	*Crumenata*	Sungai Bertedung, Endau Rompin, PM	**KC709970**
7	*Dendrobium crumenatum* Sw.	*Crumenata*	Genting Highlands, PM	**KC701378**
8	*Dendrobium setifolium* Ridl.	*Crumenata*	Sungai Bertedung, Endau Rompin, PM	**KC709971**
9	*Dendrobium truncatum* Lindl.	*Crumenata*	Cameron Highlands, PM	**KC709969**
10	*Dendrobium kentrophyllum* Hook.f.	*Strongyle*	Fraser's Hill, PM	**KC709972**
11	*Dendrobium singaporense* A. D. Hawkes & A. H. Heller.	*Strongyle*	Cameron Highlands, PM	**KC709973**
12	*Dendrobium subulatum* (Blume) Lindl.	*Strongyle*	Gunung Nuang, PM	**KC709974**
13	*Dendrobium pachyphyllum* (Kuntze) Bakh.f.	*Bolbidium*	Fraser's Hill, PM	**KC709964**
14	*Dendrobium hymenanthum* Rchb.f.	*Bolbidium*	Cameron Highlands, PM	**KC709965**
15	*Dendrobium* sp3	?	UPM Green house, No.5	**KC709967**
16	*Dendrobium* sp4	?	UPM Green house, No.5	**KC709968**
17	*Dendrobium macrophyllum* A.Rich.	*Lautoria*	NCBI	**GU004440.1**
18	*Flickingeria insularis* Seidenf.	*Crinifera*	NCBI	**GU004446.1**
19	*Diplocaulobium validicolle* (J. J. Sm.) Kraenzl.	*Diplocaulobium*	NCBI	**GU004445.1**
20	*Cadetia hispida* (A. Rich.) Schltr.	*Cadetia*	NCBI	**GU004442.1**
21	*Bulbophyllum inunctum* J. J. Sm.	*Sestochilus*	Cameron Highlands, PM	**KC709966**
22	*Bulbophyllum macranthum* Lindl.	*Sestochilus*	Gunung Jerai, PM	**KC709958**

Amplified DNA was fractionated by electrophoresis through 1% agarose gels, recovered from the gels, and purified using Wizard ® PCR Preps DNA Purification System (Promega) according to manufacturer's instructions. 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 stopcodones and then submitted to the NCBI GenBank; their accession numbers are listed in Table1. Searching of nucleotide sequences in NCBI BLAST (Basic Local Alignment Search) databases (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nuclotides) was conducted to assess sequence similarity. All the DNA sequences obtained in this study were compared with the sequences in the NCBI database. Most of the sequences showed query coverage of more than 90% with E- value= zero. All the sequences obtained from the forward and reversed sequences in this study were assembled to produce contig sequences using BioEdit ver. 7.0.2 (Hall, 1999). Multiple alignments of all of the sequences obtained in this study and those retrieved from NCBI were performed using CLUSTAL X (Thompson et al., 1997).

Molecular data analysis: parsimony and likelihood: The data for each gene region were aligned manually again using PAUP* 4.0B10 (Swofford, 2002) and MEGA5 (Tamura, 2011). To find the most parsimonious trees, maximum parsimony (MP)

analyses were run using a heuristic strategy of branch-swapping by tree bisection-reconnection (TBR) stepwise 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 for the gene region using PAUP* 4.0B10 (Swofford, 2002) and MEGA 5 (Tamura et al., 2011). Unweighted parsimony analysis resulted in 23 most parsimonious trees [tree length = 409, consistency index (CI) = 0.62, retention index (RI) = 0.65. For Maximum Likelihood (ML) analysis, to determine the optimal model, Model test 3.7 (Posada and Crandall, 1998) was used. The TrN+G substitution model was selected using a set of hierarchical Likelihood ratio tests (LRTs) implemented in Modeltest. Indeed, the best model was estimated using MEGA5 to construct ML tree was T92 (Tamura 3-parameter). The ML method was performed to find the optimal tree with a heuristic search as implemented in PAUP* 4.0B10, with TBR branch-swapping and 10 random sequence additions. Levels of support were estimated with 500 bootstrap replicates (BP), using the TBR algorithm of branch swapping for 10 random-addition replicates per bootstrap replicates. Likelihood analyses were run for the gene region using PAUP* 4.0B10 (Swofford, 2002) and MEGA5 (Tamura et al., 2011). Bayesian Inferences was carried out with MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) to calculate Posterior Probabilities of recovered clades with the optimal model of sequence evolution determined from the LRTs. MrBayes 3.0 was run with1*10^6 generations Markov chain. Graphical inspection of tree log likelihood in this sample revealed that the stationary was reached within 100,000 generations. Thus, the first 100,000 generations (1000 sampled trees) discarded as burn-in and used remaining 900,000 generations (9000 sampled trees) in all subsequent analysis. A majority rule consensus tree calculated from the 9000 remaining trees was used to determine the Posterior Probabilities of clades.

RESULTS

Analysis of *Xdh* sequence data: Complete *Xdh* sequences were analyzed for 20 *Dendrobium* species and two *Bulbophyllum* species (*Bulbophyllum inunctum* and *Bulbophyllum macranthum* as outgroup. The aligned sequences consisted of 733 nucleotide characters; 333 characters were conserved among all taxa, 341 were variable, and 131 were parsimony informative. The average percentage sequence divergence within *Dendrobium* species was 10%. The maximum divergence between groups was related to the two species of *Bulbophyllum* as outgroup with all of sections of genus *Dendrobium*, especially two sections *Aporum* and *Crumenata*. In contrast, the minimum divergence was related to *D.* sp3 with *D.* sp4. The means base composition was found to be fairly uniform among all taxa analysed (30.1% A, 23.3% C, 16.8% G, 29.8 % T). The estimated Transition/Transversion bias (*R*) was 0.88. The nucleotide frequencies are A = 30.55%, T = 28.85%, C = 22.81%, and G = 17.79%.

The MP, ML and Bayesian trees were highly congruent together (The Bayesian tree just has been shown in fig.1). The results showed that there were two main clades (A-B) with strong bootstrap (BP) and Posterior Probability (PP) 100%. The clade A was divided in to two sub-clades A1 and A2 with BP > 60% and strong PP 99%, whereas Clade B consisted of section *Lautoria.* Clade A2 with BP > 90% and PP 100% included three sections *Cadetia, Diplocaulobium* and *Crinifera.* In contrast, clade A1 consisted of four sections *Aporum, Crumenata, Strongyle* and *Bolbidium* with BP more than 90% and PP 100%. This clade was divided into two sub clades (I-II). Sub-clade I consisted of three sections (*Crumenata, Bolbidium* and *Strongyle*) (BP and PP 100%). The two sections *Crumenata* and *Bolbidium* were nested together in one clade with BP percentage more than 70% and PP 100%. The second sub-clade included section *Strongyle* and one species of section *Aporum.* This means that these two sections were nested together with a weak support of more than 60% and PP93%. Sub-clade II included section *Aporum* and two unidentified *Dendrobium* species (*D.* sp3 and *D.* sp4) BP=87% and PP= 97%. Based on the results, it confirms that sections *Crumenata, Bolbodium, Aporum* and *Strongyle* formed a well-supported monophyletic group. It also suggested that each these four sections were not all monophyletic. In addition, according to the results from MP, ML and BI, three species that were synonymous with *Dendrobium* formed a separeate clade together that was close to the four sections with BP > 60% and PP= 99%.

DISCUSSION

Low-copy nuclear genes have moderately little application in higher-level angiosperm phylogenetic. However, they hold the potential to be more informative than plastid genes because of expected higher rates of sequence divergence (Wolfe et al., 1987). Due to their biparental inheritance, nuclear DNA regions also give information about hybridization, a phenomenon of major importance in the evolution of angiosperms (Paun et al., 2009). Single and low copy nuclear genes have been increasingly used in phylogenetic reconstruction because they contain large amounts of genetic information and are biparentally inherited (Small et al., 2004); however, their application may present many difficulties (e.g.,

paralogy and copy number). In most cases, nuclear coding genes show higher evolutionary rates than neutral evolving DNA regions and are therefore of special interest to resolve relationships at low taxonomic levels and within radiations (Small et al., 2004). In the present study, the low-copy nuclear gene, *Xdh*, was used to estimate phylogenetic relationships among four sections. *Xdh* codes for xanthine dehydrogenase (XDH), which belongs to the molybdenum cofactor dependent hydroxylase class of enzymes. Sequences of *Xdh* have been used to estimate angiosperm phylogeny (Morton and Mazie, 2007). They sequenced over 200 genera, including several genera of gymnosperms. The number of informative characters in *Xdh* was greater than those reported for *rbc*L, *atp*B or *mat*K genes. Their study confirmed that *Xdh* evolves more rapidly than the previously used plastid genes in angiosperms. Gorniack et al., (2010) used *Xdh* in their study on family Orchidaceae. They indicated that this marker as low copy nuclear gene is appropriate for resolving intrafamiliar relationships. Until now, only plastid, non-coding mitochondrial and nuclear ribosomal DNA molecular markers were used to estimate phylogenetic relationships within Orchidaceae, but, to date, low-copy nuclear genes have a robust character for constructing phylogenetic relationships. In this study, *Xdh* was used for the *Dendrobium* species. The high level of substitution and around 20% PIC (Parsimony Informative Characters) were established in this region to show a high level of branch support in data analysis. The results from *Xdh* were congruent with the other DNA data in another study on these four sections using Plastid DNA (*rbc*L) and nrDNA ITS (Moudi et al, 2013, Xiang et al, 2013, Moudi and Go, 2015). The results from the preliminary study on phylogeneic analysis among these four sectionsof genus *Dendrobium* (*Aporum, Crumenata, Strongyle* and *Bolbidium*) using plastid DNA (*rbc*L) has been done by author and her co–workers showed the monophyly of these four sections as one clade in comparison with another sections o genus *Dendrobium*, however two sections, *Aporum* and *Strongyle*,were polyphyletic, whereas the other two sections, *Crumenata* and *Bolbidium*, were monophyletic. The nucleotides of the *rbcL* region into *Dendrobium* sections comprised mostly of conserved characters, therefore differentiation among sections was not successful. (Moudi et al, 2013). In the other study on these four sections using nrITS, the results showed the low level of homoplasy. 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%. In this study, *Xdh* has also been shown here to be useful to estimate the phylogenetic relationships bootstrap. According to the results, two gene regions ITS and *Xdh* showed that the nuclear genes are more reliable markers for the phylogenetic study of *Dendrobium* compared to the chloroplast DNA with a low level of resolution among the sections.

The similarities among species in section *Aporum* and *Strongyle* was noted by Schlechter in 1912 who treated the latter as a part of *Aporum*, until 2011, when indicated these two sections can be considered as one section (Schuiteman, 2011). In addition, some of authors believed that section *Strongyle* should be absorbed into section *Aporum* (Lavarack et al., 2000). The results from studies done by Yukawa in 2001 about the phylogeny of the genus *Dendrobium* showed that the two sections *Aporum* and *Strongyle* are related together with strong support (bootstrap value more than 90%). Clements in 2003 also indicated in his study that although *Dendrobium confusum*, which belongs to section *Strongyle* (Seidenfaden, 1985), it was proven nested with species of section *Aporum* within the same clade. Yien and his co-workers in 2007 showed based on their study about phylogenetic relationship of *Dendrobium* in China by AFLP Technique that the two sections *Aporum* and *Strongyle* were consisted of as a common branch compared to the other sections of this genus. Despite some diversity in the origins of the inflorescence and floral morphology, the group is held together by its vegetative characters, in particular: the possession of equitant leaves; lack of any form of thickening of the unsheathed wiry stems; production of persistent, compact, lateral and terminal inflorescences with persistent indeterminate meristematic regions from which are generated single (occasionally multiple) flowers (Clements, 2003). In this study, through most of the phylogentic trees, it is obvious that the two sections *Aporum* and *Strongyle* are closely nested. Possession of the same morphological characters and the results from molecular data from this and previous studies lead to the fact that the two sections *Aporum* and *Strongyle* should definitely be treated as one section.

Furthermore, section *Strongyle* is the link between *Aporum* and *Crumenata* (Seidenfaden and Wood 1992; Lavarack et al., 2000). Section *Crumenata* has the species that some of them sometimes occurred in section *Aporum*. This section has a long, slender stem with the basal few nodes swollen; this characteristic can help to separate section *crumenata* from *Aporum* species. The leaves are fleshy and overlapping. The flowers are produced along the stems, usually singly, and are short – lived. The lip is 3-lobed (Seidenfaden

and Wood, 1992; Lavarack et al., 2000). The inflorescences in sections *Crumenata* and section *Aporum* are similar. The flowers are usually single from a cluster of chaffy bracts and borne laterally. The flowers are small and last a few days, which is the same character among the four sections. Earlier Yukawa in 1993 presented the chloroplast DNA phylogeny of subtribe Dendrobiinae and indicated that two sections *Aporum* and *Crumenata* (they named this section as *Rhopalanthe*) were close together. Yukawa and his co-workers in 2000 based on their study on the phylogeny of Australian *Dendrobium* indicated that two sections *Crumenata* and *Strongyle* occurred in one clade. Recently, the results have been obtained from molecular study of genus *Dendrobium* from mainland Asia using plastid and nuclear sequences, suggested that the three sections *Aporum*, *Crumenata*, and *Strongyle* can be considered as one section (Xiang et al, 2013). Although only eight species were sampled in their study but the results supported the hypothesis of Schuiteman in 2011 (these three sections and section *Bolbidium* should be treated as one section) and also the current result in our study.The fourth section is section *Bolbidium*, which is the small section. The species have small with crowded pesudobulbs that are close together. There are 2 leaves, opposite each other at the apex. The flowers are produced singly by a group of bracts between the leaves. The lip is complete and they have a long mentum (Seidenfaden and Wood, 1992; Lavarack et al., 2000). Although due to the morphological classification this section is related to section *Dendrobium* (Lavarack et al., 2000). However, based on the molecular results in this study, section *Bolbidium* is close to the three sections *Aporum*, *Crumenata* and *Strongyle* and occurred in one clade with them.

Indeed, Yukawa (2001) showed that two sections *Crumenata* and *Bolbidium* were occurred in one clade compared to section *Aporum* and *Strongyle*, but four of them formed a monophyletic group with strong support and were placed in a one clade compared to the other sections of genus *Dendrobium*. However, in their study, they considered *Dendrobium kentrophyllum* under section *Aporum*. Clements in 2003, in his study on *Dendrobium* section *Pedilonum*, showed that *Dendrobium* section *Crumenata* (he maintained *Rhopalanthe*) is itself paraphyletic with representatives of sections *Aporum* and *Bolbidium* embedded within it. *Dendrobium quadrangulare* which is a representative of *D.* section *Bolbidium* (Seidenfaden 1985), was occurring in section *Crumenata*. In Schlechter's system of classification of the Dendrobiinae, section *Bolbidium* was placed in the first subgenus *Anthecebium* well removed from the third subgenus *Rhopalobium* that contained *D. crumenata*. These two sections *Crumenata* and *Bolbidium* have species possessing one to several swollen, near basal leafless internodes, the vegetative form varies from the crassulate duplicate two leaved form found in *Bolbidium*, to the conduplicate thin leaves of *D. truncatum*, and the thicker multi-leaved stems of *D. crumenatum*. Schuiteman in 2011showed his study on genus *Dendrobium* that the two sections *Crumenata* and *Bolbidium* are close together through his study on genus *Dendrobium*. He demonstrated that these four sections formed a monophyletic group and can be considered as one clade instead of four. However, he mentioned that these four sections can be also divided into two subgroups; the other having stems with a few swollen internodes, which means Section *Bolbodium* is nested within section *Crumenata*, two sections *Aporum* and *Strongyle* have stems without any swollen. In addition, Schuiteman has found that two species without swollen internodes (*D. pseudocalceolum* from New Guinea and *D. reginanivis* from Sulawesi) were clearly nested with high support inside the *Crumenata* clade based on ITS. This means that there were no morphological characters that make the two main clades recognizable. Therefore, it is best to recognize only one section to be called *Aporum* based on IBCN rules, which requires the first validly published name among the four sections to be adapted to name among the newly continued section. There were two unknown species in this study. *D.* sp3, *D.* sp4 had flattened leaves similar to section *Aporum* and *Strongyle*. Although we only had leaves from them but the leaf shapes were similar. The results based on molecular data showed that the two first species were nested in the section *Aporum* .

In Conclusion: in this study, phylogenetic relationships among the four sections of the genus *Dendrobium* were shown based on a low copy nuclear gene using MP, ML and BI analysis. The 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. 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*).

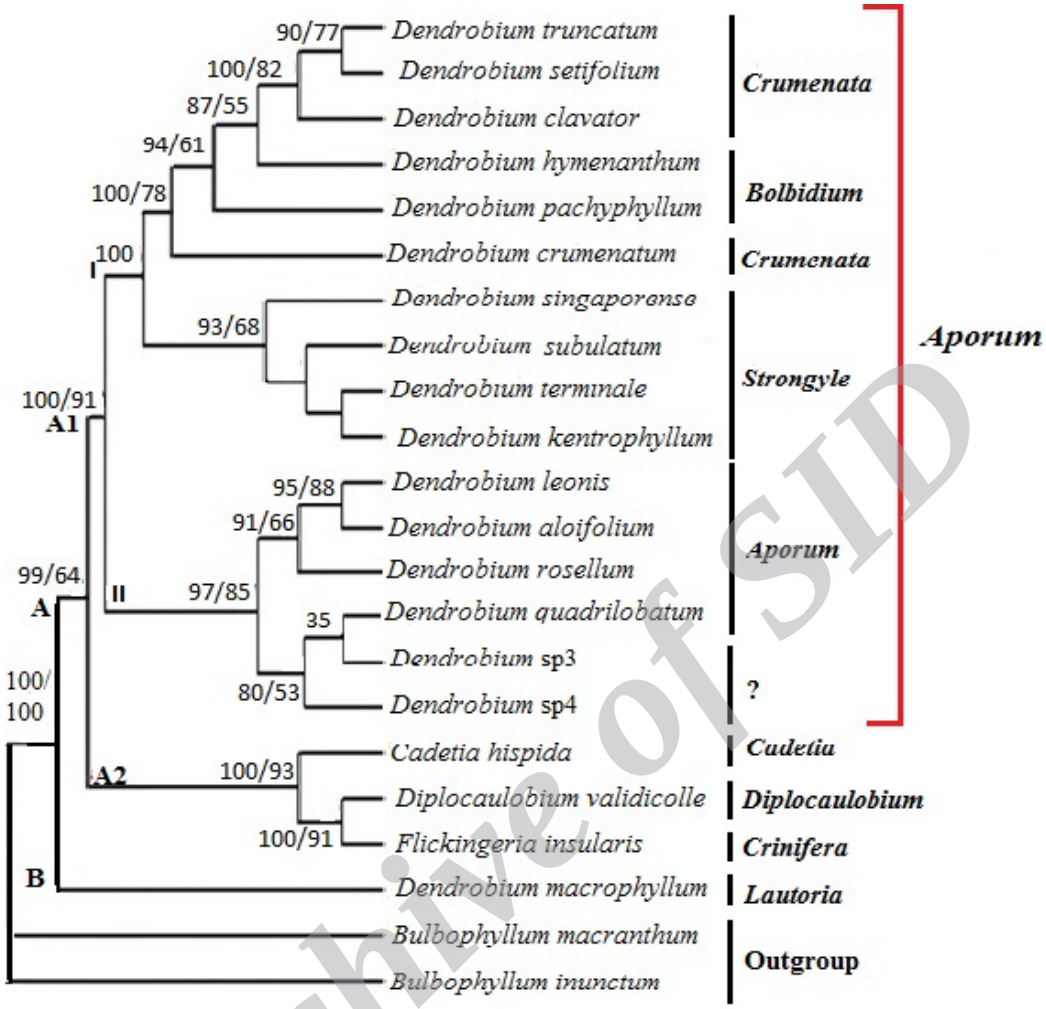

Fig. 1. Bayesian tree resulting from the *Xdh* data analysis. Posterior Probability/ Bootstrap values are indicated in the nodes. A (1-2) and B are main clades and include the different sections of genus *Dendrobium.* A1 (I and II) denote the four sections *Aporum*, *Crumenata*, *Strongyle* and *Bolbidium* form a monophyletic group and can be considered as one section named *Aporum*.

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

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

Chase, M. W. Salamin, N. Wilkinson, M. Dunwell, J. M. Kesanakurthi, R. P. Haidar, N. et al. 2005: Land plants and DNA barcods: short-term and long-term goals.- Philosophical Transactions of the Royal Society B: Biological Sciences 360: 1889-1895.

Chase, M. W. Cameron, K. M. Freudenstein, J. V. Pridgeon, A. M. Gerardo, S. Van Den Berg, C & Schuiteman, A. 2015: An updated classification of Orchidaceae. – Botanical Journal of Linnean Society 177: 151-174.

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

Dressler, R. L. & Dodson, C. H. 1960: Classification

and phylogeny in the Orchidaceae. - Annals of the Missouri Botanical Garden 47: 25-68.

Gorniak, M. Paun, O. & Chase, M. W. 2010: Phylogenetic relationship within Orchidaceae on low copy nuclear coding gene, *Xdh*: Congruence with organellar and nuclear ribosomal DNA results. - Molecular phylogenetic and evolution 56: 784-795.

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

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

Lavarack, P. S. Harris, W. & Stocker, G. 2000: *Dendrobium* and its relatives. - Oregon, Portland,Timber Press.

Li, X. Ding, X. Chu, B. Zhou, Q. Ding, G. & Gu, S. 2008 : Genetic diversity analysis and conservation of the endangered Chinese endemic herb *Dendrobium officinale* Kimura et Migo (Orchidaceae) based on AFLP. – Genetica 133: 159-166.

Mendel, R. R. & Hansch, R. 2002: Molybdoenzymes and molybdenum cofactor in plants. - Journal of Experimental Botany 53: 1689-1698.

Morton, C. M. 2011: Newly Sequenced Nuclear Gene (*Xdh*) for Inferring Angiosperm Phylogeny. - Annals of the Missouri Botanical Garden 98(1): 63-89.

Morton, C.A. & Mazie, A. 2007: Angiosperm phylogeny based on nuclear sequence information (abstract). - Botany Plant Biology Joint Congress, Available from: http://2007.botanyconference.org/engine/search/index.php?func=detail and aid =1801.

Moudi, M. & Go, R. 2015: Monophyly of four sections of genus *Dendrobium* (ORCHIDACEAE): Evidence from nuclear ribosomal DNA Internal Transcribed Spacer (ITS) sequences. – International Journal of Bioassays 4(1): 3622-3626

Moudi, M. Yong, S. Y. C. Nazre, M. Abdullah, J. O. & Go, R. 2013: Phylogenetic analysis among four sections of genus *Dendrobium* SW. (Orchidaceae) in Peninsular Malaysia using *rbc*L sequences.- International Journal of Bioassays 2(6): 932-937.

Olmstead, R. G. & Palmer, J. D. 1994: Chloroplast DNA systematics: a review of methods and data analysis. - American Journal of Botany 81: 205–1224.

Paun, O. Forest, F. Fay, M. F. & Chase, M. W. 2009: Hybrid speciation in angiosperms: parental divergence drives ploidy. - New Phytologist 182(2): 507-518.

Posada, D. & Crandall, K. A. 1998: Modeltest: testing the model of DNA substitution. – Bioinformatics 14(9): 817.

Pridgeon, A. M. Cribb, P. J. Chase, M. W. & Rasmussen, F. N. 2003: *Genera Orchidacearum 3. Orchidaceae* (Part Two), Vol*:* 3 Vanilloideae). - Oxford, UK, Oxford University Press.

Rodríguez-Trelles, F. Tarrío, R. & Ayala, F. J. 2001: Xanthine dehydrogenase (XDH): episodic evolution of a ‘‘neutral” protein. - Molecular Evvolution 53: 485–495.

Ronquist, F. & Huelsenbeck, J. P. 2003: MrBayes 3: Bayesian phylogenetic inference under mixed models. – Bioinformatics 19 (12): 1572-1574.

Sang, T. 2002: Utility of low copy nuclear gene sequences in plant phylogenetics. - Critical reviews in Biochemistry and molecular biology 37: 121-147.

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

Schuiteman, A. & Adams, P.B. 2011: New combinations in Dendrobium (Orchidaceae). - Muelleria 29(1): 67.

Seidenfaden, G. & Wood, J.J. 1992: The Orchids of Peninsular Malaysia and Singapor*e*. - Fredensborg, Olsen and Olsen.

Seidenfaden, G. 1985: Orchid genera in Thailand XII. *Dendrobium* SW. - Opera Botanica 83: 1–296.

Small, R. L. Cronn, R. C. & Wendel, J. F. 2004: L. A. S. JOHNSON REVIEW No. 2. Use of nuclear genes for phylogeny reconstruction in plants. - Australian systematic Botany 17: 145- 170.

Storfer, A. 1996: Quantitative genetics: a promising approach for the assessment of genetic variation in endangered species. -Trends in Ecology and Evolution 11: 343-348.

Swofford, D. L. 2002: PAUP*. Phylogenetic analysis using parsimony (* and other methods) Version 4b10. - Sinauer, Sunderland, Massachusetts, USA.

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

Thompson, J. D. Gibson, T. J. Plewniak, F. Jeanmougin, F. & Higgins, D. G. 1997: The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. - Nucleic Acids Research *25*(24): 4876-4882.

Wang, H. Z. Fenga, S. G. Lua, J. J. Shia, N. N. & Liub, J. J. 2009: Phylogenetic study and molecular identification of 31 *Dendrobium* species using inter-

simple sequence repeat (ISSR) markers. - Scientia Horticulture 122: 440-447.

Wang, H. Z. Wang, Y. D. Zhou, X. Y. Ying, Q. C. & Zheng, K. L. 2004: Analysis of genetic diversity of 14 species of *Cymbidium* based on RAPDs and AFLPs. - Acta Biologiae Exprimetalis Sinica 37 (6): 482–486.

Wolf, K. H. Li, W. H. & Sharp, P. M. 1987: Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. - Proceeding of the National Academy of Sciences USA 84: 9054-9058.

Xiang, X. G. Schiteman, A. Li, D. Z. Huang, W. C. Chung, S. W. Li, J. W. Zhou, H. L. Jin, W. T. Lai, Y. J. Li, Z. Y. & Jin, X. H. 2013: Molecular systemaic of *Dendrobium* (Orchidaceae, Dendrobieae) from mainland Asia based on plastid and nuclear sequences. - Molecular Phylogenetics and Evolution 69: 950-960.

Yin, B. Ying-hua, B. Wen-quan, W. Li-li, J. & Yu-ning, Y. 2007: Analysis of the Phylogenetic Relationship of *Dendrobium* in Chinaby AFLP Technique. - Acta Horticulturae Sinica 34(6): 1569-1574.

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

Yukawa, T. Kita, K. & Handa, T. 2000: 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, pp. 465–471.

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

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