*J. Orchid Soc. India, 31: 33-40, 2017* *ISSN 0971-5371*

# IDENTIFICATION OF MEDICINAL ORCHIDS OF BANGLADESH: DNA BARCODING VS. TRADITIONAL TAXONOMY

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**Abstract**

The barcoding of medicinally important orchids of Bangladesh has been done so as to confirm the traditional taxonomic identification of the species using phylogenetic analysis and match with existing gene bank sequences. Presently, twenty two medicinally important orchid species used by tribal people were collected from different parts of Bangladesh. All the species were identified and classified following traditional taxonomic methods. DNA barcodes were extracted from dried leaf sample following standard DNA extraction methods, PCR and sequencing. The maturase-coding gene (*matK*) and the ribulose 1,5-bisphosphate carboxylase-coding gene (*rbcL*) regions were used as DNA barcodes for identification and phylogenetic analysis. A total of twenty one medicinal orchid species have been sequenced with the *rbcL* gene and sixteen species with the *matK* gene. Most of the sequenced genes (17 in *rbcL* and 8 in *matK*) are new addition to the gene bank. Where the new submissions matched entries in the gene bank, they showed very similar sequences indicating the proper identification with traditional taxonomic methods. Phylogenetic trees based on *matK* data distinguish every species from each other but *rbcL* showed less species discriminating power than *matK*. Phylogenetic trees constructed on combined data showed three different groups with evolutionary trends in both terrestrial and epiphytic orchids. Importantly, DNA barcoding genes ensure proper identification of the medicinally important orchid species, in the present study. This approach is ideal as a tool for the identification of critical medicinally important species which are the origin of crude drugs. The present study may prove useful for the establishment of Intellectual Property Rights (IPR) for future drug discovery.

## Introduction

ORCHIDACEAE IS one of the largest, most diverse and highly specialized families of flowering plants with more than 24,000 species (Chowdhery, 1998). The family is species rich and the species are widely distributed from the Equator to the Arctic Circle and from lowland areas to the snowline. In Bangladesh, the family is represented by 178 species including one variety (Huda, 2007). Of these, 26 orchid species are considered to be ethno-botanically important and most of them are used for medicinal purposes (Huda *et al.,* 2006). The World Health Organization (WHO) estimates that 80 per cent of the world's population utilizes traditional medicines for healing and curing diseases (WHO, 2014). There is an increasing trend of international trade for medicinal plants, which are used both for herbal medicine and for producing pharmaceutical products (Chen *et al.,* 2010). Accurate and rapid authentication of these plants, especially the orchids and their adulterants, are very difficult and sometimes problematic to achieve at the scale of international trade in medicinal plants (Chen *et al.,* 2010).

The term *DNA barcode* for global species identification was first coined by Hebert *et al*. (2003) and has drawn the attention of scientists worldwide (Blaxter, 2003; Gregory, 2005; Miller, 2007; Schindel and Miller, 2005). The DNA barcode technique has been used for recognition of animals, plants and fungi (Hajibabaei *et al.,* 2006; Kress *et al.,* 2005; Min *et al.,* 2007; Ross *et al.,* 2008; Seifert *et al.,* 2007). The mitochondrial gene encoding cytochrome c oxidase subunit 1 (CO1) is considered a suitable region for use as a DNA barcode in most animal species (Pennisi, 2007). On the other hand, CO1 and other mitochondrial genes have limited use for identifying plant species due to the low amounts of variation in the genes, as well as the variable structure of the mitochondrial genome (Chase *et al.,* 2005, 2007; Fazekas *et al.,* 2008; Pennisi, 2007). Thus, screening for single or multiple regions favorable for DNA barcoding studies in plastid and nuclear genomes in plants have been an important research focus (Chen *et al.,* 2010).

There are two groups of potential users of DNA barcodes: Plant taxonomists/systematists and scientists of other fields (Chase *et al.,* 2005, 2007; Fazekas *et al.,* 2008; Kress *et al.,* 2008). However, DNA barcodes will be a useful and powerful tool for non-professional users such as custom officers, traditional drug producers and managers, forensic specialists, and in other relevant sectors. Therefore, a rapid and simple DNA barcoding identification system, even an imperfect one, needs to be worked out.

Recently, the CBOL (Consortium for the Barcode of Life) plant working group recommended using the two-locus combination of *rbcL* + *matK* as a plant barcode,

*Received: April 2, 2017; Accepted: June 20, 2017*

where the barcode was shown to successfully discriminate among 907 samples from 550 species at the species level with a probability of 72% (CBOL, 2009). However, this core two-locus barcode will provide a universal framework for the routine use of DNA sequence data to identify specimens and contribute toward the discovery of overlooked species of land plants. The group admits that the two-locus barcode is far from perfect due to the low identification rate, and the search is not over (Chen *et al.,* 2010).

Orchids, being highly cross pollinated have a wide range of diversity and hence characterization would be similar if DNA barcoding can be adopted. It will also help in pinpointing the genetic resources of orchids. With the recent spate of molecular data,evolutionary relationships among orchids are being re-established and re-circumscribed (Chase *et al.,* 2005). This biotechnological knowledge is becoming a more dependable method to the modern taxonomists. Orchidaceae is the most complicated family to taxonomists because of similar characteristics sharing by different species. The present investigation aims to provide a practical and powerful tool for identifying medicinal plants and their adulterants in trade and for ensuring safety in their use.

## Materials and Methods

*Taxon Sampling*

Twenty two medicinally important orchids of Bangladesh were collected from different localities of Kaptai Reserve Forest, Rangamati, Bangladesh and Chittagong University Botanic Garden so as to determine the DNA barcoding. Twenty one species were tested with universal barcoding gene *rbcL* and sixteen species were tested with *matK* gene. The voucher specimens of each species are preserved at the Chittagong University Herbarium. A list of the taxa studied with voucher information and GeneBank accession numbers is given in Table 1.

Table 1. Studied orchid species with habit, collection number, and NCBI accession number.

| Species | Habit | Collection number | Accession number | |
|---|---|---|---|---|
| | | | *rbcL* | *matK* |
| *Acampe ochracea* | E | K173 | KF421859 | KF421841 |
| *A. papillosa* | E | K185 | KF421857 | KF421842 |
| *A. rigida* | E | K170 | KF421858 | KF421843 |
| *Aerides odorata* | E | K175 | - | KF421844 |
| *Arundina graminifolia* | T | K184 | KF421860 | KF421845 |
| *Bulbophyllum lilacinum* | E | K183 | KF421861 | - |
| *Cymbidium aloifolium* | E | K190 | KF421876 | KF421846 |
| *Dendrobium aggregatum* | E | K181 | KF421862 | KF421847 |
| *D. aphyllum* | E | K172 | KF421863 | KF421848 |
| *Eria tomentosa* | E | K182 | KF421867 | - |
| *Eulophia geniculata* | T | K179 | KF421873 | KF421849 |
| *Geodorum densiflorum* | T | K188 | KF421874 | KF421850 |
| *Goodyera procera* | T | K176 | KF421864 | KF421851 |
| *Luisia filiformis* | E | K178 | KF421865 | KF421852 |
| *Nervilia scottii* | T | K182 | KF421877 | KF421853 |
| *Oberonia mucronata* | E | K185 | KF421875 | - |
| *Pelatantheria insectifera* | E | K183 | KF421868 | - |
| *Phalaenopsis cornu-cervi* | E | K177 | KF421869 | - |
| *Pholidota pallida* | E | K192 | KF421871 | KF421854 |
| *Rhynchostylis retusa* | E | K190 | KF421870 | - |
| *Robiquetia spathulata* | E | K191 | KF421866 | KF421855 |
| *Spathoglottis plicata* | T | K193 | KF421872 | KF421856 |

E, Epiphytic and T, Terrestrial

*Isolation of DNA, Amplification, and Sequencing*

Leaves were first dried in silica gel. 10 mg of each of the dried tissues was powdered for one minute at a frequency of 30 times/second in a FastPrep bead mill (Retsch MM400, Germany). Total DNA was extracted from silica-dried plant materials by DNeasy Plant Mini Kit (QIAGEN). Synthetic oligonucleotides for polymerase chain reaction (PCR) primers were obtained from Invitrogen (UK). The following primer sets for amplification of *matK* and *rbcL* genes were used:

matK_F (GAGGCGTATCTTTTGAAGC……) and

matK_R (CGACAACATGACTTCCTATACCCACT…..) for *matK*;

*rbcL a*_f (ATGTCACCACAAACAGAGACTAAAGC……) and ajf634R (GAAACGGTCTCTCCAACGCAT……) for *rbcL*

Both forward and reverse sequences were analyzed on a PE 377 automated sequencer at James Hutton Institute (Applied Biosystems Inc.), and the resulting electropherograms were edited and assembled with Sequencher versions 9.1 (Gene Codes Corp., Ann Arbor, Michigan, USA).

Sequence 4.9.1 and Bioedit software were used to verify the identification of any sequence ambiguity between forward and reverse sequences and to produce a high quality sequence for further analysis. To construct phylogenetic tree, MEGA 4 was used and phylogenetic analyses-Maximum parsimony analyses were conducted in for *matK*, *rbcL* and for combined *matK* and *rbcL*. All characters were unordered and equally weighted (Fitch, 1971). Individual gap positions were treated as missing data, the indels being separately coded and treated as additional characters. Internal support of clades was evaluated by the Bootstrap (Felsenstein, 1985), with 500 Bootstrap replicates with tree bisection-reconnection (TBR) branch swapping; saving up to 10 trees per replicate to reduce time spent swapping on large islands. The Neighbor-joining (NJ) method (Saitou and Nei, 1987) was selected for the construction of phylogenetic trees. The output data was processed using MEGA 4 (Tamura *et al.,* 2007) to draw the phylogenetic trees. A total of 1000 Bootstrap replicates were calculated for the NJ tree construction (Felsenstein, 1985).

*Assessment and Reliability*

We first retrieved all *matK* and *rbcL* sequences and constructed a reference sequence library for Bangladesh orchids. We then searched the database of NCBI with the sequences generated in this study from samples with proven taxonomic identity.

## Results and Discussion

Twenty-two species under 19 genera belonging to the family Orchidaceae from Bangladesh were studied and taxonomic position of these species following Dressler (1993) was presented in Table 2. These orchid species have also been studied so as to analyze the DNA sequence to get the barcode using both *matK* and *rbcL* genes.

Two species of the genus *Acampe* (*A. papillosa* and *A. rigida*) showed 98% similarity in the *matK* phylogenetic tree whereas *Acampe papillosa* was slightly distant from these species (Fig. 1). Morphologically, *A. ochracea* and *A. rigida* are similar but *A. papillosa* is different in their vigorness. On the other hand, the data showed that *Aerides odorata, Luisia filiformis* and *Robiquetia spathulata* are distantly related from each other. All the species were epiphytic and showed closer evolutionary phylogenetic distance. Classical classification also supports same result positioning them under same sub tribe Aeridinae. The terrestrial species *i.e. Eulophia geniculata, Geodorum densiflorum, Goodyera procera, Nervilia scottii,* have been completely isolated from epiphytic clade. Though epiphytic genus, *Dendrobium* is under same sub-clade but it showed lower (27%) similarity index. Some of the epiphytic and terrestrial orchid species are middle in evolutionary lines. The position of these species in the classical phylogeneity is in the middle which is quite similar with the current DNA sequenced phylogenetic tree. According to Dressler (1993) classification, *Goodyera* is the under sub-family Spiranthoideae which is a primitive sub-family where as *Cymbidium aloifolium, Eulophia geniculata* and *Geodorum densiflorum* under same phylad Cymbidoid. *Nervilia* is completely isolated clade in the phylogenetic tree and in traditional taxonomy it is placed in another primitive tribe Nervilieae. Two species of the genus *Dendrobium* (*D. aggregatum* and *D. aphyllum*) are in the same clade with highest similarity which is also supported by traditional taxonomy though there are distinct morphological differences in their pseudobulbs. *Eulophia* and *Geodorum* shared same clade in phylogenetic tree and taxonomically these are closely related and placed under same sub tribe Eulophiinae in traditional taxonomy. In case of *rbcL* gene, the two species (*A. ochracea* and *A. rigida*) of *Acampe* are still close to each other and the genera *Acampe, Luisia, Pelatantheria, Phalaenopsis, Rhynchostylis* and *Robiquetia* are positioned in the closer evolutionary trend and traditional classification supports genera under sub-tribe Aeridinae (Fig. 2). In the traditional classification, *Oberonia* is closely related to the genera *Cymbidium, Eulophia* and *Geodorum,* whereas in the present investigation it is distinctly placed from these genera. Only genus *Oberonia* is different considering the traditional taxonomy where *Oberonia* is placed under tribe Malaxideae. *Bulbophyllum* is isolated in

Table 2. Classification of studied orchid species of Bangladesh following Dressler's (1993) classification.

| Sub family<br>Tribe<br>Sub tribe | Genus | Species |
|---|---|---|
| Spiranthoideae Dressler<br>Cranichideae Endl.<br>Goodyerinae Klot. | *Goodyera* R. Br. | *Goodyera procera* (Ker-Gawl.) W. J. Hook. |
| Epidendroideae Lindl.<br>Nervilieae Dressler | *Nervilia* Gaudich. | *Nervilia scottii* (Reichb. f.) Schltr. |
| Epidendroideae Lindl.(Cymbidioid Phylad)<br>Cymbidieae Pfitzer | | |
| Cyrtopodiinae Benth. | *Cymbidium* Sw. | *Cymbidium aloifolium* (L.) Sw. |
| Eulophiinae Benth. | *Eulophia* R. Br. | *Eulophia geniculata* King & Pantl |
| | *Geodorum* Jacks | *Geodorum densiflorum* (Lam.) Schltr. |
| Malaxideae Lindl. | *Oberonia* Lindl. | *Oberonia falconeri* J. D. Hook. |
| Epidendroideae Lindl.<br>(Epidendroid Phylad)<br>Arethuseae Lindl. | | |
| Blettinae Benth. | *Spathoglottis* Blume | *Spathoglottis plicata* Bl. |
| Coelogyneae Pfitzer<br>Coelogyninae Benth. | *Pholidota* Hook. f. | *Pholidota pallida* Lindl. |
| Epidendroideae Lindl.<br>(Epidendroid Phylad)<br>*Dendrobioid subclade*<br>Dendrobieae Endlicher | *Bulbophyllum* Thouars | |
| Bulbophyllinae Schltr. | | *Bulbophyllum lilacinum* Ridl. |
| Dendrobiinae Lindl. | *Dendrobium* Sw. | *Dendrobium aggregatum* Roxb., *D. aphyllum* (Roxb.) Fischer |
| Podochileae Pfitzer | | |
| Eriinae Benth. | *Eria* Lindl. | *Eria tomentosa* (Koenig) J. D. Hook. |
| Vandeae Lindl. | | |
| Aeridinae Pfitzer | *Acampe* Lindl. | *Acampe ochracea* (Lindl.) Hochr.<br>*Acampe papillosa* (Lindl.) Lindl.<br>*Acampe rigida* (Buch.-Ham. ex J.E. Sm.) P.F.Hunt. |
| | *Aerides* Lour. | *Aerides multiflorum* Roxb.<br>*Aerides odoratum* Lour. |
| | *Luisia* Gaudich. | *Luisia filiformis* J.D. Hook. |
| | *Pelatantheria* Ridl. | *Pelatantheria insectifera* (Rchb. f.) Ridl. |
| | *Phalaenopsis* Blume | *Phalaenopsis cornu-cervi* (Breda) Bl. & Reichb. f.<br>*Phalaenopsis deliciosa* Reichb. f. |
| | *Rhynchostylis* Blume | *Rhynchostylis retusa* (L.) Bl. |
| | *Robiquetia* Gaudichaud | *Robiquetia spathulata* (Blume) J.J.Sm. |
| Anomalous Epidendroid<br>Arundinae Dressler | *Arundina* Blume | *Arundina graminifolia* (D. Don.) Hochr. |

phylogenetic tree but under same sub clade with *Dendrobium*, whereas traditional taxonomy placed them under same tribe with two different sub tribe. *Cymbidium, Eulophia, Geodorum, Goodyera* and *Nervilia* are in the same sub clade in the *rbcL* sequences and all these species are under the primitive tribe Cranchideae, Nervillieae, Cymbidiae, however, *Eulophia* and *Geodrum* revealed the similar consequences in the *rbcL* like *matK* became positioning in the same clade. *Bulbophyllum, Dendrobium, Pholidota, Spalthoglottis* are positioned in the middle considering DNA phylogenetic tree and Dressler classification system.

Table 3. Comparative matching of examined *matK* and *rbcL* sequences with existing NCBI Gene Bank sequences.

| Species | Accession number (and maximum identity) where species exists in Gene Bank | |
|---|---|---|
| | *mat K* | *rbcL* |
| *Acampe ochracea* | DQ091314.1 (100%) | New to GeneBank |
| *A. papillosa* | DQ091315.1 (100%) | New to GeneBank |
| *A. rigida* | New to GeneBank | New to GeneBank |
| *Aerides odorata* | EF655779.1 (100% matched with *Aerides rubescens* | - |
| *Arundina graminifolia* | EF079333.1 (100%) | AF074111.1 (99%) |
| *Bulbophyllum lilacinum* | - | New to GeneBank |
| *Cymbidium aloifolium* | JN004412.1 (99%) | New to GeneBank |
| *Dendrobium lindleyi* | HM055274.1 (100%) | AF 074145.1 (100%) |
| *D. aphyllum* | HM055174.1 (100%) | FJ 216575.1 (100%) |
| *Eria tomentosa* | - | New to GeneBank |
| *Eulophia geniculata* | New to GeneBank | New to GeneBank |
| *Geodorum densiflorum* | New to GeneBank | New to GeneBank |
| *Goodyera procera* | New to GeneBank | New to GeneBank |
| *Luisia filiformis* | New to GeneBank | New to GeneBank |
| *Nervilia scottii* | New to GeneBank | New to GeneBank |
| *Pelatantheria insectifera* | - | New to GeneBank |
| *Phalaenopsis cornu-cervi* | - | New to GeneBank |
| *Oberonia mucronata* | - | New to GeneBank |
| *Pholidota pallida* | AF302703.1 (100%) | New to GeneBank |
| *Rhynchostylis retusa* | New to GeneBank | New to GeneBank |
| *Robiquetia spathulata* | - | New to GeneBank |
| *Spathoglottis plicata* | AY368429.1 (100%) | AY 381134.1 (100%) |

The genus *Arundina* is completely an isolated group in traditional taxonomy though it is closely related with *Pholidota pallida*.

Considering the combined results of *matK* and *rbcL*, *Eulophia, Geodorum, Goodyera* and *Nervilia,* the four terrestrial orchid genera are placed in the one sub-clade, the primitive group (Fig. 3). The epiphytic orchids *Acampe, Luisia* and *Robiquetia* are in another group which are under the same sub-tribe Vandeae, the most advanced group. Two species of *Dendrobium* are in the same clade which were also found both individually for *matK* and *rbcL* gene. *Arundina* and *Pholidota* are in the last group and under same clade with distant phylogenetic relationship (54%) which is also supported by the classical classification proposed by Dressler (1993).

To estimate the reliability of species identification, using a DNA barcoding technique, two methods (BLAST and The Nearest Genetic Distance) were used (Ross *et al.,* 2008). The BLAST method determines the identity of a sample based on the best hit of the query sequence and results are presented in Table 3. Among the studied orchid species with *matK* gene, seven orchid species showed 100% match with species of existing gene bank sequences during BLAST and one showed 100% match with different species of the same genus and *Cymbidium aloifolium* showed 98% similarity, whereas, eight species did not match the gene bank sequences and represent new species additions to it. Only *Cymbidium aloifolium* showed 99% similarity and existing gene bank sequence has few N (wrong nucleotide) which revealed that our experimental

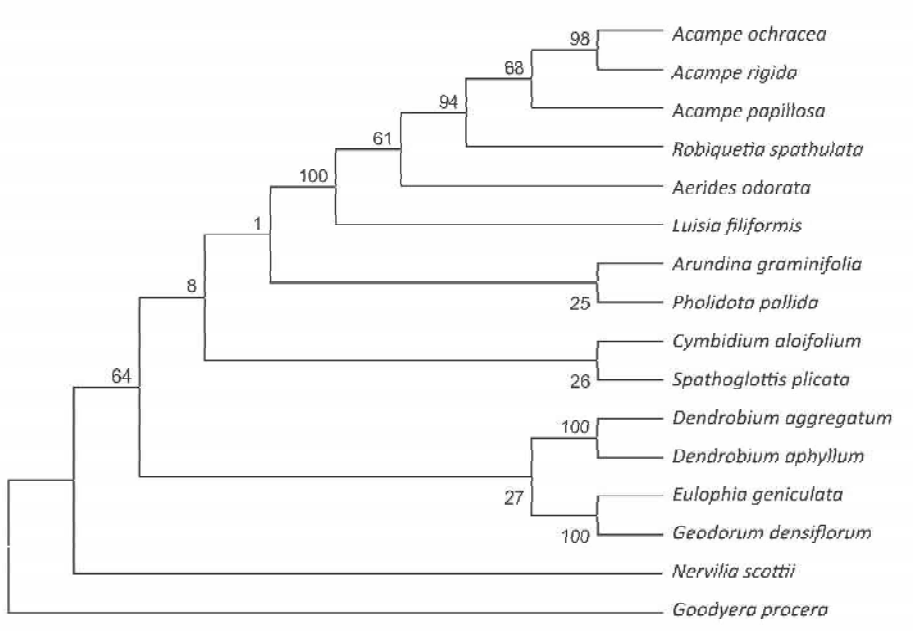


Fig. 1. Phylogenetic tree from *matK* of medicinal important orchid species of Bangladesh. Bootstrap values (%) are shown on each branch.

sequence and identification of the species was correct and other eight sequences will be the great contribution for new addition to NCBI gene bank. Matching with *rcbL* sequence of gene bank only four species out of 21 species were available. Rest 17 orchid species are new addition to NCBI gene bank. Among four species, three

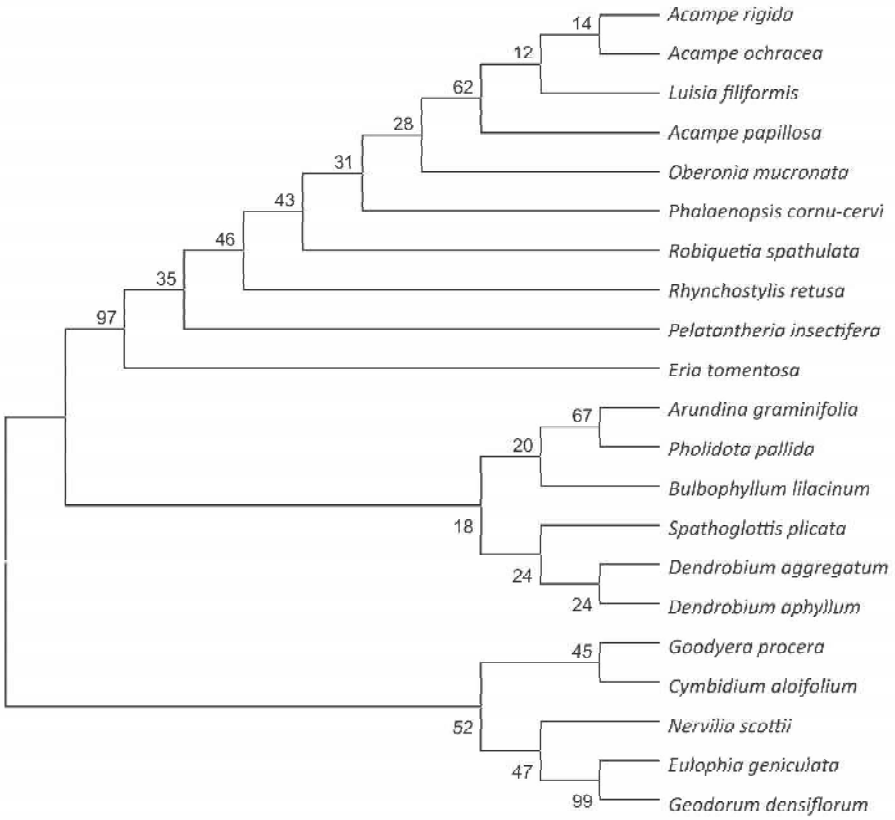


Fig. 2. Phylogenetic tree from *rbcL* of medicinal important orchid species of Bangladesh. Bootstrap values (%) are shown on each branch.

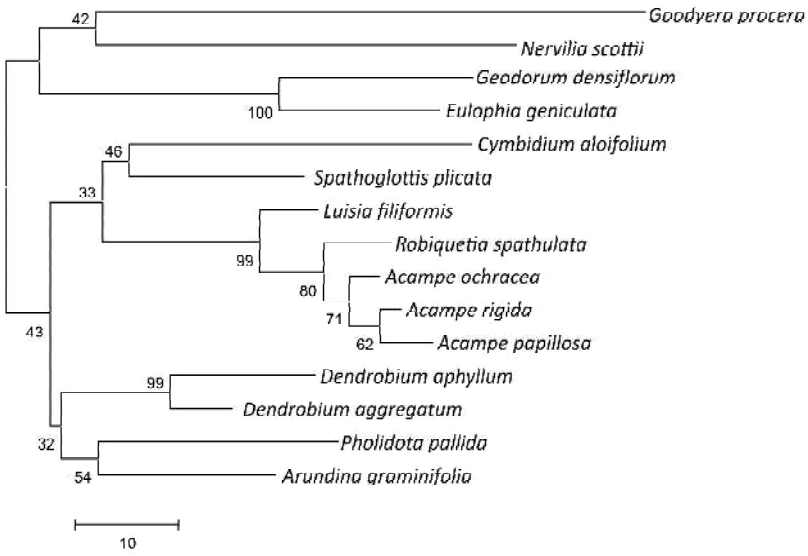


Fig. 3. Phylogenetic tree from both *matK* and *rbcL* of medicinal important orchid species of Bangladesh. Bootstrap values (%) are shown on each branch.

were 100% maximum identical and one has 99% similarity with existing gene bank. So, present DNA data show the most reliable sequences for both *matK* and *rbcL*.

The DNA sequences of the orchid species are important for their individual identity and confirmation of the species. Some useful compounds might be isolated from these species and experimentally may prove as the important sources of medicinal drugs, in future. In that case, DNA sequences of medicinally important orchids of Bangladesh will prove highly useful so as to demand Intellectual Property Right (IPR), in future.

## Acknowledgement

Authors are grateful to instrument specialists mainly to Dr. Clare Booth of SCRI, Dundee for sequencing, Ms Kirsten Brown for DNA extraction, Jim McGregor of Herbarium for providing herbarium specimens and Dr. Graeme Nicole for finalizing the data. Authors are also grateful and thankful to IDB (Islamic Development Bank) for providing fund so as to conduct this research.

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