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Differentiation of Medicinal *Dendrobium* Species (Orchidaceae) Using Molecular Markers and Scanning Electron Microscopy

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ABSTRACT

Dendrobium species have long been used as functional food supplements and herbal medicines in Asia. However, inappropriate usage of the *Dendrobium* species variants is rampant because of the considerable differences in the cost of the variants. Furthermore, the similar appearance of the dried plants of the *Dendrobium* species makes it difficult to discriminate among the individual members. In this study, simple and sensitive methods based on molecular and morphological studies were developed to verify authenticity of the *Dendrobium* species used in the preparation of medicines, particularly that of the most expensive variety, *D. huoshanense*. Molecular and anatomical differences among the 8 commonly used *Dendrobium* species (6 used for medicinal purposes and 2 for ornamental purposes) were studied. The ribosomal DNA internal transcribed spacer (ITS), chloroplast DNA *trn*L intron, and the *trn*L-*trn*F intergenic spacer (IGS) of the DNA of the 8 species were sequenced and compared. The comparison results highlighted considerable differences between the IGS region of *D. huoshanense* and that of other *Dendrobium* species to enable a clear distinction between them. A novel primer set was designed to specifically amplify the DNA of *D. huoshanense*. The leaf and stem morphologies of the 8 *Dendrobium* species were also studied by scanning electron microscopy (SEM). Granular mucilage and acicular grains in the vascular bundles were present only in the medicinal *Dendrobium* species but not in the ornamental ones.

Key words: *Dendrobium* species, ITS rDNA, cpDNA, scanning electron microscopy

INTRODUCTION

Approximately 1,600 *Dendrobium* species (Orchidaceae) are recognized worldwide, of which 15 are found in Taiwan[(1)]. Investigations of *Dendrobium* species at various taxonomic characters, including leaf and stem morphology[(2)], alkaloid content, and chemical constituents, have been carried out[(3,4)]. The traditional crude Chinese medicine "Shi-Hu," which includes *Dendrobium husohanense*, *Dendrobium officinale*, *Dendrobium tosaense*, and *Dendrobium moniliforme*, has been recorded in "Shen Nong Ben Cao Jing" as a top-grade medicine and has been mainly used as a tonic in Asian countries for over centuries. Among these varieties, *D. husohanense* exerts the best curative effect[(2)]. Recent studies have revealed that in addition to its known effects of this plant species, *D. husohanense* exerts antitumor, anti-angiogenic, anti-platelet aggregation, anti-inflammation, and immunoregulatory effects[(5,6)]. The similar appearance of the dried stems of various species makes it difficult to distinguish between them, resulting in the use of incorrect ingredients in medicines. Therefore, the intergenic spacer (IGS) sequences of 8 selected *Dendrobium* species were compared with those published to better establish the phylogenetic relationships among the various species.

Several molecular techniques have been developed for the identification of the species on the basis of the genotypic pattern including restriction site comparative sequencing and polymerase chain reaction (PCR)-based techniques. These techniques are based on multiple species-specific genomic DNA (gDNA) probes (MSSPs)[(7)], PCR amplification of the ribosomal DNA internal transcribed spacer (ITS)[(8-11)], and intersimple sequence repeats (ISSRs)[(12)]. Phylogenetic analysis using chloroplast *rbc*L sequences was performed to determine

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the relationship between the subtribe Dendrobiinae and sister group candidates[13]. In addition, chemical methods have been used to classify the *Dendrobium* species. For example, studies have used high-performance liquid chromatography with diode array detection (HPLC–DAD), a method capable of simultaneously identifying 11 phenols to classify these species[14]. However, identification of the botanical origins of the different Shi-Hu samples and assessment of the medicine quality on the basis of morphological and chemical studies remain difficult. Because IGS regions are highly variable among different genera and species, IGS regions have been recently adopted as molecular markers to identify medicinal *Dendrobium* species. The IGS regions of 8 *Dendrobium* species were sequenced and compared to explore the possible use of differentiating species.

Scanning electron microscopy (SEM) is an effective technique for examining plant surfaces at high resolution. *D. huoshanense* stems are the most common ingredients of the *Dendrobium* species used in the preparation of herbal medicines in Taiwan. However, similar morphologies of the stems of the different *Dendrobium* species make difficult to differentiate among the various *Dendrobium* species. This difficulty is further compounded by the fact that both authentic and false substitutes of *Dendrobium* are available in Chinese markets[15].

In this study, we analyzed the molecular marker DNA sequences of the complete ITS1-5.8S-ITS2 region, *trn*L intro/*trn*L-*trn*F gene sequences, and the morphology of studied 8 *Dendrobium* samples, which included species used for medicinal and ornamental purposes, obtained from various localities in Taiwan. For the rapid and accurate identification of *Dendrobium* species, we developed a primer pair specific to *D. huoshanense* to differentiate it from other *Dendrobium* species.

MATERIALS AND METHODS

I. *Collection of Materials*

The detailed sources of the 8 *Dendrobium* samples used in this study are summarized in Table 1. Fresh leaves of the samples were snap-frozen in liquid nitrogen and stored at -80°C until DNA isolation.

II. *DNA Extraction*

The total DNA from the prepared fresh leaves of the 8 samples was extracted using a modified cetyltrimethylammonium bromide (CTAB)[16,17] method. Approximately 0.1 g of dried leaf powder was resuspened in DNA extraction buffer [2% CTAB, 1.4 M NaCl, 20 mM ethylenediaminetetra acetic acid (EDTA), 100 mM Tris-HCl (pH 8.0)]. The mixture was incubated at 60°C for 30 min and centrifuged at 10,000 ×*g* for 5 min. The pellet was removed and 250 μL of chloroform/isoamyl alcohol (24:1, v/v) was then added. The mixture was then centrifuged at 12,000 ×*g* for 5 min. In order to precipitate DNA, 180 μL of isopropanol was added, and the mixture stored at –80°C for 30 min. The DNA precipitate was washed twice with 70% ethanol and dissolved in 30 μL sterile water after vacuum drying for 15 min. This DNA stock was stored at –80°C until further use. The approximate DNA concentration was determined using a spectrophotometer (Beckman Coulter™ DU®640, Minnesota, USA), and the concentration of each sample was adjusted to 100 ng/μL.

III. *PCR Amplification and Sequencing*

From the total genomic DNA, a DNA segment containing ITS1, 5.8S rDNA, and ITS2 was amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)[18]. The PCR steps were as follows: denaturation for 1 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, 1.5 min at 72°C, and then a final extension for 7 minutes at 72°C. The *trn*L intron/*trn*L-*trn*F IGS region segments were amplified by PCR using the primers TrnL (5′-CGAAATCGGTAGACGCTACG-3′) and TrnF (5′-ATTTGAACTGGTGACAC GAG-3′)[19]. The PCR conditions were 96°C for 1 min, followed by 30 cycles of 1 s at 96°C, 1.5 min at 54°C, 2 min at 72°C, and then a final extension for 10 min at 72°C. A *D. huoshanense*-specific primer set was designed according to the results obtained from the abovementioned reactions. A 596 bp species-specific fragment of the DNA of *D. huoshanense* was amplified by PCR using the primers DENS (5′-TCGAAATGACAGAAAGGA-3′) and DENA (5′-GTGCATCATCCCTAGTTT-3′) and the following

Table 1. Collected taxa and sources of the Dendrobium species used in this study

Species	Sample ID	Source county/ city	Origin
D. officinale	D1	I-lan, Taiwan	cultivated
D. tosaense	D2	Taipei, Taiwan[a]	cultivated
D. cumulatum	D3	Taipei, Taiwan	cultivated
D. linawianum	D4	Taipei, Taiwan[a]	cultivated
D. moniliforme	D5	Chia-yi, Taiwan[b]	cultivated
D. aurantiacum	D6	Taipei, Taiwan	wild
D. huoshanense	D7	Taipei, Taiwan[c]	cultivated
D. nindii	D8	Taipei, Taiwan	wild

[a] Provided by Professor C. N. Chang, Department of Horticulture, National Taiwan University.

[b] Provided by IHSIN orchid.

[c] Provided by Professor K.W. Yeh, Institute of Plant Biology, National Taiwan University.

protocol: 3 min at 95°C, 35 cycles of 1 min at 95°C, 30 s at 40°C, 1.5 min at 72°C, and a final extension for 10 min at 72°C. The PCR products were separated by gel electrophoresis on 2% agarose in Tris-actetate-EDTA (TAE) buffer with ethidium bromide and observed under UV light. The PCR products of the 8 *Dendrobium* species were sequenced by Mission Biotech Co. Taiwan on an ABI PRISM 377-96 DNA sequencer (Perkin-Elmer, Minnesota, USA).

IV. *Data Analysis*

A total of 40 sequences of the ITS1-5.8S-ITS2 region and 3 sequences of the *trn*L-*trn*F IGS region of *Dendrobium* species were collected from the GenBank. Sequences were aligned using the GCG software (GCG Command 11.1). Genetic distances between the populations were calculated using the two-parameter method. The populations were clustered into a dendrogram on the basis of their pair-wise values determined using the unweighted pair group methods with the averaging (UPGMA), neighbor-joining (NJ) method, and parsimony (PA) method. To test the robustness of the results, a bootstrap analysis was performed with 1000 replicates. Bootstrapping and dendrogram constructions were performed using the PHYLIP software.

V. *Preparations for SEM*

For the anatomical study[20-22], leaf blade samples were carefully cut and individually fixed overnight in 2.5% glutaradehyde in phosphate buffer (pH 6.8) at 4°C. The samples were dehydrated through acetone-graded series. Samples were coated with gold/palladium in an ion sputter (Bio-Rad SC502, Hertfordshire, UK) and observed by standard techniques using a Tapcon ABT-60 scanning electron microscope (Tokyo, Japan).

RESULTS AND DISCUSSION

I. *Sequence Analyses*

The ITS regions of the *Dendrobium* species examined were aligned and analyzed (Figure 1). Length of the ITS region varied from 628 to 1299 bp; the lengths were 666 bp and 645 bp in *F. comata* and *P. carnea*, respectively. Among the 770 aligned positions, the polymorphic sites of *Dendrobium* species were 300 bp in ITS1, 115 bp in 5.8S rDNA, and 355 bp in ITS2. The sequences were observed to vary with the species. The length of the phylogenic sites was 239 bp (34%) in the 6 *Dendrobium aurantiacum* sequences, 43 bp (6%) in the 2 *Dendrobium chrysanthum* sequences, 135 bp (21%) in the 3 *Dendrobium fimbriatum* sequences, 36 bp (5.6%) in the 2 *Dendrobium hancockii* sequences, 9 bp (1.4%) in the 4 *Dendrobium linawianum* sequences, 137 bp (20%) in the 4 *Dendrobium moniliforme* sequences, and 9 bp (1.4%) in the 2 *Dendrobium nobile* sequences. Genetic distances of the 48 *Dendrobium* sequences ranged from 0.515 to 1 for the in-group taxa and 0.483 to 0.863 between the in-group taxa and the outgroup taxon *F. comata*. The decreased genetic distance between *D. linawianum* and *D. nobile* indicated that they have a close phylogenetic relationship.

The *trn*L intron/*trn*L-*trn*F IGS regions of cpDNA in the 8 *Dendrobium* species and lengths of the corresponding PCR products ranged from 849 to 1182 bp (Figure 2). The sequences that were identified to contain the *trn*L intron/*trn*L-*trn*F IGS regions were aligned. In addition, the variable sites among the 11 samples, including the outgroup *B. lobbii* and 2 *Dendrobium* species sequences recorded in the GenBank, were analyzed. A total of 561 variable sites in the 614 aligned positions were identified in the *trnL* intron (91%), and 332 variable sites in the 424 aligned positions were identified in the *trn*L-*trn*F IGS (78%). The similarity matrix of *D. officinale, D. tosaense*, and *D. linawianum* revealed a close phylogenetic relationship (0.985, 0.985, and 0.987, respectively). *D. huoshanense* and *D. moniliforme* also exhibited a close phylogenetic relationship (0.829 and 0.849, respectively), and they are the most important species used as ingredients in the preparation of traditional herbal medicines in Asia.

II. *Dendrobium Clustering Determined on the Basis of the ITS and trnL Intron/ trnL-trnF IGS Region Sequence Data*

The topology of the ITS region tree, constructed by the NJ method, exhibited 6 clusters (Figure 3). The major medicinal *Dendrobium* species, *D. moniliforme* and *D. huoshanense*, were grouped with *D. officinale, D. tosaense*, and *D. linawianum*. In 2004, Tsai *et al.* suggested that *D. moniliforme*, *D. tosaense*, and *D. linawianum* are grouped with *D. aurantiacum* in the same cluster[9]. However, *D. aurantiacum* was grouped with *D. hancockii* in cluster IV. Both ornamental species, *Dendrobium cumulatum* and *Dendrobium nindii*, were in cluster VIII with 100% cluster support. Three sequences of *D. fimbriatum* in different studies were dispersed in clusters IV, VI, and VII. Sequence analysis indicated that these sequences have high divergence, which might arise due to hybridization or different growth conditions of the samples[10].

Analysis of the *trn*L intron/*trn*L-*trn*F IGS regions for the various species revealed that the 10 *Dendrobium* species can be grouped into 6 clusters (Figure 4). An interior branch test revealed that *D. tosaense*, *D. officinale*, and *D. linawianum* were grouped into cluster V with 99.5% support. On the basis of the ITS regions, *D. cumulatum* and *D. nindii* were grouped into a single cluster (cluster IV). On the other hand, *D. moniliforme, D. huoshanense,* and *Dendrobium kingianum* were in cluster VI with 100% support, as determined by an interior branch test.

There were more instances of identical *trn*L intron/

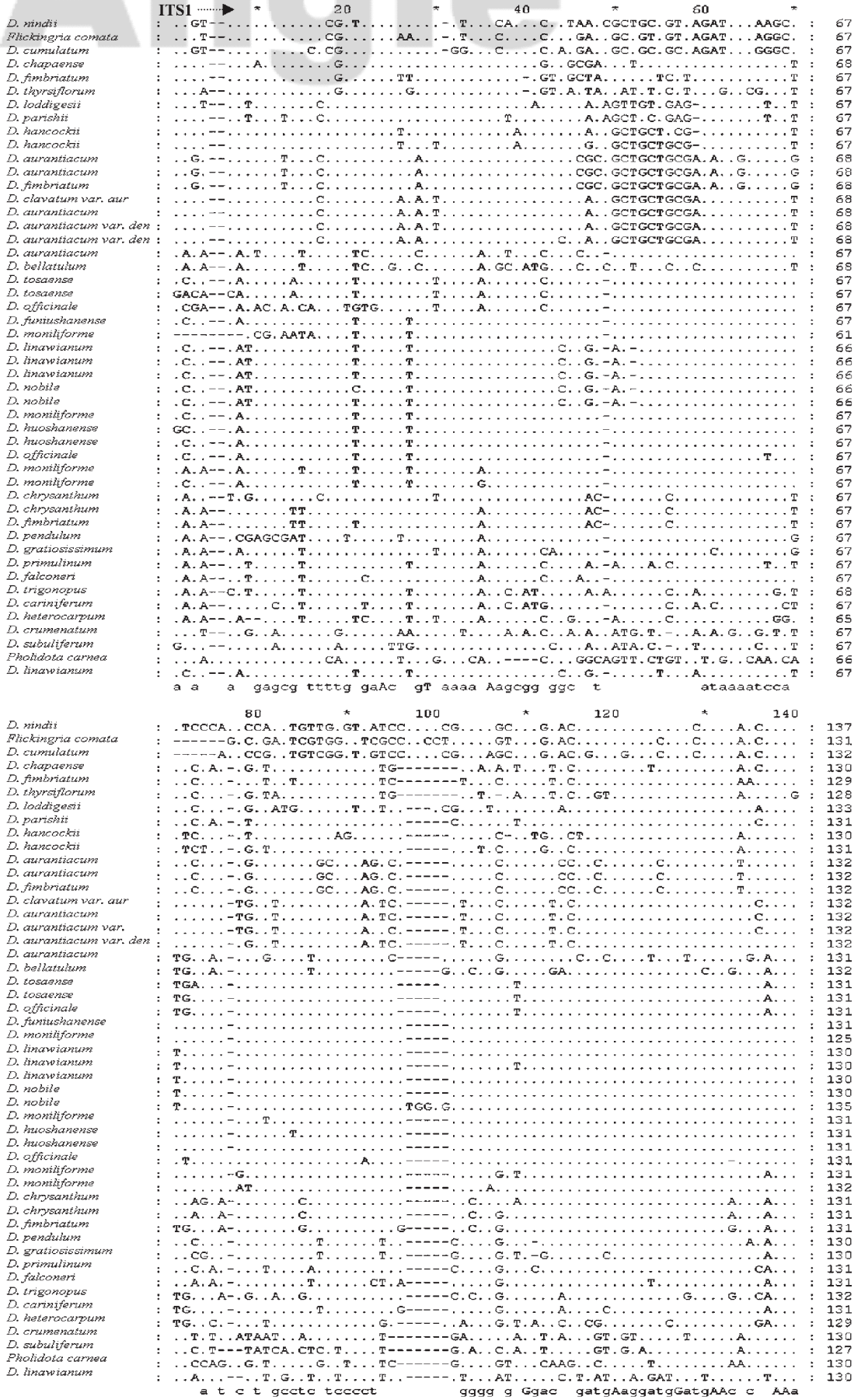
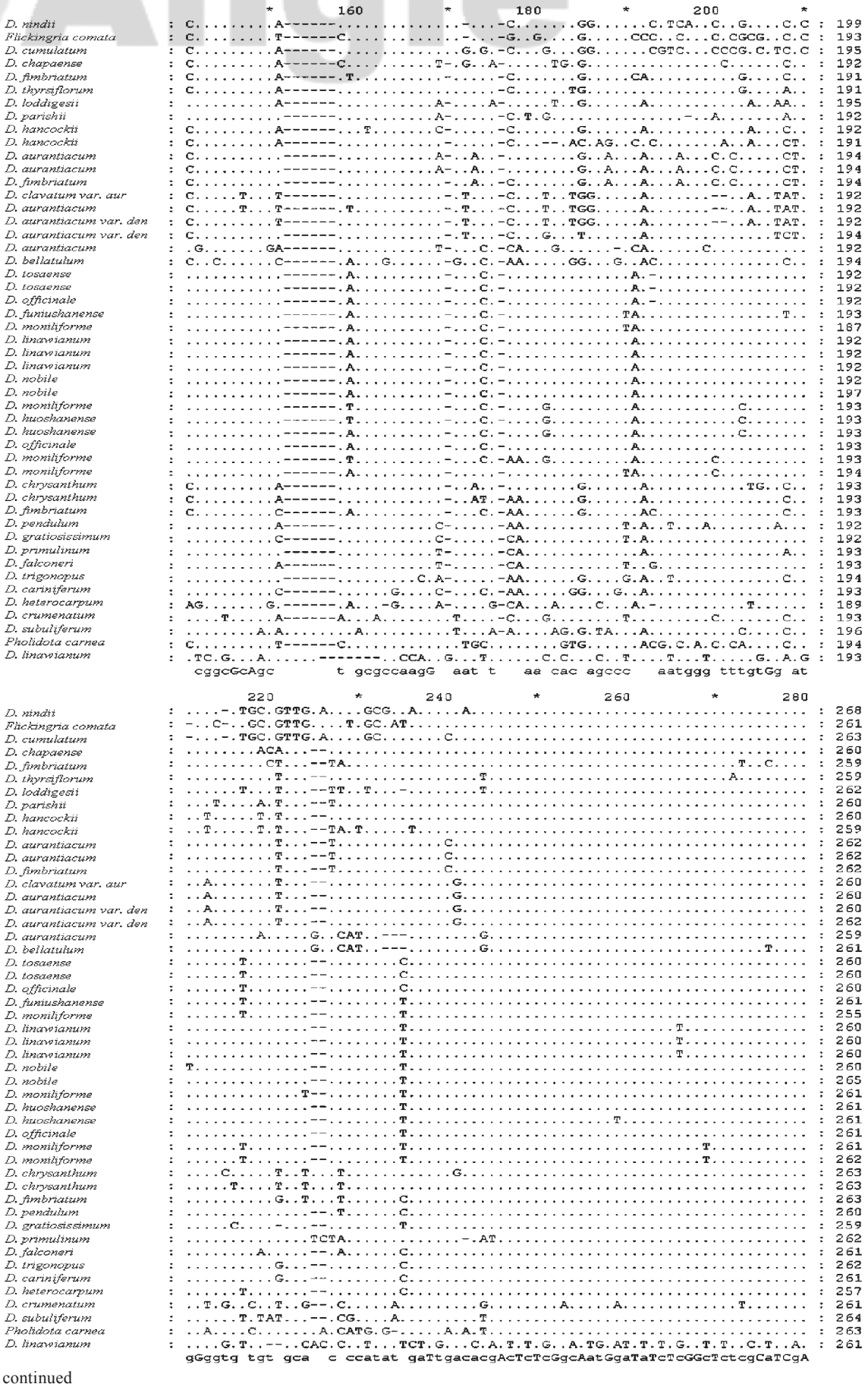


Figure 1. Sequence alignment of rDNA ITS1-5.8S-ITS2 fragments.



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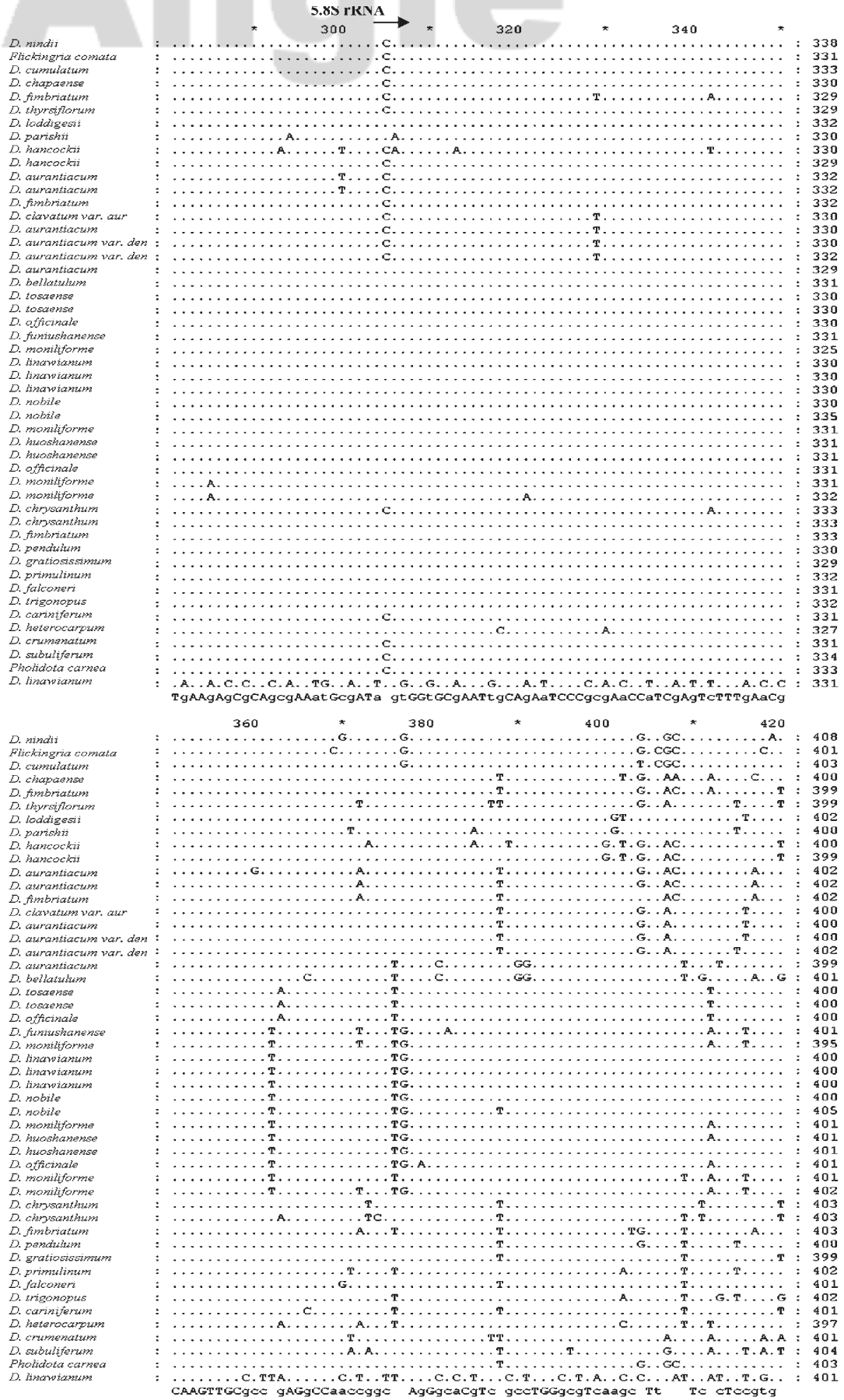

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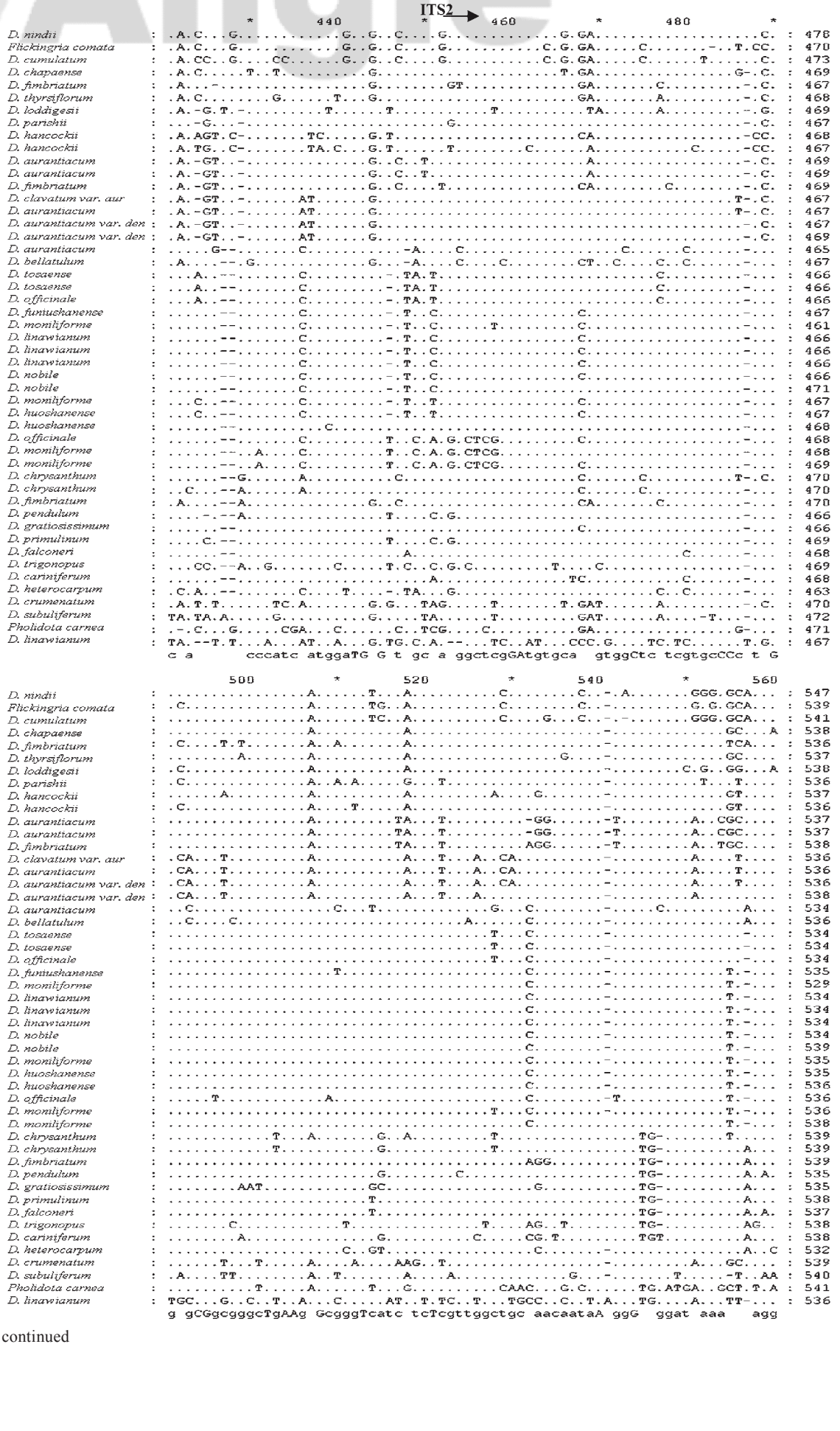

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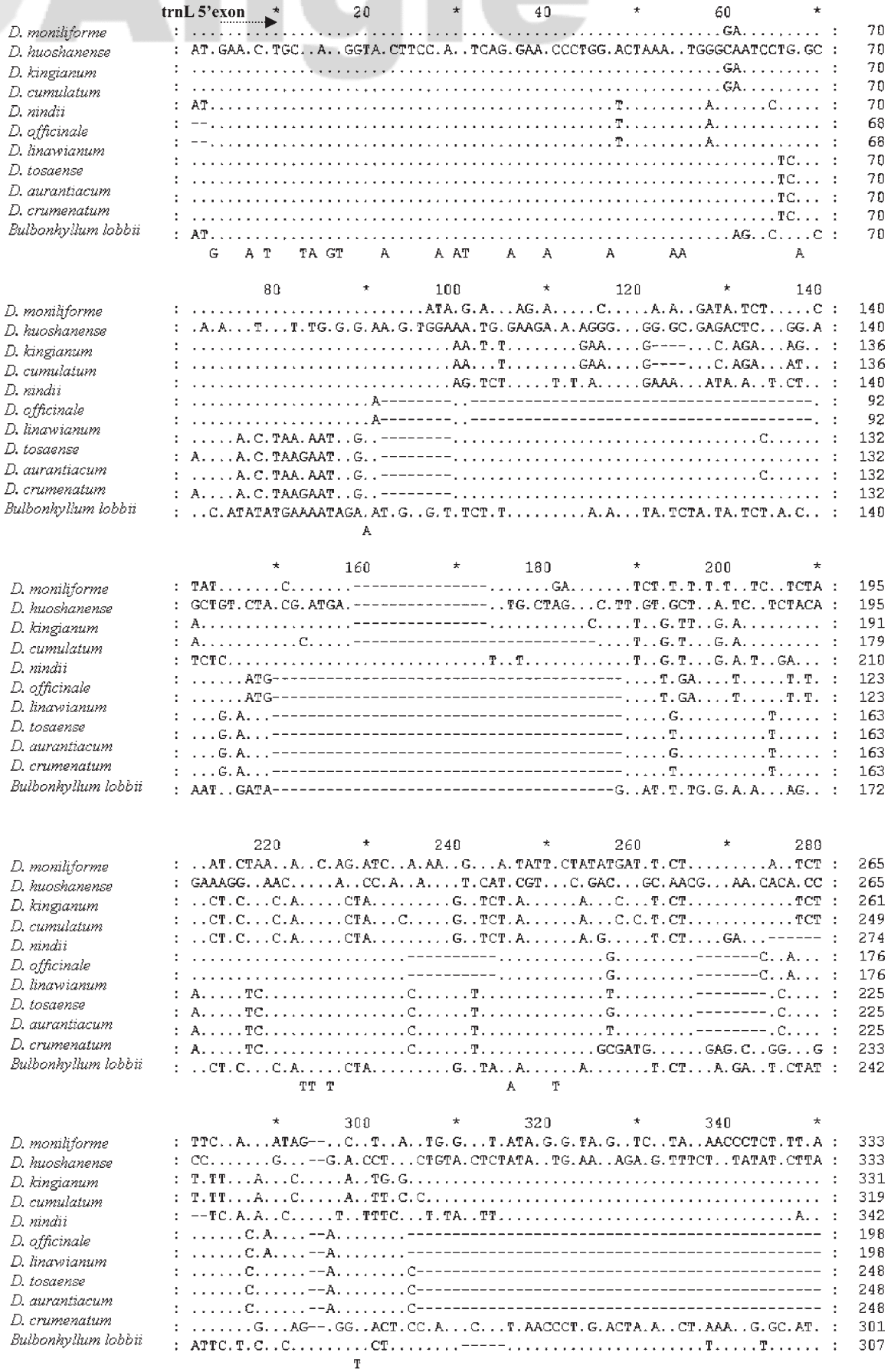

Figure 2. Sequence alignment of cpDNA trnL intron and trnL-trnF fragment from Dendrobium species.

*trn*L-*trn*F IGS regions than ITS regions among the *Dendrobium* species. The differences in the ITS sequences of a particular species observed in various studies further confirm the difficulty in identifying *Dendrobium* species. Many individual markers were observed in the *trn*L intron sequences of *D. huoshanense* and *D. moniliforme*, and the results were applied to investigate authenticity of the medicinal *Dendrobium* species[8].

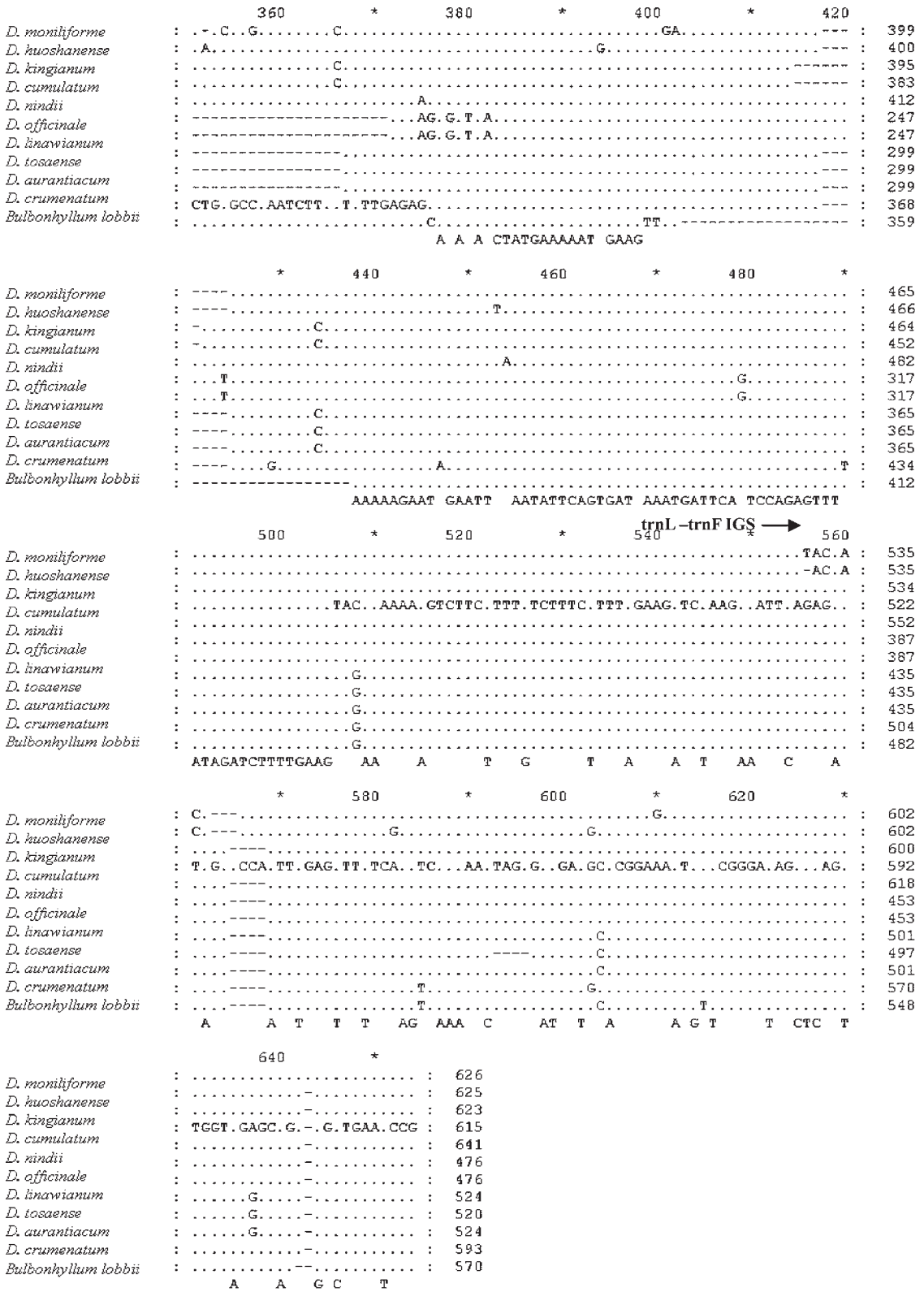

Figure 2. continued

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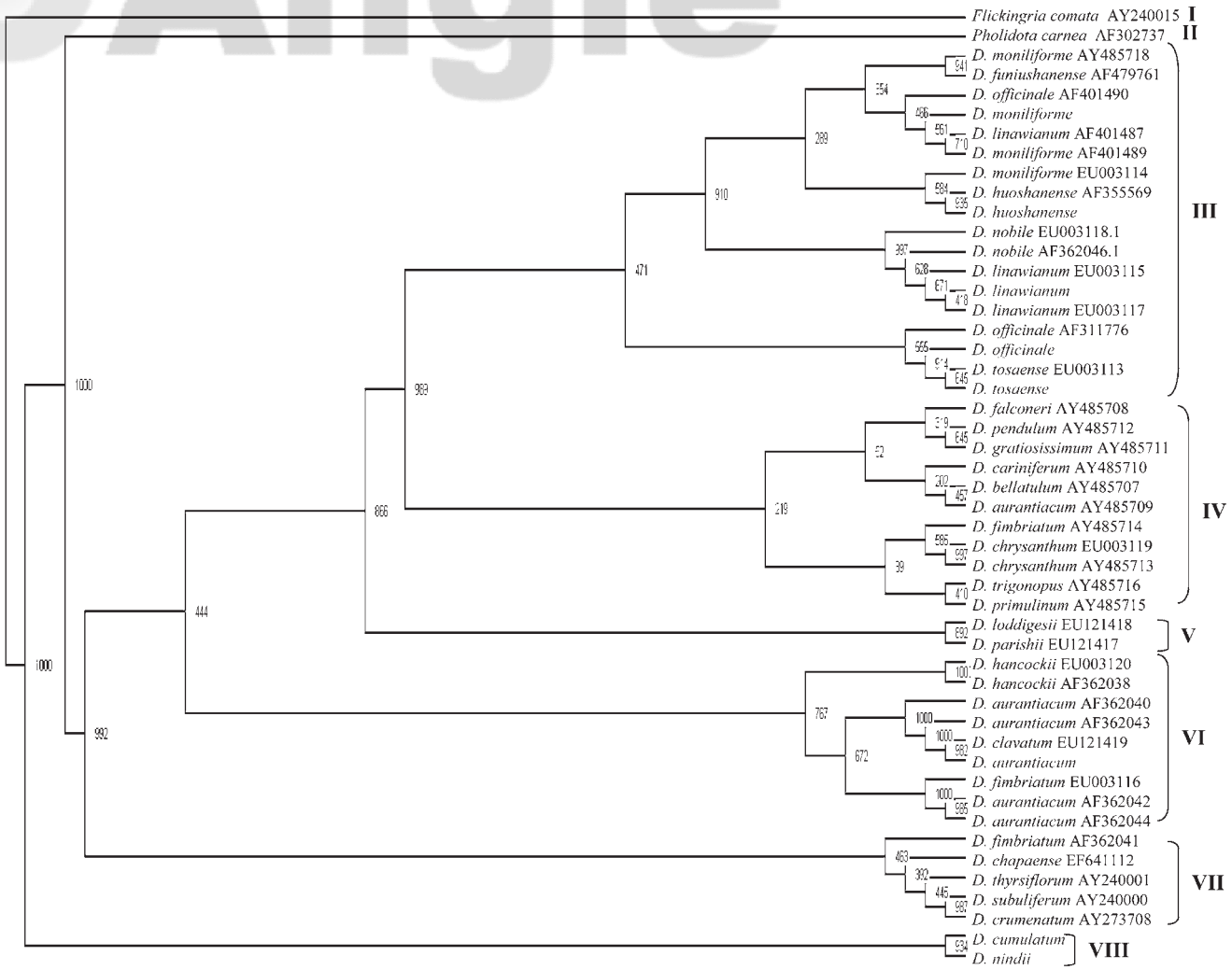

Figure 3. Phylogenetic tree based on the sequences of rDNA ITS1/ITS4 regions from distinct *Dendrobium* species. Neighbor-join method (NJ) was used in this analysis (bootstrapping number = 1000).

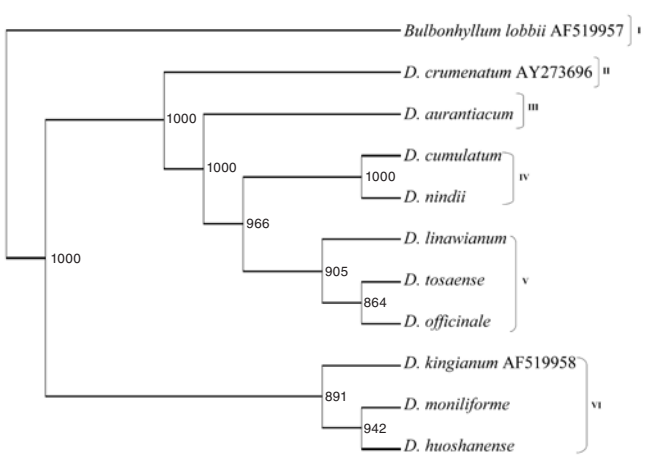

Figure 4. Phylogenetic tree built by using the sequences of cpDNA *trnL* intron and *trnL-trnF* region. Maximum-likelihood (ML) algorithm was employed (bootstrapping number = 1000).

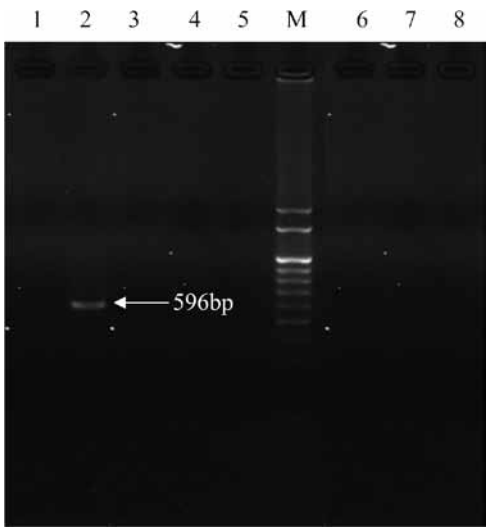

Figure 5. PCR amplifications using the *D. huoshanense*-specific primers.
Lane 1: *D. nindii,* lane 2: *D. huoshanense,* lane 3: *D. aurantiacum,* lane 4: *D. moniliforme,* lane 5: *D. linawianum,* lane 6: *D. cumulatum,* lane 7: *D. tosaense,* lane 8: *D. officinal*.M: 100 bp marker.

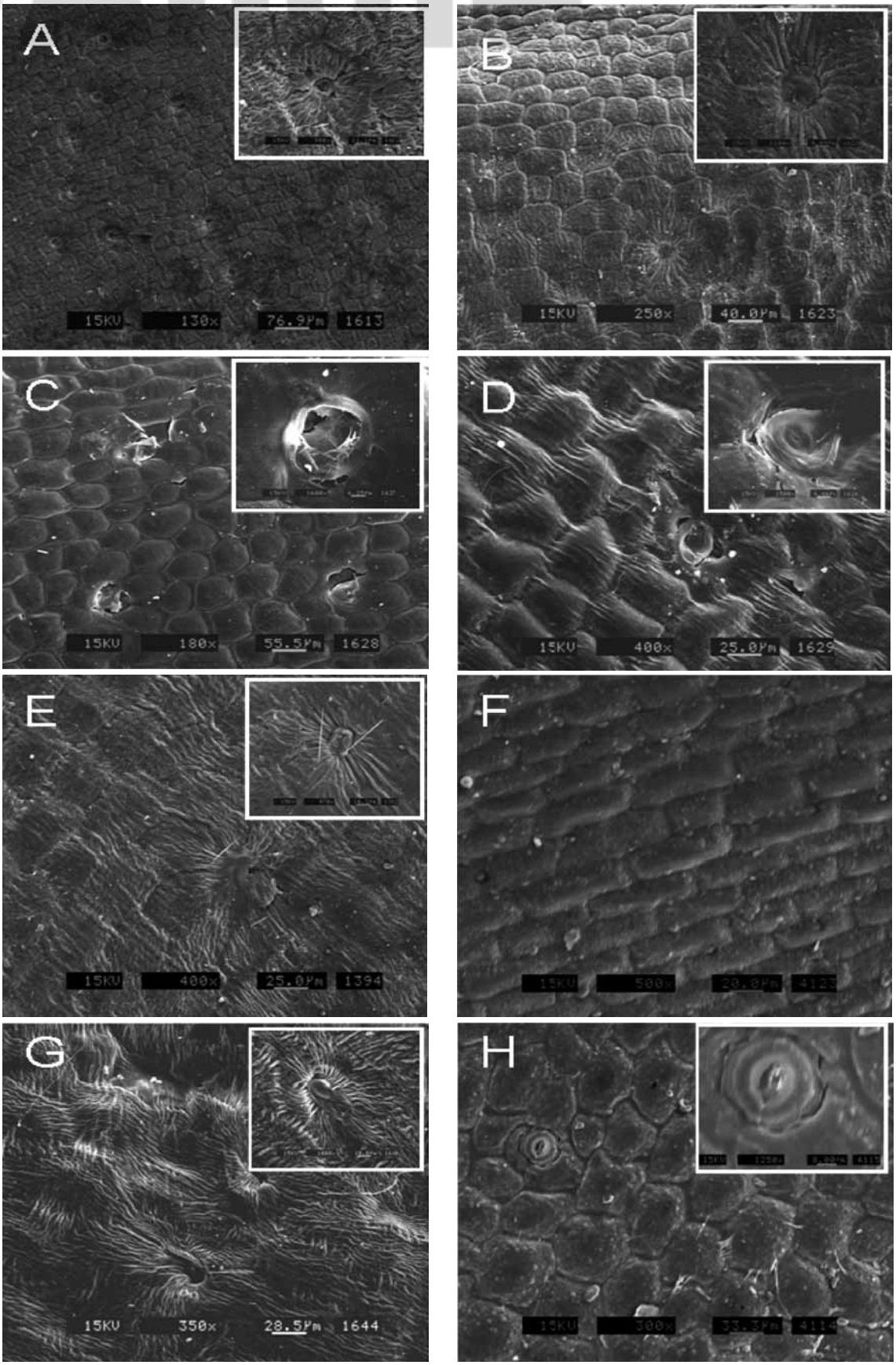

Figure 6. Characteristics of upper epidermal cells observed under a scanning electro microcope (SEM).
A, *D. officinale*; B, *D. tosaense*; C, *D. cumulatum*; D, *D. linawianum*; E, *D. moniliforme*; F, *D. aurantiacum*; G, *D. huoshanense*; H, *D. nindii.*

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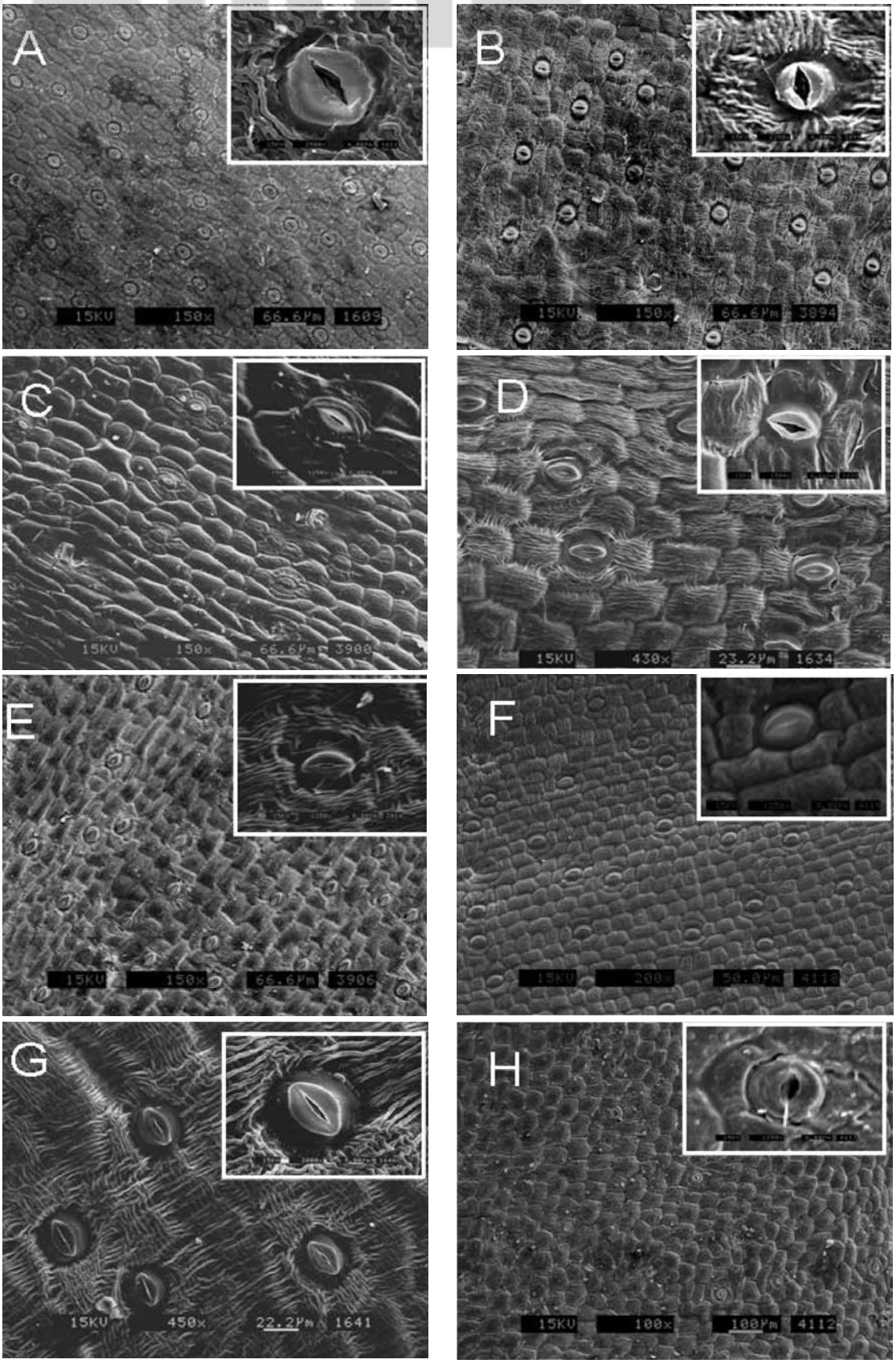

Figure 7. Characteristics of epidermal cells under scanning electro microcopy (SEM).
A, *D. officinale*; B, *D. tosaense*; C, *D. cumulatum*; D, *D. linawianum*; E, *D. moniliforme*; F, *D. aurantiacum*; G, *D. huoshanense*; H, *D. nindii*.

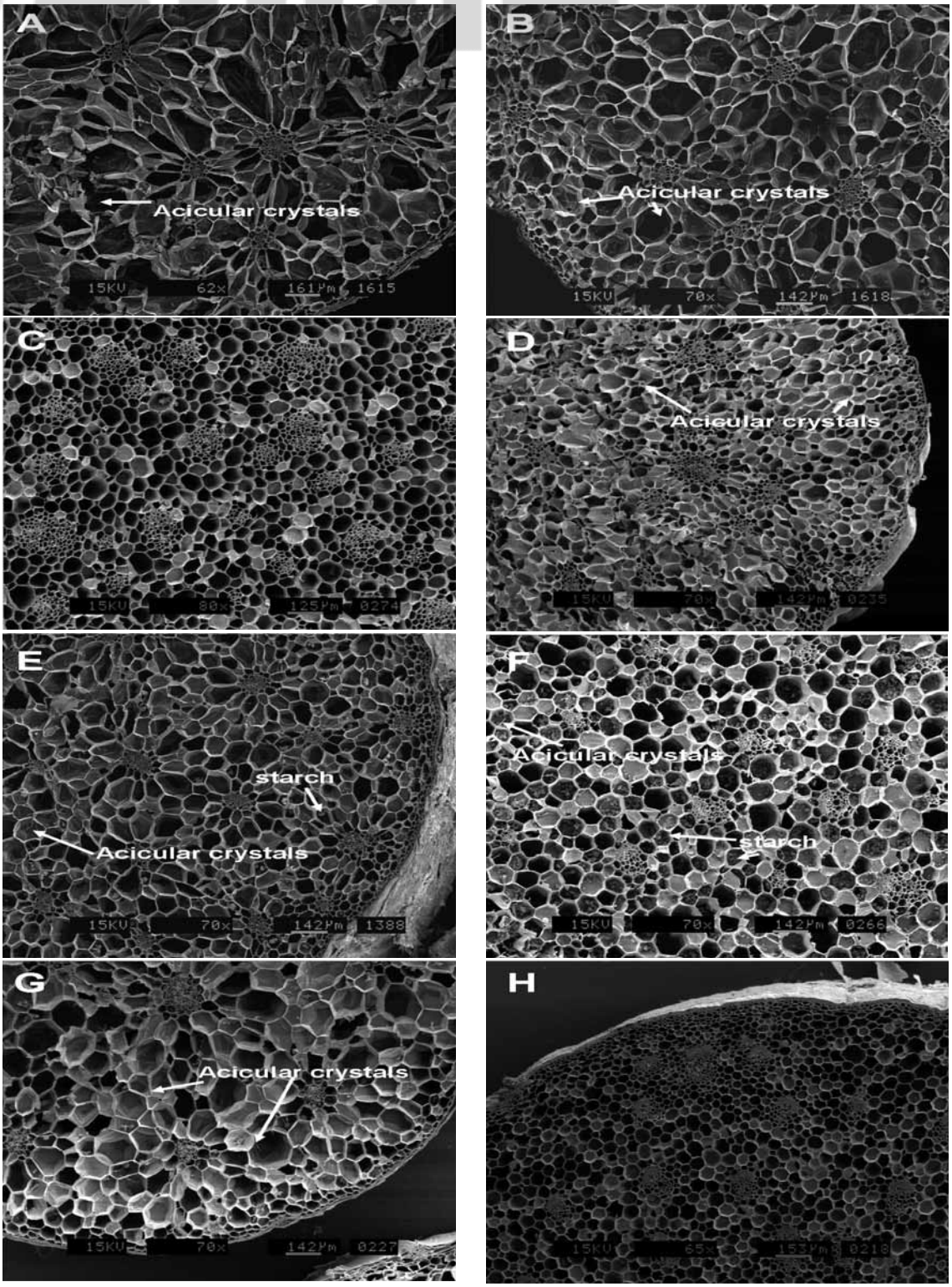

Figure 8. Observation of stem structure of *Dendrobium* species of by *s*canning electro microcopy (SEM).
The arrow indicates the raphides crystal and starch grain in the stem structure. A, *D. officinale*; B, *D. tosaense*; C, *D. cumulatum*; D, *D. linawianum*; E, *D. moniliforme*; F, *D. aurantiacum*; G, *D. huoshanense*; H, *D. nindii.*

The sequence alignment suggests that *D. huoshanense* has a specific and unique fragment in the cpDNA *trn*L intron/*trn*L-*trn*F IGS region. The reproducibility and consistence of this newly developed marker were confirmed in our work. Five authentic *Dendrobium huoshanese* from different sources were tested and a

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unique 596 bp band can be detected from all *Dendrobium huoshanese*. The existence of the unique fragment in *D. huoshanense* allows for the detection of adulteration in medicines that contain *Dendrobium* species (Figure 5).

III. *Morphological Analysis by Scanning Electron Microscopy (SEM)*

Characteristics of the stems and epidermal structures of *Dendrobium* species were observed by SEM. The cells in the upper epidermis of *Dendrobium officinalem*, *D. tosaense*, *Dendrobium crumenatum*, *D. linawianum*, and *D. aurantiacum* were polygonal (Figure 6), while those of *D. moniliforme*, *D. huoshanense*, and *D. nidii* were irregular. Diameter of the stoma of *D. officinalem*, *D. tosaense*, *D. linawianum*, and *D. aurantiacum* was approximately 25.61 μm; the stoma size in *D. crumenatum* and *D. nidii* was 27 μm, while it was 17 μm in *D. moniliforme* and *D. huoshanense* (Figure 7). All the guard cells in *Dendrobium* species were ellipsoidal. The vascular bundles of the stems were full of starch grains and acicular crystals in many *Dendrobium* species, such as *D. officinalem*, *D. tosaense*, *D. linawianum*, *D. moniliforme*, *D. aurantiacum*, and *D. huoshanense* (Figure 8). The epidermis of the stem of *D. moniliforme* and *D. huoshanense* has a thick golden cuticle; this finding was in agreement with that observed by Li *et al*.[7]. On the basis of the morphologies of the epidermal cells, stomata, guard cells, and stem, the medicinal and ornamental *Dendrobium* species can be divided into different groups. The main morphological difference between medicinal and ornamental *Dendrobium* species is the number of starch grains and acicular crystals in the vascular bundles of the stem.

CONCLUSIONS

The phylogenetic relationship of the 8 commonly used *Dendrobium* species was determined based on the patterns of ribosomal DNA ITS regions, chloroplast DNA *trn*L intron and *trn*L-*trn*F IGS. To distinguish among the various *Dendrobium* species, a novel *D. huoshanense*-specific primer set was designed. SEM revealed that the number of mucous cells and acicular crystals in vascular bundles could be a useful feature to align putatively related groups and differentiate medicinal *Dendrobium* species from ornamental ones.

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