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Phylogenetic study of some major *Dendrobium* species of Eastern Himalaya using internal transcribed spacer marker

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ABSTRACT

The entire internal transcribed spacer (ITS) region sequencing was used to determine the phylogenetic relationship of 44 Eastern Himalayan *Dendrobium* species using the Sanger method and MEGA 11. The combined length of ITS-I and ITS-II varies between 623 and 644 base pairs (bp). Among them, there are 403 polymorphic, 91 unique, and 223 conserved nucleotides. In addition, 337 nucleotides are parsimony informative, with a mean of 0.51 that supports the presence of genetic variation in the genome of the selected species. Overall composition distance is 0.32, transition and transversion bias (R) 1.444, and mean evolutionary divergence (d) 0.16. The length of ITS-2 sequences is bigger than that of ITS-I. ITS-II exhibits a higher G+C% content, and the K2+G model is the most suitable, with the lowest penalty for nucleotide substitutions. The average consistency, retention, composite value, and parsimony-informative sites (in parentheses) are 0.5028 (0.4571), 0.6545 (0.6545), and 0.3290 (0.2992), respectively. The mean value of the disparity indices is 0.192, which measures the variation in evolutionary trends between two sequences. In addition, the ratio of non-synonymous to synonymous substitutions (dN/dS) is −0.33 promotes purifying species selection. The prevalence of the TGT codon is abundant across the sequences, with the UCG codon exhibiting the highest relative synonymous codon usage (RSCU). Conversely, the GAC codon has the lowest RSCU value. The greatest genetic divergence occurred between *Dendrobium aphyllum* and *Dendrobium denudans*. The *Breviflorus* species group has the most genetic diversity, whereas *Formosae* has the least. Between Grastidium and *Stachyobium* group sequences had the highest evolutionary distance, whereas *Dendrobium* and Grastidium had the least. The negative value of Tajima's *D*, Fu and Li's D*, F*, and Fu's Fs tests indicated current population is growing after facing a recent bottleneck. This study suggests that ITS sequencing could be a major focus of comparative sequencing at the generic level in *Dendrobium* plants and could provide valuable phylogenetic reconstruction for clarifying the evolutionary relevance of the taxa studied.

1. INTRODUCTION

Dendrobium with over 1500 species present in the eastern and southeastern countries of the world is the second-largest genus of the orchid family [1]. Since that genus is broad, biologically diversified, and taxonomically complicated, various scientists have divided it into many sections and sub-sections at different times [2]. There are 124 different *Dendrobium* species in India, making it the country's second-biggest orchid genus [3]. The widespread distribution, enormous diversity, and great commercial and horticultural importance of *Dendrobium* species and hybrids all contribute to the plant's widespread fascination [4]. On the other hand, modern research shows that polysaccharides, alkaloids, amino acids, trace elements, and other active ingredients found in *Dendrobium* are pharmaceutically important [5]. Due to the huge diversity at different levels of expression, nowadays *Dendrobium* is considered a model for the study of biodiversity *in situ* and for biotechnological improvement [6]. Based on morphological important features, *Dendrobium* species variability and diversity were widely examined. However, the morphological characters are insufficient to clearly separate individuals. Therefore, the best way to measure the variability and diversity within a genus or a population is a resolution by molecular marker, particularly DNA marker. One notable benefit of employing a molecular marker lies in its exceptional purity, as it is unaffected by environmental factors. At present, there exists a wide array of DNA molecular markers. Among these markers, the internal transcribed spacer (ITS) of nuclear rDNA is commonly employed as a molecular tool. This is due to its ability to offer a substantial amount of information for systematic classification [7]. In addition, it is easy to analyze current variations within and among species.

Clusters of hundreds or thousands of tandemly repeated copies of the genes for plant major ribosomal RNA (rRNA) are found on nuclear ribosomal DNA (nrDNA) in the cell nucleus. The ITS sequences

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are the most important segments of nrDNA and can be used for the identification of biological organisms in lower taxonomic hierarchical levels [8]. The 18S-5.8S and 5.8S-28S regions of ribosomal DNA make up ITS-I and ITS-II, respectively, which are shorter than 300 base pairs. During transcription of rRNA genes, ITS-I and ITS-II are transcribed as RNA transcripts, but they are neither translated as protein nor incorporated as a structural component of the ribosome, so it is free to vary. They seem to be involved in the development of nuclear rRNAs by placing the large and tiny components of the RNAs close together in a processing area [9]. Clements (2003) utilize ITS to do an evolutionary study on the subtribe *Dendrobiinae*. Numerous studies have used the ITS conserved sequence to characterize and establish the genetic relationships between diverse *Dendrobium* species [10-15]. Taken together, these results point to a complicated and very diverse range of genetic history at the species level in *Dendrobium* spp. This article shows the findings of a study of nrDNA sequences from 44 species of Eastern Himalayan *Dendrobium*. The sequences cover the 5.8S segment and two gaps on either side which are ITS-I and ITS-II. Based on the conserved sequences, the variability of sizes and sequences in a base pair, the percentage of alignment, and the G+C content of the conserved genome of *Dendrobium* species were studied and usefully exploited to correlate such findings to understand their genetic or phylogenetic relatedness and the probable cladistic position within the selected species of that genus.

2. MATERIALS AND METHODS

Forty-four different epiphytic species of *Dendrobium* were collected during their new growth time, i.e., sprouting of new and fresh leaves taken from the different elevations of Darjeeling, Sikkim, Arunachal Pradesh, Assam, and Manipur Hills of the Indian part of Eastern Himalaya. The 44 members are derived from nine sections, i.e., Dendrobium (21) Grastidium (1), Breviflores (2), Densiflorum (6), Holochrysa (5), Stachyobium (2), Calcarifera (1), Formosae (5), and Aporum (1) [Table 1]. *Bulbophyllum inunctum* and *Bulbophyllum macranthum* xanthine dehydrogenase gene sequences were collected from the gene bank and utilized as outgroups [16] in the analyses. Gene bank reference numbers of the ITS regions of each specimen studied here except the out-group used in the analyses are the outcome of our research work.

2.1. Genomic DNA Isolation

0.260 mg of young and fresh leaf tissue were collected in triplicate and ground with mortar and pestle. The hexadecyl trimethyl ammonium bromide procedure was used to extract DNA from the tender leaves [17].

2.2. Primer Designing and Polymerase Chain Reaction (PCR) Amplification

The quality of the genomic DNA was analyzed by injecting it into an agarose gel with a concentration of 1.2%. PCR amplification with two bilateral degenerate primers was used to amplify the whole nrDNA ITS region from each species' genomic DNA. Both the forward and reverse primer sequences are as follows 5'-GGAAGGAGAAGKCGKARCWASG-3' and 5'-TCCTCCGCTTATWGRTMYKC-3' for ITS5 and ITS4, respectively. Every cycle of PCR requires separation of the template DNA (94°C), annealing of the primers (52°C), and finally, extension of primers (72°C) to continue up to 35 cycles. The solution mixture for PCR amplification included genomic DNA (1 µL), ITS forward and reverse primers (200 ng each), and dNTPs (2.5 mM each). For the reaction, add 2 µL, ×10 Taq DNA polymerase assay buffer (0.5 µL), Taq DNA polymerase enzyme (3 U/µl) 0.5 µL, water was added, and adjust the total volume to 50 µL. Quality was checked by loading PCR products on 1.2% agarose gel [Figure 1] where the first (L_1) is 100 bp and the last lanes (L_2) of the gel have 1Kb DNA ladders, and finally bi-directionally sequenced the PCR products.

2.3. Sequencing of DNA

The sequencing reaction mixture consisted of Bigdye terminator V.3.1. ready reaction mix (0.4 µL/µL), template (0.1 µg/µL), 2 µL primer (10 pmol/λ), and sterile water (3 µL). For further sequencing, the PCR procedures were initially denaturation at 96°C for 1 min, denaturation continued for 10 s (96°C), hybridization for 5 s (50°C), and finally, elongation was done for 4 min at 60°C. The PCR reaction was achieved as 25 thermal cycles. The PCR outcomes were sequenced (~760 bp) on an ABI 3500 XL Genetic Analyzer.

2.4. Sequencing Data Alignment, Construction of Pairwise Distance Matrix, Phylogenetic Tree, and other Statistical Analysis

The sequence data were aligned by the MUSCLE alignment algorithm. Sequence statistics, nucleotide diversity, selected model-based evolutionary analyses, construction of pairwise distance matrixes, and dendrogram preparation were conducted in MEGA 11 using the Maximum Composite Likelihood model, neighbor-joining algorithm, Tamura-Nei model-based Maximum Likelihood (ML) Technique, Kumar method, and Bayesian-based parsimony method [18]. The neutrality test statistics, specifically Fu and Li's and Tajima's *D*, were computed using the software DnaSP V.5.10.01 [19].

3. RESULTS

3.1. Nucleotide Based Diversity

Purified genomic DNA was amplified using ITS-compatible primer and the quality was checked by loading PCR sequences on 1.2% agarose gel, usually yielding a single band in all cases for each sample [Figure 1]. The ITS fragment (in-between the 18S and 28S rDNA) that was amplified includes segments of the 18S and 5.8S rDNA. The total size of the first internal transcribed spacer (ITS-I) ranged from 216 to 234 base pairs (bp), with a mean of 230.09 bp. The second internal transcribed spacer (ITS-II) had lengths ranging from 237 to 248 bp, with a mean of 244.66 bp. The 5.8S rDNA was 163 bp in length, and this length was shown to be stable and conserved across all species [Table 1]. ITS-I (G+C %) ranged from 43.53 to 54.58 across taxa. ITS-I has an average G+C content of 50.05%. ITS-II had a mean G+C content of 52.11% and a range of 44.31% to 55.28%. The ITS-II region has a comparatively higher percentage (G+C) than the ITS-I region [Table 1] with an overall composition distance of GC% 52.82 [Supplementary Table 1]. Each *Dendrobium* species was discovered to have a distinct sequence in the ITS-I and ITS-II regions, allowing for easy distinction between them genetically, as this data shows that interspecific DNA sequence variation is relatively strong. *Dendrobium* is a molecularly natural taxon since the lengths of the ITS-I to ITS-II sections in the 44 *Dendrobium* species ranged from 623 to 644 bp with an average of 637.7 bp, which is in agreement with earlier research [Supplementary Table 1]. All selected *Dendrobium* species have 403 polymorphic, 223 conserved, and 337 parsimony informative nucleotides. Single-ton bases number 91 and informative site substitutions average 0.51. Overall composition distance is 0.32, transition and transversion ratio 1.63, and mean evolutionary divergence (d) 0.16 [Supplementary Table 1]. In total, ML fits were performed on 24 nucleotide substitution models. The K2+G model was the only one with a minimum BIC value

Table 1: Sizes (bp), and G+C% of ITS-I, ITS-II (individually), and cumulative ITS-I, 5.8s, and ITS-II of 44 *Dendrobium* species of Eastern Himalaya.

S. No.	Accession No.	Name of Plant	Sections	Size (bp)			G+C %		
				ITS I	ITS II	ITS I-5.8s-ITS II	ITS I	ITS II	ITS I-5.8s-ITS II
1.	KX600499	*Dendrobium aduncum* Lindl.	Breviflores	232	242	637	51.72	52.89	53.85
2.	KX600515	*Dendrobium amoenum* Wall. ex Lindl.	Dendrobium	229	246	638	51.96	54.47	54.7
3.	KX600514	*Dendrobium aphyllum* R. Brown.	Dendrobium	231	244	638	45.89	52.05	50.94
4.	KX600501	*Dendrobium bellatulum* Rolfe.	Formosae	230	247	640	53.04	52.63	54.37
5.	KX792018	*Dendrobium bicameratum* Lindl.	Breviflores	230	245	639	51.3	52.6	53.8
6.	KX522648	*Dendrobium capillipes* Rchb. f.	Holochrysa	228	247	638	50.88	49.39	51.72
7.	KX792016	*Dendrobium cathcartii* (Hook. f.) M.A.Clem. and D.L. Jones *Dendrobium salaccense* Lindl. (Synonym)	Grastidium	228	246	637	52.1	54.4	54.7
8.	KX522638	*Dendrobium chrysanthum* Wallich ex Lindley.	Dendrobium	233	241	638	51.50	52.70	53.60
9.	KX522645	*Dendrobium chrysotoxum* Lindley.	Densiflorum	231	245	640	52.81	55.10	55.31
10.	KX522644	*Dendrobium crepidatum* Lindl.	Dendrobium	225	244	632	48.44	50.41	51.42
11.	KX600510	*Dendrobium cumulatum* Lindl.	Calcarifera	230	237	631	53.48	52.32	53.72
12.	KX600511	*Dendrobium denneanum* Kerr. *Dendrobium clavatum* Wall. ex Lindl.(Synonym)	Holochrysa	233	244	640	49.78	50.82	52.34
13	KX522633	*Dendrobium densiflorum* Lindl. ex Wall.	Densiflorum	232	247	643	50.00	52.23	53.18
14.	KX600504	*Dendrobium denudans* D. Don.	Stachyobium	232	246	641	43.53	46.75	48.05
15.	KX522643	*Dendrobium devonianum* Paxton.	Dendrobium	233	244	641	44.63	49.18	49.76
16.	KX792014	*Dendrobium draconis* Rchb. f.	Formosae	229	247	640	52.4	51.0	53.0
17.	KX600498	*Dendrobium falconeri* Hook. f.	Dendrobium	231	245	639	48.48	51.02	51.96
18.	KX600516	*Dendrobium farmeri* Paxton.	Densiflorum	229	248	643	47.6	50.4	50.7
19.	KX522634	*Dendrobium fimbriatum* Hooker.	Holochrysa	232	244	640	47.41	46.31	49.53
20.	KX522642	*Dendrobium formosum* Roxb. ex Lindl.	Formosae	230	244	638	53.48	53.69	54.70
21.	KX522636	*Dendrobium gibsonii* Paxton.	Holochrysa	229	243	635	49.78	51.44	52.44
22.	KX522646	*Dendrobium gratiosissimum* Rchb. f.	Dendrobium	229	246	639	54.58	54.88	55.87
23.	KX600513	*Dendrobium heterocarpum* Wall. ex Lindl.	Dendrobium	232	242	637	51.29	50.41	52.75
24.	KX509992	*Dendrobium hookerianum* Lindl.	Dendrobium	216	244	623	48.61	50.82	52.01
25.	KX600506	*Dendrobium jenkinsii* Wall. ex Lindl.	Densiflorum	234	246	643	46.58	48.37	50.23
26.	KX522640	*Dendrobium lindleyi* Steud.	Densiflorum	228	247	638	48.68	48.58	51.10
27.	KX792015	*Dendrobium lituiflorum* Lindl.	Dendrobium	233	243	639	52.7	51.4	53.9
28.	KX509991	*Dendrobium longicornu* Lindl.	Formosae	230	247	640	53.48	52.23	54.22
29.	KX792017	*Dendrobium moniliforme* (L.) Sw. *Dendrobium candidum* Wall. ex Lindl.(Synonym)	Dendrobium	233	244	640	52.7	51.6	53.9
30.	KX522635	*Dendrobium moschatum* Sw.	Holochrysa	232	244	640	47.41	46.31	49.53
31.	KX600497	*Dendrobium nobile* Lindl.	Dendrobium	227	246	636	53.3	55.28	55.5
32.	KX509993	*Dendrobium nobile* var. pendulum	Dendrobium	231	242	636	50.22	52.89	52.99
33.	KX495131	*Dendrobium nobile* var. varginalis	Dendrobium	231	242	636	49.78	52.89	52.83
34.	KX522637	*Dendrobium ochreatum* Lindl.	Dendrobium	231	244	639	45.45	52.87	51.33
35.	KX522639	*Dendrobium parishii* Rchb. f.	Dendrobium	229	244	637	46.72	54.51	52.75
36.	KX600502	*Dendrobium pendulum* Roxb.	Dendrobium	228	246	637	49.56	52.84	52.9
37.	KX600505	*Dendrobium porphyrochilum* Lindl.	Stachyobium	231	246	640	44.15	44.31	47.19
38.	KX522641	*Dendrobium primulinum* Lindley.	Dendrobium	231	244	639	45.45	52.87	51.33
39.	KX600507	*Dendrobium ruckeri* Lindl.	Dendrobium	230	246	639	52.61	54.88	55.09
40.	KX522647	*Dendrobium terminale* Par. and Rchb. f.	Aporum	232	248	644	48.71	53.63	52.95
41.	KX600503	*Dendrobium thyrsiflorum* Rchb. f.	Densiflorum	231	247	641	49.35	53.44	52.89
42.	KX600508	*Dendrobium transparens* Wall.	Dendrobium	228	246	637	53.07	52.84	54.47
43.	KX600500	*Dendrobium wardianum* Warner.	Dendrobium	231	242	636	49.78	52.89	52.83
44.	KX522632	*Dendrobium williamsonii* J. Day and Rchb. f.	Formosae	229	243	635	51.96	52.26	53.38

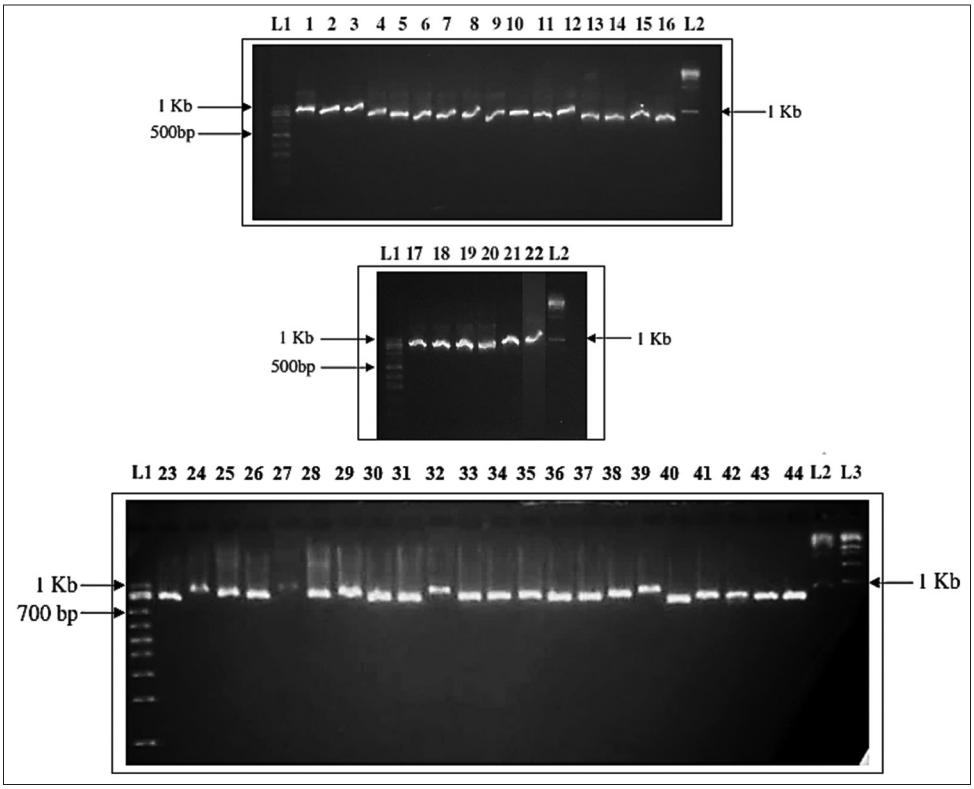

Figure 1: nrDNA internal transcribed spacer polymerase chain reaction region amplification results of *Dendrobium* spp. 1. *Dendrobium nobile* var. *varginalis*, 2. *Dendrobium longicornu*, 3. *Dendrobium hookerianum*, 4. *Dendrobium nobile* var. *pendulum*, 5. *Dendrobium draconis*, 6. *Dendrobium williamsonii*, 7. *Dendrobium densiflorum*, 8. *Dendrobium fimbriatum*, 9. *Dendrobium moschatum*, 10. *Dendrobium gibsonii*, 11. *Dendrobium ochreatum*, 12. *Dendrobium chrysanthum*, 13. *Dendrobium parishii*, 14. *Dendrobium lituiflorum*, 15. *Dendrobium primulinum*, 16. *Dendrobium formosum*, 17. *Dendrobium devonianum*, 18. *Dendrobium crepidatum*, 19. *Dendrobium chrysotoxum*, 20. *Dendrobium gratiosissimum*, 21. *Dendrobium terminale*, 22. *Dendrobium capillipes*, 23. *Dendrobium nobile*, 24. *Dendrobium falconeri*, 25. *Dendrobium aduncum*, 26. *Dendrobium wardianum*, 27. *Dendrobium bellatulum*, 28. *Dendrobium cathcartii*, 29. *Dendrobium thyrsiflorum*, 30. *Dendrobium denudans*, 31. *Dendrobium porphyrochilum*, 32. *Dendrobium jenkinsii*, 33. *Dendrobium moniliforme*, 34. *Dendrobium transparens*, 35. *Dendrobium lindleyi*, 36. *Dendrobium cumulatum*, 37. *Dendrobium denneanum*, 38. *Dendrobium bicameratum*, 39. *Dendrobium heterocarpum*, 40. *Dendrobium aphyllum*, 41. *Dendrobium amoenum*, 42. *Dendrobium farmeri*, 43. *Dendrobium pendulum*, 44. *Dendrobium ruckeri*. L_1 = 100bp DNA ladder, L_2 = 1kb DNA ladder, DNA ladder run on 1.2% gel electrophoresis.

(13172.666) to be considered the top nucleotide substitution pattern model [Supplementary Table 2]. Molecular evolution among chosen *Dendrobium* species was calculated by estimating the ML substitution matrix to see the substitution probabilities that led to the most likely model of evolution [Supplementary Table 3]. A tree topology was automatically constructed for Maximum Likelihood (ML) estimation. This calculation yielded the highest log-likelihood of −8102.528. Comparisons of immediate r value ($\sum r = 100$) were performed throughout the assessment and revealed nucleotide percentages of A = 23.70, T = 23.48, G = 29.11, and C = 23.71 [Supplementary Tables 1 and 3] and the transitions exceed transversionsal nucleotide replacements [Table 4]. The total transition/transversion bias (R) is 1.444, with pyrimidine substitution (k_2) being more common than purine (k_1) [Supplementary Table 1]. Using Maximum Parsimony, we were able to piece together the evolutionary past. All sites and parsimony-informative sites (enclosed in brackets) have indices of 0.5028 (0.4571), 0.6545 (0.6545), and 0.3290 (0.2992), respectively, for consistency, retention, and composite [Supplementary Table 1]. Both ML and Baseyan analysis methods yielded great congruence. Genome sequence substitution patterns were tested for homogeneity. The disparity indices range from 0.00 to 1.89 with a mean of 0.192 which quantifies the present evolutionary differences between sequences [Supplementary Table 1]. The yellow color reading indicated a significant $P \leq 0.05$ rejecting the null hypothesis of homogeneous substitution [Supplementary Table 8]. Codon-based neutrality screening was performed among sequences with an estimated mean value (d_N/d_S) −0.33 [Supplementary Table 1]. As shown in Supplementary Table 9, there is a good chance that ruling out the possibility that dN=dS, the rigorous neutrality null hypothesis ($\alpha \leq 0.05$, marked as yellow color). In the same way, an elevated dN–dS ratio and positive readings of the statistic indicate an excessive occurrence of nucleotide base substitutions. Among the species that were selected for analysis, it was observed that the TGT triplet codon (Codon Omega reading) exhibited a notably higher dN–dS value of 4.973, indicating significant ($P < 0.05$) non-synonymous substitutions [Supplementary Table 10]. The codon use bias of the chosen *Dendrobium* genomes was determined by calculating relative synonymous codon usage (RSCU) values for 64 synonymous codons. Overrepresented (RSCU average > 1.6) was the codons UUG, CUC, AUC, GUC, UCG, UAU, CAA, GAU, and GAA whereas underrepresented (RSCU average <0.6) was the codons UUC, UUA, CUU, CUA, GUA, UCU, UCA, CCU, ACU, UAC, UAG, CAC, CAG, GAC, GAG, CGG, and AGU [Supplementary Table 11]. The other 38 codes were moderately expressed. The occurrence of triplet codes ending with A and C is more frequent compared to those ending

with G and U/T. In addition, the RSCU values (3rd codon of triplet) for the codons C, A, G, and U are 17.65, 16.75, 14.93, and 14.64, respectively. Notably, the codon UCG exhibits the highest RSCU value of 2.68, whereas the codon GAC has the lowest value of 0.16. The codon with the lowest frequency seen is AGU, which occurs at a rate of 0.4. Conversely, the codon with the highest frequency observed is GGC, which occurs at a rate of 7.5 [Supplementary Table 11]. Comparing the relative fixing rates of synonymous and non-synonymous alteration helps explain molecular sequence evolution.

3.2. Comparing Evolutionary Variations

The calculation of genetic distance arises when the dissimilarity between two genes is proportional to the duration of their divergence from a shared ancestral population. The amount of substituted nucleotide per site between sequences is displayed. Calculations used the Maximum Composite Likelihood model. This investigation examined using ITS sequence of 44 *Dendrobium* species where minimum divergence was calculated (0.000) among *Dendrobium moschatum* and *Dendrobium fimbriatum*, *Dendrobium ochreatum* and *Dendrobium primulinum*, *Dendrobium nobile* var. *varginalis,* and *Dendrobium wardianum.* The maximum genetic divergence (0.276) was found between *Dendrobium aphyllum* and *Dendrobium denudans* [Supplementary Table 12]. Intra-section (within a group) and inter-section (among different groups) genetic differences can be calculated using the Jukes–Cantor pairwise genetic distance analysis algorithm. The *Breviflores* species group exhibits the highest level of genetic diversity (0.171), whereas the *Formosae* section of *Dendrobiums* displays the lowest level (0.045) of genetic diversity [Supplementary Table 4]. The calculation of evolutionary diversity within the taxonomic groups *Aporum*, *Calcarifera*, and Grastidium is hindered by the limited number of species available for analysis. The evolutionary distance between *Dendrobium* and Grastidium group sequences was the smallest (0.117), whereas the distance between Grastidium and *Stachyobium* group sequences was the greatest (0.285) [Supplementary Table 5]. Species belonging to the Grastidium section exhibit the highest genetic diversity when compared to the *Stachyobium* and the minimum when compared to the *Dendrobium* groups. This diversity is reflected in their distinct physical features. These features include elongated and rounded stems, thin and lens-shaped flat leaves, and short peduncles from which inflorescences emerge at leafy stem nodes found in the members of Grastidium [20].

3.3. Analyzing Phylogenetic Tree

The dendrogram [Figure 2] shows that 21 species were included in the section Dendrobium and other members belong to sections Grastidium (1), Breviflores (2), Densiflorum (6), Holochrysa (5), Stachyobium (2), Calcarifera (1), Formosae (5), Aporum (1). The gene bank supplied the outgroup species *Bulbophyllum inunctum* and *Bulbophyllum macranthum* for the studies. There are five distinct sub-clades within the *Dendrobium* section. The first subclade is comprised the species *Dendrobium amoenum*. *Dendrobium nobile* makes up the second subclade. The third subclade includes *Dendrobium transparens*, *Dendrobium gratiosissimum*, *Dendrobium nuckeri*, *Dendrobium pendulum*, and *Dendrobium falconeri* whereas *Dendrobium heterocarpum*, *Dendrobium nobile* var. *pendulum*, *Dendrobium nobile* var. *varginalis*, *D. wardianum*, *D. aphyllum*, *Dendrobium parishi*, *D. ochreatum*, *Dendrobium primilinum*, *Dendrobium chrysanthum*, *Dendrobium crepidatum*, *Dendrobium devonianum*, *Dendrobium moniliforme,* and *Dendrobium lutiforum* are included in the fourth subclade. The fifth subclade consists of only *Dendrobium hookerianum* [Figure 2]. The ML-based ancestral tree reveals the presence of four distinct subclades within the *Dendrobium* section. It is seen that both *D. amoenum* and *D. nobile* have a common ancestor. The shared progenitor of the *Dendrobium* species exhibits the nucleotide "A" in the third position of the codon, a modification that occurred in a more recent ancestor, replacing the nucleotide "C." In ancient times, the original foundation was referred to as "A" [Supplementary Figure 1]. However, the second subclade of *Dendrobium* species exhibits a nucleotide substitution from "A" to "C," which bears similarity to the molecular features observed in section Grastidium. In *D. chrysanthum*, a notable transition occurs in the nucleotide base from its immediate ancestor, where the base "A" undergoes an abrupt alteration to "C" [Supplementary Figure 1]. Section Grastidium contains a single species, i.e., *Dendrobium cathcartii* shearing the common ancestor of *D. amoenum* and *D. nobile* [Figure 2].

Section Breviflores consists of two subclades containing a single species in each subclade, i.e., *D. aduncum* and *Dendrobium bicameratum*, respectively. Three subclades are noted in the section *Densiflorum*. *Dendrobium chrysotoxum* forms a subclade. However, *Dendrobium jenkensii* and *Dendrobium lindeyi* constitute together the second subclade. The third subclade includes *Dendrobium densiflorum*, *Dendrobium thyrsiflorum,* and *Dendrobium farmerii*. Section Holochrysa includes three subclades. *Dendrobium denneanum*, *D. fimbriatum*, and *D. moschatum* belong to the first subclade. *Dendrobium capillipes* is in the second subclade. The third subclade contains a single species, i.e., *Dendrobium gibsonii.* Section *Stachyobium* has no subclade in the present investigation and contains only two species *D. denudans* and *Dendrobium porphyrochilum.* Section *Calcarifera* has only the species *Dendrobium cumulatum* and has no other subclades. Section *Formosae* includes five species *Dendrobium williamsonii*, *Dendrobium longicornu*, *Dendrobium bellatum*, *Dendrobium draconis*, and *Dendrobium formosum* under one subclade. Only one species *Dendrobium terminale* belongs to section *Aporum* [Figure 2]. The first, second, and third subclades of the section *Dendrobium* and section Grastidium are monophyletic. On the other hand, the first subclade of the section Breviflores and *D. heterocarpum*, *D. nobile* var. *pendulum*, *D. nobile* var. *varginalis*, *D. wardianum* of the fourth subclade under section *Dendrobium* also constitute the second monophyletic group. However, *D. aphyllum*, *D. parishi*, *D. ochreatum*, and *D. primilinum* of the fourth subclade under section *Dendrobium* make a separate monophyletic cluster. *D. chrysanthum*, *D. crepidatum*. Moreover, *D. crepidatum, and D. devonianum* under the fourth subclade of the section *Dendrobium* are polyphyletic. *D. moniliforme*, *D. lutiforum,* and *D. bicameratum* of the second subclade of Breviflores make the fifth monophyletic group. *D. chrysotoxum* (section *Densiflorum*), *D. denneanum*, *D. fimbriatum*, and *D. moschatum* (Section Holochrysa) constitute together the sixth monophyletic lineage. Moreover, *D. hookerianum* is a polyphyletic group. *D. capillipes* is also a polyphyletic group. *D. jenkensii*, *D. lindeyi*, *D. denudans*, *D. pophyrochilium*, and *D. cumulatum* make up the seventh monophyletic group but *D. pophyrochilium,* and *D. cumulatum* are polyphyletic. The ninth monophyletic group consists of *D. williamsonii*, *D. longicornu*, *D. bellatum*, *D.draconis*, and *D. formosum*, whereas *D. gibsonii* branches out as a polyphyletic group. *D. terminale*, *D. densiflorum*, *D. thyrsiflorum*, and *D. farmerii* make them the tenth monophyletic group. It is known that *D. capillipes* and *D. densiflorum* do not group with most of the other species in section *Dendrobium* which they resemble with previous data obtained from ITS. *Dendrobium* and Grastidium subclades I, II, and III clustered together to create a single phylogenetic unit. This finding demonstrated the close genetic relatedness between groups. Similarly, the subclades I and II of the section Breviflores and *Dendrobium*

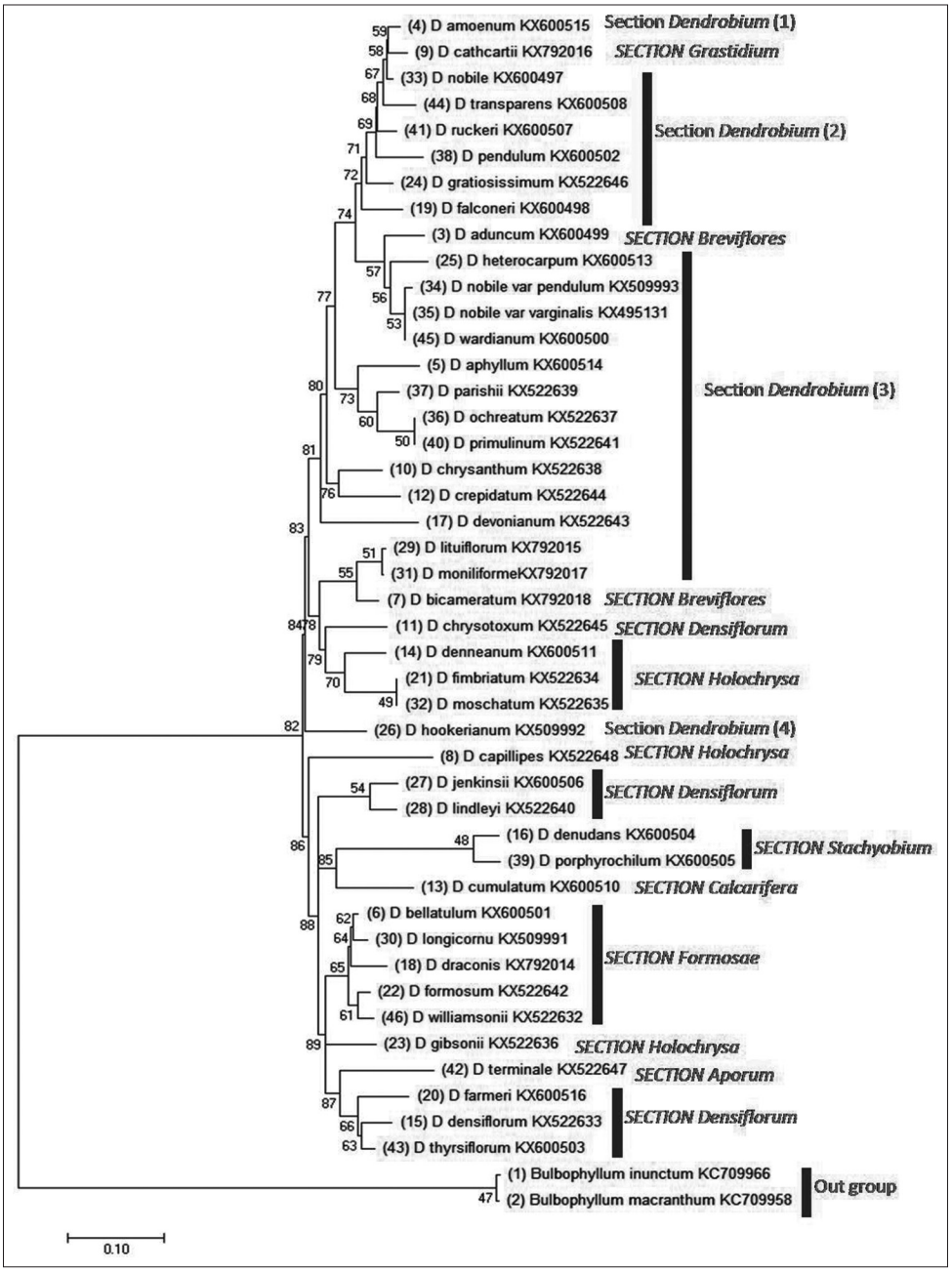

Figure 2: Analysis of molecular phylogeny using the Tamura-Nei model and the Maximum Likelihood approach. Taxa evolution is represented by the bootstrap consensus tree estimated from 1000 repetitions. Branches collapsed for partitions with fewer than 50% bootstrap replicates. The bootstrap test 1000 replicates a proportion of replicate trees with associated taxa clustered next to branches. The Neighbor-Joining approach was used to build initial tree(s) for the heuristic search from a matrix of pairwise distances computed by the Tamura-Nei model. This investigation examined 44 nucleotides. 1st+2nd+3rd+non-coding were codons. Complete deletion removed all gaps and missing data. The final dataset has 548 positions. MEGA11 performed evolutionary analyses.

were nested together indicating their high phylogenetic relatedness. Monophyletic clustering of *D. chrysotoxum* of the subclade I of section *Densiflorum* and species of the first subclade of the section Holochrysa was noted in the dendrogram and they related together with the strong support of the bootstrap value of 79%. *D. hookerianum* of the fifth subclade of the section *Dendrobium* was located in the dendrogram in between the first and second subclade of the section Holochrysa as a polyphyletic group [Figure 2]. This species is distantly related to other *Dendrobium* species [Supplementary Figure 1]. The species under the second subclade of the section *Densiflorum* are also polyphyletic. Section *Calcarifera* and *Aporum* as a paraphyletic group are related to the polyphyletic group of section *Stachyobium* and *Densiflorum,* respectively, [Figure 2 and Supplementary Figure 1]. *D. longicornu* and *D. draconis* under the section of *Formosae* are polyphyletic whereas *D. longicornu, D. bellatum, D. draconis* are monophyletically linked. On the other hand, *D. williamsonii* and *D. formosum* are polyphyletic. The selected orchid species, specifically *D. gibsonii*, belongs to the third subclass within the section Holochrysa and exhibits a direct relationship with the common ancestors of these orchid species [Supplementary Figure 1]. Convergent evolution may have occurred

in this particular orchid. *D. terminale* belonging to section *Aporum* and the rest of the species under the third subclade of *Densiflorum* are genetically closely related and form a monophyletic group.

3.4. Tests for Neutrality

According to the neutrality study, Tajima's *D* result of −0.1697 was not statistically significant ($P > 0.10$) among the selected species [Supplementary Table 6], which is consistent with a neutral evolution model. The results of the D*, F*, and Fu's Fs tests for Fu and Li were −2.80834 ($P < 0.05$), −2.19041 ($0.10 > P > 0.05$), and −4.677 ($P < 0.01$), respectively [Supplementary Table 7]. The findings of Tajima's *D* and Fu and Li's D* test results show a statistical anomaly in the neutral evolution theory. Fu's Fs is highly sensitive to recent population growth and an excess of alleles, therefore its negative value and statistical significance here make logical sense. A value of *D* below 0 indicates that natural selection has recently been at work, and the population has grown since the last bottleneck. While there is substantial evidence indicating that the chosen species are undergoing a selective sweep, it is important to consider the significant D* score.

4. DISCUSSION

Many species of *Dendrobium* are recognized to share a similar appearance. As a result, identifying them dependent on morphological features is challenging, unless they are in bloom. Many *Dendrobium* morphological features appear homoplasious in several molecular investigations [21]. In this investigation, we observed that the DNA segment of ITS may be used to identify individual species, taxonomical markers, and germplasm preservation in *Dendrobium*. Most ITS nucleotides are variable and provide extensive systematic data. ITS-I and ITS-II lengths are retained in one population. It was found that the lengths of ITS-I and ITS-II in angiosperms are typically 187–298 base pairs and 187–252 base pairs, respectively, whereas the length of 5.8S is consistently 163 or 164 base pairs and in angiosperm, ITS nucleotide sequence divergence ranges from 1.2% to 10.2% among species and 9.6–28.8% among genera [9]. The lengths of both the ITS area and the 5.8S region in the current study are consistent with the previously reported standard findings. ITS-I and ITS-II were genetically divergent between *Dendrobium* species, as stated in earlier research and it also indicated better taxonomic identification [22,23]. The ITS sequences have been widely used because of their significance in molecular studies of the orchid family [24]. It is a common tool for tracing the evolutionary history of *Dendrobium* [11,25] and related families across a wide range of taxonomic groups [26,27]. The result shows 223 (34.97%) conserved bases, 337 (52.85%) parsimony informative sites, and 91 (14.27%) single-ton bases number. The mean percentage of the polymorphic site (63.20%), conserved sites (34.97%), and low mean disparity index (0.192) provide evidence that all species are closely related. However, singleton bases serve as the naturally occurring sites for selection or mutation. The transition (S) and transversion (V) ratio (1.63) shows extremely reactive to saturating mutations and the result differs from conventional Orchid ts/tv ratios ranging from 0.66 to 1.02 [28]. The CI, RI, composite index, and parsimony-informative sites (in bract) make it clear that all species can keep evolving and make changes to their genomes that are either synonymous or non-synonymous to fit in with their new surroundings. A small deviation (R = 1.444) from perfectly balanced transition and transversion contents indicates that transition substitutions are more common than transversion [Supplementary Table 1 and 3], as is usual for advanced taxa, and confirms the amino acid conservative theory [29]. The d_N/d_S results support the purifying selection (allelic elimination through selective means) of the selected species. The top ML-based DNA models evaluated the K2+G technique as the best nucleotide substitution pattern model because it had the least BIC values among the 24 models [Supplementary Table 2]. The transition-and-transversion-based Tamura-Nei model (K2+G) hypothesized differences between the species. This suggests that transversional substitution rates are similar and purine-purine and pyrimidine-pyrimidine transitions are employed [30]. Mutations detected through multiple sequence alignment supported these findings. Nucleotide composition showed moderate differences (0.32) among sequences [Supplementary Table 1]. However, the base composition may increase GC% due to ambient nitrogen overabundance [31]. These character distributions are optimum for the maximum log-likelihood (−8102.528) phylogenetic tree. The use of particular codons varies greatly not only between species but also between genes and even within genes at different places. Natural codon use variance for UCG (RSCU= 2.68) can be explained by either mutation or natural selection [Supplementary Table 11]. The variability of the GAC codon is comparatively lower inside the ITS sequences. A sensitive indicator of pressure for selection at the amino acid level is provided by Codon Omega results [Supplementary Table 10]. The TGT codon, which exhibits a significant dN–dS value of 4.973 ($P < 0.05$), is responsible for encoding the amino acid cysteine. Cysteine, encoded by the TGT codon, is an antioxidant that, in *Cymbidium goeringii*, prevents browning and stimulates rhizome growth by blocking the actions of catalase and polyphenol oxidase [32].

The species of *Breviflores* exhibit the greatest level of genetic variety (d = 0.171), as indicated in Supplementary Table 4. In addition, the maximum genetic distance (d = 0.285) is observed between the Grastidium and *Stachyobium* groups [Supplementary Table 5] confirming the finding by their almost opposite morphological characteristics the plant belongs to the group Grastidium and *Stachyobium* [20]. The study of the data has revealed a high degree of branch support, as evidenced by the presence of a large number of parsimony instructive characters. The resolution of clades was high, and species boundaries were clearly defined. The dendrogram [Figure 2] agrees well with results from previous studies [11,12,14]. In phylogenetic tree analysis, the values at each node are bootstrap. These values can be correlated with branch lengths and conclusions on the evolutionary position of taxa can be drawn. The species of *Dendrobium* which have high bootstrap values and relatively smaller branch length are considered the most recent origin and advanced. However, those species that have low bootstrap values but long-branch lengths can be correlated as phylogenetically primitive [33]. All of these species (apart from *D. falconeri*) belong to the first or second subclade of the *Dendrobium* section, the Grastidium section, and *D. chrysanthum* has an unstable AC (adenine and cytosine) intermediate as compared to their ancestors [Supplementary Figure 1]. However, the unsteady AC intermediate is extremely rare and the substitution frequencies were highly variable over the two-step procedure, demonstrating that AC is subject to distinct selective forces from those exerted on GU/T which supports compensatory mutation [34]. The monophyletic status of sects. *Stachyobium*, Grastidium, and *Dendrobium* are supported in the present study. However, Grastidium was poorly represented due to a lack of data. All reasons viewed, and acknowledging that monophyly is one of the limited objective standards for delimiting genera, it seems better to widen *Dendrobium* to encompass all previous segregate genera [27]. In contrast, many infra-generic taxa within the genus *Dendrobium*, namely section *Dendrobium*, section Holochrysa, and section Densiflora exhibit paraphyly or polyphyly. Consequently, a thorough reassessment and redefinition of their taxonomic classification is warranted. The efficacy of ITS sequencing

in *Dendrobium* taxonomy is still little explored, thus we studied the DNA sequencing methodologies and variability of the ITS-I, 5.8S, and ITS-II locations in 44 wild species and their impact on phylogenetic reconstruction. The present findings corroborate those of other research reporting that current sequence data show that the ITS sections of those species exhibit several differentiations, which match with published sequences. This provides further evidence that *Dendrobium* species can be distinguished from one another based on the sequence of their ITS-I and ITS-II segments [35]. A negative value of Tajima's *D* (−0.1697) denotes an overabundance of low-to-moderate polymorphisms (mean 63.20%), in comparison to expected, signifying the size of the population increase following a bottleneck and the observed nucleotide diversity (π) of 0.1377 [Supplementary Table 6] exhibits a close resemblance to previous research findings in the genus *Dendrobium* [36]. In summary statistics (Tajima's *D* and Fu and Li's test), probabilities are simulated values, not calculated. Since these p-values are approximate. The Fu's Fs significant value [Supplementary Table 7] indicates an overabundance of alleles and a possible selective sweep [37]. At present, population growth is the most acceptable explanation for the deviations from neutrality, as supported by the negative values of Tajima's *D*, Fu and Li's D*, F*, and Fu's Fs.

5. CONCLUSION

This study evaluated the ITS sequences of 44 *Dendrobium* species. A significant amount of variation is present in the ITS sequences among the selected species of *Dendrobium*. ML tree analysis strongly supports the possible use of the ITS region for distinguishing morphologically most similar *Dendrobium* species. In the future, more numbers of *Dendrobium* species should be analyzed to confirm the suitability of the ITS region for identifying all *Dendrobium* species and other orchid genera.

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7. AUTHOR CONTRIBUTIONS

Animesh Mondal: Sample collection, laboratory work, assessment of data, and early manuscript writing. Kalyan Kumar De: oversaw the experimentation, verification, review, and editing of the updated manuscript.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the raw data regarding the research are available within this manuscript.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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SUPPLEMENTARY MATERIAL

The supplementary material can be accessed at the journal's website:
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