RESEARCH ARTICLE

Phylogenetic analysis and DNA barcoding of *Luisia tristis* (Orchidaceae): Implications for species identification and biodiversity preservation

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ABSTRACT

Background: Understanding and preserving biodiversity requires effective methods for plant identification. The study aimed to analyze the genetic relatedness of *Luisia tristis*, a species of the Orchidaceae family, and assess the potential of DNA barcoding as a reliable identification technique. *Methods:* In this study, DNA was isolated from leaf samples, amplified, and sequenced for the chloroplast gene *matK* (maturase K). The sequence was uploaded to the NCBI database and subjected to multiple sequence alignment and BLAST analysis to assess its similarity to other close taxa. Using the Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods, their phylogenetic relationships were investigated using MEGA X version 7.0. To verify the statistical robustness, 1000 bootstrap repetitions were used. *Results:* The results were further validated by constructing a phylogenetic tree utilizing sequence data from other orchid species. With sequence similarity values of nearly 98.5%, the analysis revealed that the species clustered into two distinct clades, indicating a close genetic relationship. Based on *matK* divergence patterns, the derived phylogenetic trees indicate that the taxa most likely have a monophyletic origin. A *matK*-specific QR code was generated using Bio-Rad's barcode generator, providing a convenient method for encoding and retrieving genetic information. This study shows the effectiveness of DNA barcoding as a powerful tool for identifying species and preserving orchid taxa that face serious threats. Additionally, the molecular function of the *matK* protein from *L. tristis* was clarified by utilizing EMBOSS software to predict its 3D structure models. *Conclusion:* The result of this study was essential for the easy identification and protection of such orchid species for the future.

Keywords: *Luisia tristis*, Orchid, *matK*, DNA barcoding, phylogenetic tree analysis.

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INTRODUCTION

In the kingdom of plants, the orchid family, scientifically known as Orchidaceae, is one of the largest and most diverse plant groups. This family, comprising more than 800 genera and 25,000 to 30,000 species, is renowned for its visual appeal, ecological adaptability, and floral complexity. A fascinating collection of mostly terrestrial orchids, the Orchidinae is a subtribe of the Orchideae, a large family distinguished for its morphological diversity, adaptive radiation, and unique pollination strategies. Notable genera, including *Orchis*, *Dactylorhiza*, *Habenaria*, and *Platanthera,* are among the Orchidinae, which are found in a variety of climates, ranging from temperate to tropical.[3] It has long been difficult to unravel their evolutionary history due to intergeneric hybridization and morphological convergence. However, discoveries in molecular phylogenetics have fundamentally altered our understanding of this group by shedding light on its taxonomic relationships, biogeography, and speciation.

The orchid species *Luisia tristis* is a member of the genus *Luisia*, a member of the Orchidaceae family, and one of the biggest and most varied flowering plant families. Orchids of this genus are epiphytic and usually grow on other plants, typically trees, instead of soil. The epiphytic herbaceous plant *L. tristis* has a wiry, slender stem that can grow slightly erect or become pendulous.[4] To help the plant receive moisture and nutrients from the atmosphere, its stems are frequently branched and have aerial roots. An essential characteristic of epiphytic orchids is their fleshy, terete (cylindrical), and linear leaves, which are adapted for water conservation[5]. They are frequently found alternating along the stem and are dark green. The name "Tristis" comes from the comparatively small and frequently dull or purplish flowers, which are typically greenish or yellowish. The inflorescence usually arises from the leaf axils. The floral morphology is typical of an orchid, with two petals, three sepals, and a characteristic labellum (lip) that is frequently lobed and aids in attracting pollinators.[6] Unlike many other orchids, the labellum is placed on the

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upper side of the flowers because they are non-resupinate, meaning the flowers do not undergo the typical 180-degree twist. Orchid aficionados, particularly those interested in botanical or miniature orchids, occasionally cultivate *L. tristis*. It needs to be grown in a mounted or well-drained basket to replicate its native epiphytic environment.

Plant systematics has made molecular phylogenetics a mainstay by examining DNA sequences to determine evolutionary lineages.[7] Nuclear ribosomal regions, such as ITS, low-copy nuclear genes, and chloroplast genes (*matK, rbcL,* and *trnL-F*) were important molecular markers employed in plant species.[8] These indicators are essential for identifying cryptic species, resolving both shallow and deep phylogenetic clades, and detecting reticulate evolutionary events, such as hybrid speciation.[9] The *matK* gene (maturase K, found in chloroplast DNA)is notable among these because of its broad applicability in DNA barcoding and quick nucleotide substitution rate, which is especially useful for differentiating closely related species and elucidating genus-level relationships.[10] An important stage in the maturation of chloroplast RNA is the splicing of group II introns, which is accomplished by the maturase enzyme produced by the *matK* (maturase K) gene, which is encoded within the chloroplast genome and located in the intron of the *trnK* gene.[11,12] The phylogenetic investigation of *matK* in *L. tristis* has shed important light on its taxonomic classification, evolutionary relationships, and lineage divergence within the orchid family as well as among angiosperms in general. DNA barcoding is a successful molecular technique for identifying species by comparing reference material from reputable genomic databases with brief, standardized genetic sequences.[13] The method is beneficial in conservation biology, forensics, and biodiversity monitoring since it can produce accurate identification from even very little genetic material.[14,15] Particularly encouraging is its application to *L. tristis*, which may aid in species conservation efforts and combat the illicit trade in rare orchids. The *matK* gene in *L. tristis* serves a purpose beyond phylogenetic analysis. As a direct contributor to the processing and maturation of vital RNA transcripts, the *matK* protein is essential for regulating gene expression and chloroplast metabolism.[16] Understanding the functional dynamics, evolutionary conservation, and diagnostic potential of this protein can be enhanced with the help of 3D structure modelling.[17,18] Although the linear gene sequence reveals the underlying genetic framework, the three-dimensional structure may reveal protein functions that facilitate RNA splicing activities within the chloroplast.[19]

Through a combination of phylogenetic analysis, structural modeling, and DNA barcoding, this study aims to investigate the species discrimination potential of the *matK* gene in *L. tristis*, providing essential insights for molecular evolution, restoration genomics, and orchid systematics.

MATERIALS AND METHODS

Sample Collection

The Southern Regional Centre, the National Orchidarium, and the Experimental Garden of the Botanical Survey of India, situated in Yercaud, Salem District, Tamil Nadu, India, provided the plant samples of *L. tristis* for the sole purpose of research analysis and conservation study.

DNA Isolation

The genomic DNA was extracted from 5 g of fresh leaf tissue of *L. tristis* using a modified version of the C-TAB extraction process. [20] Using a mortar and pestle, the plant's tender young leaves were finely pulverized in liquid nitrogen before being placed in an Oak Ridge tube with pre-heated C-TAB extraction buffer. After thoroughly mixing the homogenate, it was incubated for an hour at 65°C. After adding and carefully mixing a solution of chloroform and isoamyl alcohol (24:1), the mixture was centrifuged for 10 minutes at 10,000 rpm at 4°C. By inversion, the resultant aqueous phase was carefully mixed with an equivalent volume of cold isopropanol. After two hours of incubation at -20°C, the sample was centrifuged again at 10,000 rpm for 10 minutes at 4°C. After rinsing the resultant DNA pellet with 70% cold ethanol, it was allowed to air-dry for approximately an hour. Lastly, 50 µL of 1X TE buffer was added. Samples should be stored at -20°C until they are needed again after being incubated at 50°C for one to two hours to guarantee full re-suspension.

DNA quantification

Using a UV spectrophotometer, absorbance at 260 and 280 nm wavelengths was measured to assess the purity of the extracted DNA. Samples of DNA with an absorbance ratio of 260/280 near 1.8 were considered to be of high quality and purity.

PCR amplification and sequencing of the *matK* region

A total reaction volume of 25 µL was used for amplification of the *matK* gene region. This contained 2 µL of genomic DNA, 12.5 µL of GoTaq PCR master mix, 1.25 µL of each *matK* forward and reverse primer, and 8 µL of nuclease-free water. According to Table 1, the primers were used at

Table 1: Details of the *matK* primer used for amplification

Primer used	*Tm of Primer*	*Annealing temperature*	*Concentration of primer (pmol/µL)*	*Primer sequence*
matK Forward (F)	48	52°C	150	TAATTTACGATCAATTCATTC
matK Reverse (R)	53	52°C	198	ACAAGAAAGGCGAAGTAT

final concentrations of 0.958 µM for *matK*-R and 1.236 µM for *matK*-F. The Veriti 96-well Thermal Cycler from Applied Biosystems was used to perform the polymerase chain reaction (PCR). Before 35 cycles of denaturation at 94°C for 60 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 1-minute, the program started with an initial denaturation at 94°C for 4 minutes. The last extension stage was carried out for seven minutes at 72°C by loading 5 µL of the PCR product onto a 1% agarose gel, electrophoresing, and viewing under a UV transilluminator, which helps verify the success of amplification. The ABI 3730 XL sequencer (by Bioserve Biotechnologies Pvt. Ltd.) was used for sequencing the PCR products.

Sequence submission

After being modified following the guidelines established by NCBI, the sequence was compared to pre-existing global entries in the GenBank database using NCBI-BLAST. To evaluate the similarities between nucleotides, BLASTn was carried out sequentially. Following validation, the sequences were uploaded via the BankIt submission tool to the NCBI database. (https://www.ncbi.nlm.nih.gov/WebSub/). The assigned accession number for the uploaded sequence was obtained and listed in Table 2.

Sequence analysis

ClustalW, a combined pairwise and multiple sequence alignment technique, was used to align the DNA sequences. Then, using the MEGA 7 software, clustering algorithms such as the Neighbor Joining (NJ) method[21] and the Maximum Likelihood (ML) method[22] were used to evaluate the evolutionary relationships of the sequences in MEGA X software.[23] The first ten similarity sequences with (98.5 %) of the species were thoroughly examined.

Protein structural analysis

Using EMBOSS (https://www.ebi.ac.uk/Tools/emboss/), a 3D structural model of the *matK* gene protein was generated. The 3D model depicted is likely generated through homology modeling using software like SWISS-MODEL, I-TASSER, or Phyre2.

RESULTS

Amplification and sequencing of *matK*

The *matK* gene was successfully amplified by the designed primers in *L. tristis* sample that was analyzed. The size of the generated PCR products ranged from 800 to 850 base pairs (bp). Table 2 lists the details and accession numbers of the sequences submitted.

Table 2: Accession number and details of the submitted sequence retrieved from NCBI

Plant specimen name	*NCBI accession*	*Base pairs*
L. tristis	MZ666899	824

Molecular phylogenetic analysis by the NJ method

The *matK* gene is commonly used in plant phylogenetics due to its high rate of evolution and effectiveness in resolving species-level relationships. NJ is a distance-based phylogenetic method that constructs a tree based on pairwise genetic distances. In this case, the NJ method[25] was employed to generate a phylogenetic tree, calculating the genetic distances among different orchid species, along with *L. tristis* (MZ666899). It is closely related to several *Luisia* species based on branch length and clustering in the NJ tree. The relatively short branch lengths connecting *L. tristis* with other *Luisia* species suggest low genetic divergence within this group.[26,27]

These accession groups, *L. morsei* (KY966903), *L. magniflora* (KJ733583), *L. cordata* (KJ733581), *L. tristis* (EF655788), and *L. tristis* (MT518506), form a tight cluster with *L. tristis*, indicating a monophyletic clade, meaning they share a common ancestor. This indicates high genetic similarity within the *Luisia* genus, especially within the *matK* region. *Papilionanthe hookeriana* (AB972329), *P. teres* (KC823036), *P. hookeriana* (KC823035), and *L. trichorhiza* (EF655800) are more distantly related, forming separate clades, and serve as a comparative group (Outgroup) to demonstrate divergence from *L. tristis*. Longer branches in this group indicate higher genetic distance from the *Luisia* species (Figure 1). These genetic distances are calculated based on nucleotide substitutions per site, using a suitable model (Tamura-Nei).

Molecular phylogenetic analysis by the ML method

To evaluate the evolutionary relationships between *L. tristis* (MZ666899) and other available vouchers, a matrix of pairwise genetic distances was examined. It was discovered that the genetic gap between *L. tristis* and *L. morsei* (KY966903) was extremely small, ranging between 0.003 and 0.006. A tight evolutionary link and potentially recent speciation are indicated by the high degree of genetic similarity suggested by this minimal divergence.

Furthermore, supporting the idea of tight relatedness and minimal mutation accumulation within the *matK* gene, low genetic distance is found in the comparison between *L. tristis* and *L. magniflora* (KJ733583). On the other hand, there was a considerable degree of divergence shown by the significantly greater genetic distance between *L. tristis* (MZ666899) and *P. hookeriana* (AB972329), *P. teres* (KC823036), *P. hookeriana* (KC823035), and *L. trichorhiza* (EF655800), which was between 0.008 and 0.010. This greater distance, although still within the bounds of intrageneric variation, may reflect modest evolutionary changes resulting from ecological differentiation, geographic isolation, or longer periods of divergence (Figure 2). Overall, the data support the conclusion that *L. tristis* shares recent evolutionary origins with several congeners, with varying levels of genetic separation consistent with ongoing diversification within the genus.

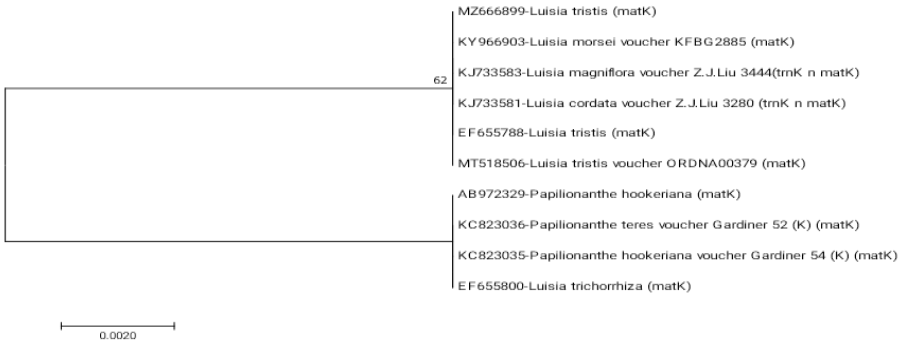

Figure 1: Genetic distance of the *matK* sequence calculated using the Neighbor-Joining (NJ) method for *L. tristis* (MZ666899)

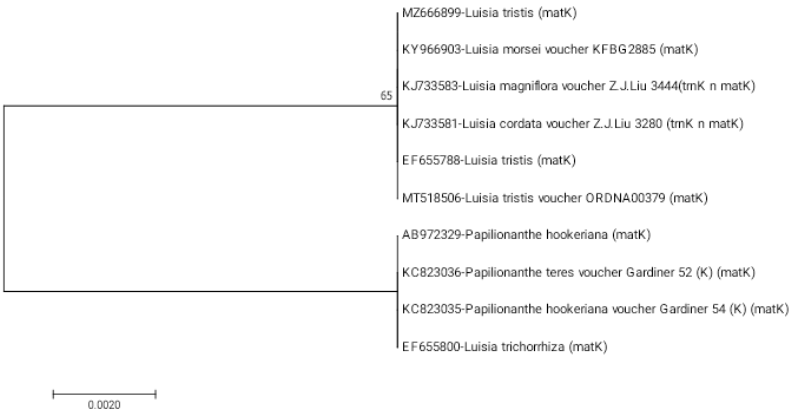

Figure 2: Genetic distance of the *matK* sequence calculated using the maximum composite likelihood (ML) method for *L. tristis* (MZ666899)

Sequence Analysis

The genetic relatedness of *L. tristis* with other available samples was analyzed using cluster analysis based on a similarity matrix, and the results were visualized using a dendrogram generated by MEGA7 software. The analysis revealed that all isolates were grouped into two separate cluster groups, based on the results obtained. The NJ and ML procedures showed almost similar associations (Figures 1 and 2).

L. tristis (MZ666899) shares the exact consensus sequence with most other accessions, indicating its sequence is highly conserved (Figure 3). It may belong to a core lineage within the sampled group. It differs from (AB972329), (KC823036), (KC823035), and (EF655800) by one base, which could be phylogenetically informative.[28]

DNA barcode is derived from the *matK* gene sequences of the plant species, likely from the *L. tristis* orchid. This visually striking format is part of modern DNA barcoding techniques used in taxonomy and biodiversity studies.[29,30] DNA barcode is a short, standardized region of DNA used for species identification. In plants, the *matK* gene is a popular barcode marker because of its high substitution rate (informative for closely related species), conserved flanking regions (useful for designing universal primers), and its role in intron splicing, which adds evolutionary constraint and phylogenetic utility.

Figure 4 is a color-coded representation of the nucleotide sequence. Each vertical-coloured bar corresponds to a single nucleotide at a particular position. Colors typically map to Adenine (A) – green, Thymine (T) –red, Guanine (G) – black or blue, Cytosine (C) – yellow or orange. The barcode acts as a genetic fingerprint, where repeated patterns suggest conserved (evolutionarily stable) regions. High variation (colour diversity) indicates polymorphic or informative sites, which are helpful in distinguishing species.

The similarity range is 98.5%. The DNA barcode was generated using the sequences in Biorad's Barcode Generator (http://biorad-ads.com/DNABarcodeWeb/) and the *matK* QR Code Generator. Figure 4 provides the barcode and QR code. The QR code's capacity to decode as DNA sequences makes it a convenient tool for genetic research.

Protein Structural Analysis and Validation

The predicted 3D protein structure of the *matK* (maturase K) protein from *L. tristis* provides valuable insights into the functional and evolutionary characteristics of the *matK* gene product. *matK* is encoded in the chloroplast genome and plays a key role in RNA splicing of group II introns during chloroplast gene expression. Acts as a maturase enzyme, which facilitates the correct folding and splicing of intron-containing precursor RNAs

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KJ733581.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTT
EF655800.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTT
KJ733583.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTT
MT518506.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTT
EF655788.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTT
KY966903.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTT
AB972329.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTA
KC823036.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTA
KC823035.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTA
MZ666899.1    TTGGTTCAAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGCATTTATTGCGATTT

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Figure 3: Consensus region of the aligned sequences showing nucleotide substitution as highlighted

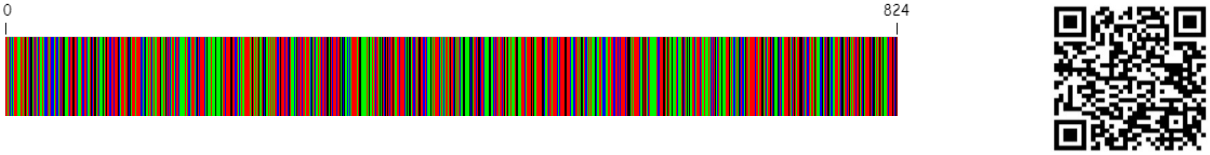

Figure 4: *MatK* sequences represented illustratively as a barcode and a QR code

The tertiary structure is determined based on known protein templates. The α-Helices (Red), these spiral regions are characteristic of helical secondary structures, often involved in protein-protein or protein-RNA interactions. The long α-helix on the left suggests a stable core domain, potentially key to enzymatic or binding activity. The β-Sheets (Cyan/Blue) represent parallel or antiparallel strands connected by hydrogen bonds. Typically found in the core of protein folds, contributing to the overall stability of the structure. Loops and Turns (White/Green): these connect helices and sheets, allowing for flexibility and surface interactions. The loops often form active or binding sites and might interact with RNA substrates during intron splicing. Color Mapping corresponds to different secondary structure motifs for clarity.

The nucleotide sequences were converted into amino acid sequences using the EMBOSS transeq tool (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). The 3D structural models of the *matK* gene protein were generated using EMBOSS (https://zhanglab.ccmb.med.umich.edu/I TASSER/). The protein models indicate a higher level of confidence and more reliable predictions.[32] The structural models of the *matK* gene protein from the *L. tristis* sample, as displayed in Figure 5, were selected for analysis.

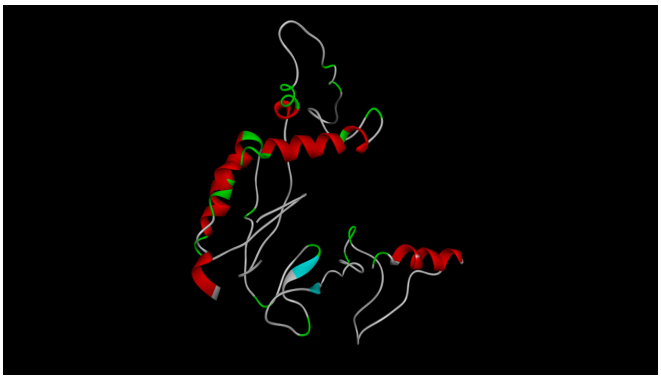

Figure 5: The 3D protein structural models for the *matK* protein of *L. tristis* were predicted through EMBOSS

DISCUSSION

The genetic distance analysis of *L. tristis* using its *matK* gene sequence (Accession No. MZ666899) provides valuable insight into the evolutionary relationships of this orchid species with other taxa. The phylogenetic trees based on NJ and ML methods offer complementary perspectives rooted in different computational approaches.[33-35] Sequencing results revealed *matK* sequences[36,37] with lengths of approximately 824 bp in *L. tristis.*

The NJ tree supports a well-defined monophyletic grouping of *Luisia* species. The minimal genetic distance among *L. tristis* and other *Luisia* members reflects evolutionary conservatism in the *matK* region within this genus, and the finding is similar to the results of.[38] The strength of the NJ-derived correlations is confirmed by the ML method, which also accurately accounts for sequence variability. According to Ronquist *et al*., evidence for the monophyly of *Luisia* and the distinctiveness of *L. tristis* within the genus was reported[39], and this observation supports our findings.

Strong evidence of *L. tristis's* close genetic relationships with other species in the *Luisia* genus, especially *L. tristis* and *L. morsei,* is provided by both the NJ and ML phylogenetic analyses based on the *matK* gene sequence. The high divergence of *Papilionanthe* further confirms the usefulness of the *matK* gene as a reliable marker for intergeneric differentiation in Orchidaceae. The combined insights from both approaches enhance the robustness and dependability of phylogenetic interpretations in plant molecular taxonomy. The *matK* gene and its product are essential for normal chloroplast development and function. It is also widely used in phylogenetic and DNA barcoding studies.[40]

The *matK* gene sequences represented by DNA barcodes are a useful tool in molecular taxonomy and plant systematics.[12,36] Each colour band in this barcode represents a distinct

sequence pattern, visually encoding the genetic information. This enables quick visual comparisons between several accessions or species. A practical and educational visual aid for genetic variation is the barcode image of *matK* sequences (Figure 4). When used in conjunction with phylogenetic techniques, this structure provides valuable insights into plant taxonomy, species identification, and evolutionary biology. It reflects the dynamics of the sequence among species.[41-43] International barcoding initiatives, primarily Barcode of Life Data Systems (BOLD), utilize a standardized format that facilitates data sharing, expedites diagnosis, and enhances comparative analysis.

A structurally conserved and functionally robust maturase, crucial for chloroplast RNA processing, is revealed by the 3D structural model of the *matK* protein in *L. tristi*[44-46] Visible helices, sheets, and flexible areas demonstrate its ability to interact intricately with RNA substrates. Besides advancing our understanding of chloroplast gene molecular biology, this model offers a crucial structural framework for comparative evolutionary research in angiosperms, including orchids.

The close genetic affinities of *L. tristis* (MZ666899) with other species in the *Luisia* genus were confirmed by the *matK*-based NJ and ML phylogenetic analysis. Additionally, the tree highlights, rather than emphasizes, evolutionary separation from allied orchid taxa, such as *Papilionanthe* spp. and *L. trichorrhiza*. In the Orchidaceae family, this not only supports the taxonomic placement but also the usefulness of *matK*-based phylogenetic analyses.

With one significant alteration (T→A) at the 60th position in a few accessions, the consensus area demonstrates the excellent conservation of the *matK* sequence across species. Although small, this alteration can have a significant impact on phylogenetic refinement of orchid molecular taxonomy, DNA barcoding, and the ability to distinguish closely related species.

A visual representation of easy genetic identification based on the *matK* gene is provided by the barcode image. It is an effective method for quickly identifying species' genetic diversity and aiding evolutionary and phylogenetic research. The dominant α-helices and β-sheets are visible in the predicted tertiary protein structure of *matK* from *L. tristis*. The identification and future preservation of the medicinally significant plant species in this area depend heavily on the findings of this phylogenetic and structural analysis.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.