

ITS AND RBCL DNA BARCODING FOR MOLECULAR AUTHENTICATION OF *THYMUS LINEARIS* (LAMIACEAE) FROM THE KASHMIR HIMALAYA: A FIRST REPORT

Ishtiyak Ahmad Peerzada^{1*}, PA Sofi¹, Arfa ji¹, Suraya Shabir¹, Irtiqah Mohammad¹, L M Gupta², A R Malik¹, Jauhar Rafeeq¹

¹Centre of Excellence on Herbal Technology, Faculty of Forestry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, 191201, India

² Division of Forest Products and Utilization, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, 180009, India

*Corresponding author : Ishtiyak Ahmad Peerzada, E-mail: drishtiyak@skuastkashmir.ac.in

ABSTRACT

Background: DNA barcoding is an important molecular method for identifying species, particularly in taxonomically complicated genera like *Thymus* (Lamiaceae), where physical similarities might lead to misidentification. Molecular markers such as the chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit(*rbcL*) gene and the nuclear Internal Transcribed Spacer (ITS) region are commonly used in authentication and phylogenetic research. The current study aims to authenticate *Thymus linearis* from the Kashmir Himalaya using *rbcL* and ITS DNA barcodes and to establish reference sequences for future taxonomic and phylogenetic studies.

Methods and Results: This study used an integrated taxonomic and DNA barcoding approach to authenticate *Thymus linearis* from the Kashmir Himalaya. Morphological identification was confirmed at the KASH Herbarium, while chloroplast *rbcL* and nuclear ITS regions were amplified and sequenced. Sequence similarity with authenticated GenBank records confirmed species identity. The ITS sequences generated represent the first publicly available barcode records for *T. linearis*. Neighbor-Joining phylogenetic analysis further validated the identification by clustering all accessions into a well-supported monophyletic clade.

Conclusion: The study establishes the first ITS barcode references for *Thymus linearis* and was authenticated using *rbcL* sequences as well. These results support molecular authentication and offer useful genetic resources for future phylogenetic and taxonomic research in the genus *Thymus*.

KEYWORDS: Medicinal plants, *Thymus*, DNA barcoding, ITS, *rbcL*

1. INTRODUCTION

The family Lamiaceae (Labiatae) is one of the world's largest and most distinctive group of flowering plants, with roughly 236 genera and over 7,000 species. This family is well-known for its vast species diversity and close relationship with medicinal and aromatic characteristics, which have been used by human civilizations from prehistoric times (Bekut et al., 2018; Bartolucci et al., 2013). Among these is *Thymus linearis* (*T. linearis*) Benth., or "Himalayan Thyme," a fragrant perennial herb. Despite its Mediterranean origins, it has become a staple of the Kashmir Himalaya's high-altitude flora, flourishing in alpine and subalpine environments (Auger et al., 2018). *T. linearis* has substantial nutritional and pharmacological significance because of its high concentration of bioactive components, particularly thymol and carvacrol, which are usually identified as the major components of its essential oil (Verma and Shukla 2015). The aerial parts of *T. linearis* have long been used for a variety of reasons around the world, including herbal tea, antitussive tonic, and an effective antiseptic against skin disorders such as dermatitis and psoriasis (Bashir et al., 2019). Traditional medicine uses infusions and decoctions of the plant for its carminative, digestive, antispasmodic, and emmenagogic characteristics. This makes it a valuable source for food additives and functional ingredients (Petrovic et al., 2017). Modern pharmacological investigations have verified these traditional assertions by investigating a diverse variety of biological functions. *T. linearis* has been shown in studies to exhibit antiproliferative (Bashir et al., 2019; Hussain et al., 2013), antioxidant (Chnadra et al., 2016), anti-inflammatory, antipyretic, analgesic, and antibacterial properties (Hussain et al., 2013; Kumar et al., 2020). Despite its economic significance, reliable species identification remains difficult. The genus has high chemical polymorphism and morphological plasticity, which is impacted by climatic, seasonal, and geographical factors (Tohidi et al., 2019; Chen et al., 2024). This flexibility, combined with frequent hybridization, has traditionally resulted in taxonomic confusion and widespread misidentification (Nazar et al., 2022). To overcome these identification challenges, a dual approach combining classical morphological taxonomy and current molecular approaches is needed. DNA barcoding has developed as a new technology for identifying biological materials by utilizing short, standardized DNA sequences from the nuclear or organelle genomes (Hollingsworth et al., 2011; Kress and Erickson, 2012). It provides a fast, objective, and repeatable method for species-level authentication when classical morphology fails (Kress, 2017). Plant barcoding commonly uses genomic regions such as the

chloroplast *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase) and *matK* regions, as well as the nuclear Internal Transcribed Spacer (ITS). Among these, ITS2 is the most favourable region for determining variation among plant species. Because of its great diversity and mutation rate, ITS2 is regarded as a key DNA barcode for identifying and discriminating a diverse variety of plant taxa (Alvarez and Wendel, 2003). The use of barcoding in plants presents significant challenges (Hollingsworth et al., 2011; Mattia, 2011). Several species in commercial plant samples, including Lamiaceae species, have been successfully identified using DNA barcoding (Mattia, 2011; Theodoridis et al., 2014). However, due to significant morphological variability, frequent hybridization, and a lack of reliable reference sequences, its application for infrageneric classification in *Thymus* has proven inconclusive (Federic et al., 2013; Brauchler et al., 2010). A significant contributor to this constraint has been the lack of publicly available Internal Transcribed Spacer (ITS) reference sequences for *Thymus linearis*, which has hampered accurate molecular identification and comparative analysis. In the current study, this essential gap was addressed using an integrated taxonomic method that included *rbcL* and ITS region sequencing. Initially, the plant material was classified taxonomically. The gathered specimens were initially identified at the KASH Herbarium (Centre for Biodiversity and Taxonomy, University of Kashmir) with conventional taxonomic keys and voucher authentication. This morphological identification was later corroborated using chloroplast *rbcL* gene sequencing, which is a reliable and widely accepted DNA barcode for genus-level confirmation. The nuclear ITS region of *Thymus linearis* was amplified and sequenced after herbarium-based taxonomy and *rbcL* sequencing analysis confirmed their identity. The produced ITS sequences were submitted to the NCBI GenBank database, resulting in the first publicly available ITS barcode entries for this species. This study bridges a critical gap in molecular reference databases and establishes a validated framework for future DNA barcoding, phylogenetic, and authentication research in the genus *Thymus*.

2. MATERIALS AND METHODS

2.1. Plant Material Collection

Thymus linearis accessions were obtained from six distinct locations in the Kashmir Himalaya, including Tulail Gurez (T₁), Verinag, Anantnag (T₂), Haril, Kupwara (T₃), Doodipatheri, Budgam (T₄), MohandMarg, Ganderbal (T₅), and Sheeshnag Shajimarg, Kagan (T₆). The gathered specimens were then sent to the Centre for Biodiversity and Taxonomy (CBT), Department of Botany, University of Kashmir, India, for taxonomic identification and the assignment of voucher specimen numbers.

2.2. Molecular examinations

2.2.1. DNA Extraction amplification and sequencing

Genomic DNA was extracted from young, fresh leaves using the standard cetyltrimethylammonium bromide buffer CTAB extraction procedure, with minor modifications (Doyle and Doyle, 1987). In brief, 100mg of sample was pulverized in 1mL of 2% CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA) at room temperature, followed by organic phase separation (Chloroform: Isoamyl alcohol) and precipitation with cold isopropanol. DNA was eluted in 30 µl of TE(Tris-EDTA) buffer and kept at 4°C for future use. Electrophoresis on 0.7% agarose gels made in 1x TAE (Tris-Acetate-EDTA) buffer was used to analyze DNA quality and integrity, which were then examined under UV illumination with a Gel Documentation System (Bio-Rad).

2.2.2. PCR amplification

Using universal primer sets, targeted amplification of the *rbcL* and ITS regions was carried out. 60 ng/µl template DNA (1µl), 10µM forward primer (0.5µl), 10µM reverse primer (0.5µl), 25 mM dNTPs (1µl), 10xbuffer with MgCl₂ (2.7 µl), Taq polymerase (0.5µl), and nuclease-free water (18.8 µl) comprised the reaction mixture used for PCR reaction, which were carried out in a volume of 25 µl. PCR amplification was carried out using the Biorad real-time PCR machine. For both markers, the thermal cycling conditions were as follows: an initial denaturation at 94 °C for three minutes, followed by thirty cycles of denaturation at 94 °C for thirty seconds, annealing at 55 °C for forty-five seconds, and extension at 72 °C for two minutes. Final extension step was carried out at 72 °C for 10 minutes for ITS and 15 minutes for *rbcL*. The primer sequences are given below.

2.2.3. Sequence data analysis

The sequences (Table 1) were examined using a variety of bioinformatics tools. BLAST and Clustal Omega were used to conduct a comparative sequence analysis of the query and database sequences. Unique sections of the query sequences were found and tagged. The similarity of the acquired sequences to previously published sequences in GenBank was assessed using the BLASTN similarity search. Additionally, Clustal Omega was used to do multiple sequence alignment among the query sequences. A phylogenetic tree was created using MEGA 11 software based on the Neighbor-Joining (NJ) method for evolutionary connection research, and the resulting dendrogram depicted the genetic links between the sequences.

Table 1: Sequences and T_m values for Forward and Reverse Primers

Primer		Sequence (5'-3')	T _m °C
ITS	Forward primer	ATGCGATACTTGGTGTGAAT	53.2
	Reverse primer	TCCTCCGCTTATTGATATGC3	55.3
<i>rbcL</i>	Forward primer	ATGTCACCACAAACAGAGACTAAAGC	61.6
	Reverse primer	TCGCATGTACCTGCAGTAGC	59.3

2.2.4. Evolutionary relationships of taxa

The Neighbor-Joining approach was used to deduce the evolutionary history (Saitou and Nei 1987). The ideal tree is displayed. Above the branches is the percentage of duplicate trees where the related taxa clustered together in the bootstrap test (1000 replicates) (Felsenstein, 1985). The tree is scaled with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree (Nei and Kuamr, 2000). The rate variation across locations was modelled using a gamma distribution (shape parameter = 1). In this study, eighteen nucleotide sequences were employed. All ambiguous locations were removed for each pair of sequences (pairwise deletion option). There were 709 places in all in the final dataset. Evolutionary studies were conducted in MEGA11(Tamura et al., 2021).

3. Results

3.1. Herbarium based identification

All obtained accessions were thoroughly inspected and authenticated at the KASH Herbarium (Centre for Biodiversity and Taxonomy, University of Kashmir) and NBPGR (National Bureau of Plant Genetic Resources) using standard taxonomic keys and regional floristic literature. The specimens consistently displayed *Thymus linearis* diagnostic characteristics, such as linear-lanceolate leaves, peculiar inflorescence arrangement, and distinct floral morphology unique to the genus *Thymus* (Lamiaceae). Voucher specimens were given official accession numbers and stored in the herbarium repository to ensure traceability and future taxonomic verification. The herbarium-based identification provided a good morphological baseline for future genetic validation. Table 2 shows the accession numbers that were provided.

Table 2: Collection sites and Voucher numbers of *Thymus linearis*

Place of collection	KASH Voucher no	Herbarium	NBPGR Voucher no
T ₁	9907-KASH		IC655096
T ₂	9909-KASH		IC655090
T ₃	9911-KASH		IC655092
T ₄	9917-KASH		IC655099
T ₅	9910-KASH		IC655091
T ₆	9916-KASH		IC655099

3.2. ITS and rbcL sequence-based characterization

In the present study, both the nuclear ITS region and the chloroplast *rbcL* gene were sequenced to ensure accurate species identification. The PCR amplification of the nuclear ITS region and the *rbcL* gene produced producible amplicons with fragment sizes of around 400 bp for ITS and 600 bp for *rbcL* (Fig 1-A,B).BLAST analysis of the *rbcL* sequences showed a high similarity with *Thymus linearis*, thereby providing reliable molecular evidence for species identity. In contrast, ITS sequences did not yield a direct match with *T. linearis* in the database, which can be attributed to the absence of previously deposited ITS reference sequences for this species in GenBank. However, the combined evidence from *rbcL*-based molecular identification and taxonomic characterization of the collected specimens unequivocally supported their identification as *Thymus linearis*. Therefore, to the best of our knowledge the ITS sequences generated in the present study represent the first molecular reference for this species and contribute valuable genetic information for future phylogenetic and barcoding studies within the genus *Thymus*. The effective amplification of *rbcL* and ITS markers in this study demonstrates their suitability for molecular systematics in the Lamiaceae family.

Table 2: Accession data of *Thymus linearis* based on rbcL and ITS Markers

Place of collection	Barcode region	Accession no	Species identified
T ₁	rbcL	PX409199	<i>Thymus linearis</i>
	ITS	PX248636	
T ₂	rbcL	PX391229	<i>Thymus linearis</i>
	ITS	PX276547	
T ₃	rbcL	PX519192	<i>Thymus linearis</i>
	ITS	PX459835	
T ₄	rbcL	PX519193	<i>Thymus linearis</i>
	ITS	PX459823	
T ₅	rbcL	PX519191	<i>Thymus linearis</i>
	ITS	PX459824	
T ₆	rbcL	PX519194	<i>Thymus linearis</i>
	ITS	PX459825	

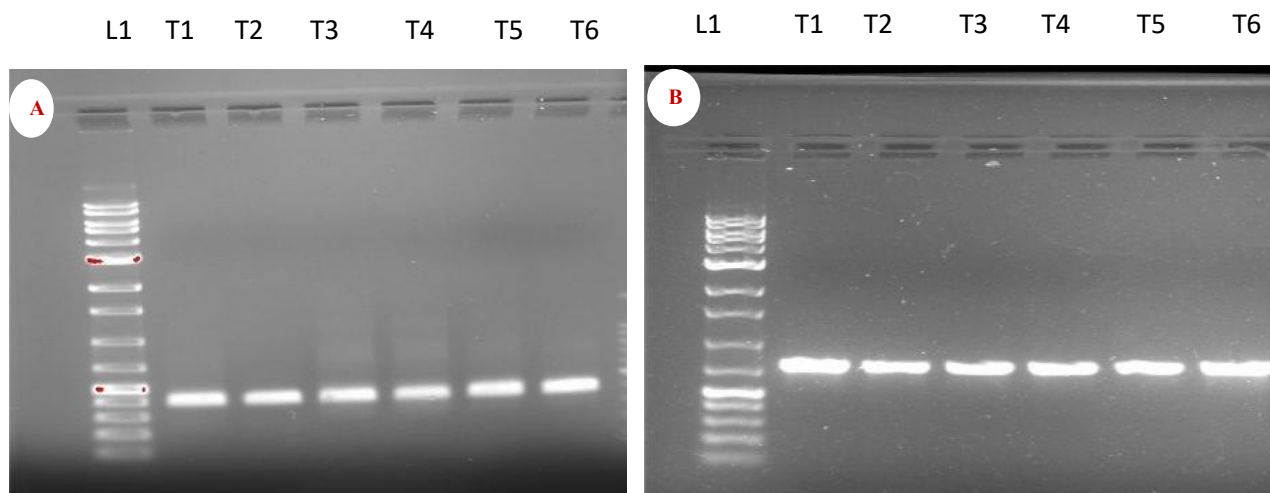


Fig 1: Gel profile of PCR amplification using (A)-ITS, (B)-rbcL markers of *Thymus linearis* from the indicated locations T₁, T₂, T₃, T₄, T₅ and T₆.

3.3. Phylogenetic analysis:

To further confirm taxonomic placement, phylogenetic analysis was performed. The newly generated sequences were aligned with representative *Thymus* taxa retrieved from GenBank, and phylogenetic reconstruction was performed using standard tree-building algorithms with bootstrap analysis to assess nodal support. *Mentha spicata* was designated as the outgroup to root the topology (Fig. 2). The resulting phylogeny resolved the generated sequences as a distinct and well-supported monophyletic lineage, supported by bootstrap values. The minimal branch lengths within the clade indicate low intraspecific nucleotide divergence and high sequence homogeneity among the sampled individuals. The strong statistical support and stable clustering pattern across bootstrap replicates demonstrate the robustness of the inferred topology. The exclusive formation of a coherent lineage corresponding to *Thymus linearis* confirms the taxonomic identity of the analyzed material. As no prior ITS sequences of *T. linearis* were available in public repositories, the sequences generated in the present study constitute the first molecular reference for this species based on the DNA ITS marker. These data provide a foundational framework for future phylogenetic, taxonomic, and DNA barcoding studies within the genus *Thymus*. The phylogenetic inference provide definitive molecular confirmation of the identity of *Thymus linearis*.

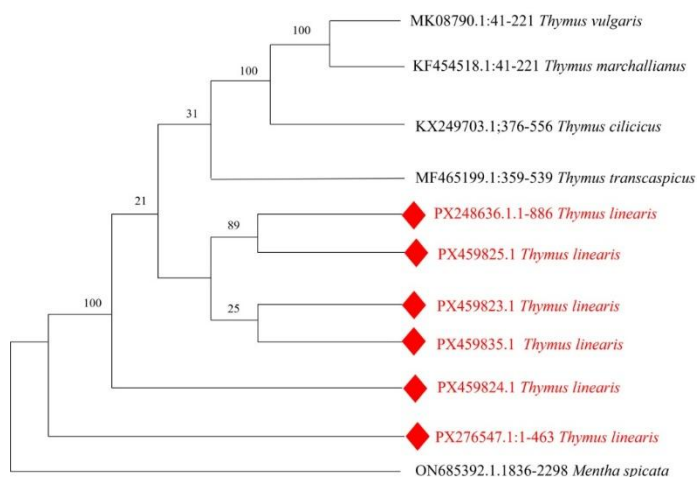


Fig 2: Neighbour joining phylogenetic tree showing the evolutionary relationships between closely related *Thymus* species obtained from GenBank and *Thymus linearis* sequences developed in the present work (highlighted in red).

4. DISCUSSION

4.1. Herbarium based identification

Morphological examination remains a fundamental step for accurate plant identification. In the present study, the examined specimens consistently displayed diagnostic characteristics of *Thymus linearis*, including linear-lanceolate leaves, characteristic inflorescence structure, and typical floral morphology of the genus *Thymus*. Similar studies on Lamiaceae have emphasized that morphological traits, together with floristic keys, remain essential for reliable species delimitation and authentication of medicinal plants (Llorent et al., 2022). The

deposition of voucher specimens in recognized herbaria such as KASH and NBPGR ensures proper documentation and traceability of plant material. Herbarium collections provide permanent reference specimens that enable verification of taxonomic identity and support future systematic studies (Rostanski, 2024). Furthermore, recent studies highlight that herbarium vouchers are crucial for linking morphological observations with molecular data, thereby improving the accuracy and reproducibility of biodiversity and taxonomic research (Heberling et al., 2022; Culley, 2013). Thus, the herbarium-based identification in this study established a reliable morphological foundation for subsequent molecular validation of *T. linearis*.

4.2. ITS and *rbcL* sequence-based characterization

In the present study, successful amplification of the nuclear ITS region (~400 bp) and the chloroplast *rbcL* gene (~600 bp) confirmed the suitability of these markers for molecular identification of *Thymus linearis*. BLAST analysis of the *rbcL* sequences showed high similarity with *T. linearis*, providing reliable molecular evidence for species authentication. Similar studies have demonstrated that *rbcL* is a conserved chloroplast marker widely used for plant DNA barcoding and taxonomic verification (Aneva et al., 2002). However, ITS sequences did not show a direct match in GenBank, likely due to the absence of previously reported ITS sequences for this species. Recent studies have also highlighted gaps in reference barcode databases for many medicinal plants, which can limit direct species identification using ITS markers (Sun et al., 2002). Nevertheless, the combined use of *rbcL*, ITS, and morphological characterization provided strong evidence supporting the identification of the collected specimens as *Thymus linearis*. Integrative approaches combining chloroplast and nuclear markers are widely recommended for accurate species discrimination and phylogenetic studies in Lamiaceae and other angiosperms (Sun et al., 2016; Herbert et al., 2003). Therefore, the ITS sequences generated in this study represent the first molecular reference for *T. linearis*, contributing valuable genetic data for future phylogenetic and DNA barcoding studies within the genus *Thymus*.

4.3. Phylogenetic analysis

The phylogenetic reconstruction in the present study revealed that the analyzed sequences formed a well-supported monophyletic clade, confirming the taxonomic placement of *Thymus linearis*. Similar findings have been reported in recent molecular studies of the genus *Thymus*. For instance, a DNA barcoding study on several *Thymus* species demonstrated that ITS and chloroplast markers can effectively resolve phylogenetic relationships and group closely related taxa into distinct clades, supporting species-level identification [30]. Likewise, a recent phylogenetic investigation of Lamiaceae using ITS, *matK*, and *rbcL* markers showed that molecular phylogenetic trees with bootstrap support provide reliable frameworks for species authentication and resolving taxonomic complexities within the family (Endresser et al., 2003). In addition, genome-based phylogenetic analysis of *Thymus mongolicus* confirmed the evolutionary placement of *Thymus* species within Lamiaceae and highlighted the usefulness of molecular markers in clarifying interspecific relationships (Na et al., 2025). These findings are consistent with the present study, where strong bootstrap support and stable clustering patterns provided reliable molecular confirmation of *T. linearis*.

5. CONCLUSION

The present study demonstrates the effectiveness of combining DNA barcoding using the *rbcL* gene with detailed morphological analysis for the accurate identification of *Thymus linearis*. In the absence of species-specific primers, the *rbcL* marker successfully confirmed species identity, underscoring its applicability in medicinal plant authentication. The morphological characterization and formal submission of specimens to the Centre of Biodiversity and Taxonomy of Kashmir contribute to reliable taxonomic documentation and support future conservation and research efforts on this valuable Himalayan medicinal plant.

REFERENCES

1. Alvarez, I. J. F. W., & Wendel, J. F. (2003). Ribosomal ITS sequences and plant phylogenetic inference. *Molecular phylogenetics and evolution*, 29(3), 417-434.
2. Aneva, I., Zhelev, P., Bonchev, G., Boycheva, I., Simeonova, S., & Kancheva, D. (2022). DNA barcoding study of representative *Thymus* species in Bulgaria. *Plants*, 11(3), 270.
3. Auger, C., Chabert, P., Lugnier, C., Mushtaq, M. N., & Schini-Kerth, V. B. (2018). Mechanisms underlying vasorelaxation induced in the porcine coronary arteries by *Thymus linearis*, Benth. *Journal of ethnopharmacology*, 225, 211-219.
4. Bartolucci, F., Peruzzi, L., & Passalacqua, N. (2013). Typification of names and taxonomic notes within the genus *Thymus* L.(Lamiaceae). *Taxon*, 62(6), 1308-1314.
5. Bashir, R., Zargar, Q. P. O., & Hamid, R. (2019). *In vitro* antiproliferative activity of *Thymus linearis* essential oil from five ecozones of Kashmir valley. *Pharma Innovation Journal*, 8, 205-210.
6. Bekut, M., Brkic, S., & Kladar, N. (2018). Potential of selected Lamiaceae plants in anti(retro) viral therapy. *Pharmacology Research*, 133, 301-314.
7. Bräuchler, C., Meimberg, H., & Heubl, G. (2010). Molecular phylogeny of Menthinae (Lamiaceae, Nepetoideae, Mentheae)—taxonomy, biogeography and conflicts. *Molecular phylogenetics and evolution*, 55(2), 501-523.

8. Chandra, M., Prakash, O., Bachheti, R. K., Kumar, M., & Pant, A. K. (2016). Essential oil composition, phenolic constituents, antioxidant and pharmacological activities of *Thymus linearis* Benth. collected from Uttarakhand region of India. *Journal of essential oil bearing plants*, 19(2), 277-289.
9. Chen, Y., Zhang, J., Liu, J., Hu, H., Wang, L., & Jin, L. (2024). Comparative study on morphological features and chemical components of wild and cultivated *Angelica sinensis* based on bionic technologies and chemometrics. *ACS omega*, 9(40), 41408-41418.
10. Culley, T. M. (2013). Why vouchers matter in botanical research. *Applications in Plant Sciences*, 1(11), 1300076.
11. Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical bulletin*.
12. Endlessa, C., Hidayat, T., & Sriyati, S. In *Silico Phylogenetic Analysis of Lamiaceae Based on ITS, matK, and rbcL DNA Barcodes*.
13. Federici, S., Galimberti, A., Bartolucci, F., Bruni, I., De Mattia, F., Cortis, P., & Labra, M. (2013). DNA barcoding to analyse taxonomically complex groups in plants: the case of *Thymus* (Lamiaceae). *Botanical Journal of the Linnean Society*, 171(4), 687-699.
14. Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *evolution*, 39(4), 783-791.
15. Heberling, J. M. (2022). Herbaria as big data sources of plant traits. *International Journal of Plant Sciences*, 183(2), 87-118.
16. Hebert, P. D., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 270(1512), 313-321.
17. Hollingsworth, P. M., Graham, S. W., & Little, D. P. (2011). Choosing and using a plant DNA barcode. *PloS one*, 6(5), e19254.
18. Hussain, A. I., Anwar, F., Chatha, S. A., Latif, S., Sherazi, S. T., Ahmad, A., ... & Sarker, S. D. (2013). Chemical composition and bioactivity studies of the essential oils from two *Thymus* species from the Pakistani flora. *LWT-Food Science and Technology*, 50(1), 185-192.
19. Kress, W. J. (2017). Plant DNA barcodes: Applications today and in the future. *Journal of systematics and evolution*, 55(4), 291-307.
20. Kress, W. J., & Erickson, D. L. (2012). DNA barcodes: methods and protocols. In *DNA barcodes: Methods and protocols* (pp. 3-8). Totowa, NJ: Humana Press.
21. Kumar, A., Kamal, A., Singh, S., Padalia, R. C., Tandon, S., Chauhan, A., ... & Verma, R. S. (2020). Chemical composition, antimicrobial activity, kinetics and mechanism of action of Himalayan-thyme (*Thymus linearis* Benth.). *Journal of Essential Oil Research*, 32(1), 59-68.
22. Llorent-Martínez, E. J., Ruiz-Medina, A., Zengin, G., Ak, G., Jugreet, S., Mahomoodally, M. F., ... & Chiavaroli, A. (2022). New biological and chemical evidences of two Lamiaceae species (*Thymbra capitata* and *Thymus sipyleus* subsp. *rosulans*): in vitro, in silico and ex vivo approaches. *Molecules*, 27(24), 9029.
23. Mattia, F., Bruni, I., Galimberti, A., Cattaneo, F., Casiraghi, M., & Labra, M. (2011). A comparative study of different DNA barcoding markers for the identification of some members of Lamiaceae. *Food Research International*, 44(3), 693-702.
24. Na, N., Wu, Z., Wang, Z., Yang, Y., Tian, C., Zhu, L., ... & Li, Z. (2025). The complete mitochondrial genome of *Thymus mongolicus* and its phylogenetic relationship with Lamiaceae species. *Biomolecules*, 15(3), 343.
25. Nazar, N., Howard, C., Slater, A., & Sgamma, T. (2022). Challenges in medicinal and aromatic plants DNA barcoding—lessons from the Lamiaceae. *Plants*, 11(1), 137.
26. Nei, M., & Kumar, S. (2000). *Molecular evolution and phylogenetics*. Oxford university press.
27. Petrović, S., Ušjak, L., Milenković, M., Arsenijević, J., Drobac, M., Drndarević, A., & Niketić, M. (2017). *Thymus dactyloides* as a new source of antioxidant and antimicrobial metabolites. *Journal of functional foods*, 28, 114-121.
28. Rostański, A., Gerold-Śmiałowska, I., & Tokarska-Guzik, B. (2024). Historical and contemporary herbaria as a source of data in plant taxonomy and phytogeography research: An example from Poland. *Diversity*, 16(8), 434.
29. Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, 4(4), 406-425.
30. Sun, K., Chen, X. L., Ma, R. J., Li, C., Wang, Q., & Ge, S. (2002). Molecular phylogenetics of *Hippophae* L.(Elaeagnaceae) based on the internal transcribed spacer (ITS) sequences of nrDNA. *Plant Systematics and Evolution*, 235(1), 121-134.
31. Sun, W., Li, J. J., Xiong, C., Zhao, B., & Chen, S. L. (2016). The potential power of Bar-HRM technology in herbal medicine identification. *Frontiers in Plant Science*, 7, 367.
32. Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. *Molecular biology and evolution*, 38(7), 3022-3027.
33. Theodoridis, S., Stefanaki, A., Tezcan, M., Aki, C., Kokkini, S., & Vlachonasios, K. E. (2012). DNA barcoding in native plants of the Labiatae (Lamiaceae) family from Chios Island (Greece) and the adjacent Çeşme-Karaburun Peninsula (Turkey). *Molecular Ecology Resources*, 12(4), 620-633.

34. Tohidi, B., Rahimmalek, M., & Trindade, H. (2019). Review on essential oil, extracts composition, molecular and phytochemical properties of *Thymus* species in Iran. *Industrial Crops and Products*, 134, 89-99.
35. Verma, N., & Shukla, S. (2015). Impact of various factors responsible for fluctuation in plant secondary metabolites. *Journal of applied research on medicinal and aromatic plants*, 2(4), 105-113.