Pak. J. Bot., 59(4): April 2027.

ARTICLE | DOI: http://dx.doi.org/10.30848/PJB2027-4(3)

DNA BARCODING OF LAMIACEAE FROM POONCH VALLEY, AZAD JAMMU AND KASHMIR, PAKISTAN

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Abstract

Lamiaceae is the sixth-largest angiosperm family and the largest one in the order 'Lamiales'. The family members are widely used for medicinal purposes and to produce perfumes, oils, and culinary products. There are 21 Genera and approximately 36 species of Lamiaceae in Poonch valley, Azad Jammu and Kashmir (AJK), Pakistan. It is important to identify the Lamiaceae species to explore its taxonomical status. In current research DNA barcoding of Lamiaceae was performed to ensure correct identification of Lamiaceae species from Poonch valley, Azad Jammu and Kashmir (AJK), Pakistan. For this purpose, Lamiaceae plants were collected from different regions of Poonch valley, high quality DNA was extracted and subjected to amplification by three plastid barcoding loci (rps11, rbcLa, and trnL-trnF). The amplified products were purified and sequenced. Electropherograms were assembled and edited by Geneious 6.1.8. The novel sequences were compared against previously available data using the Basic Local Alignment Search Tool (BLAST).

The consensus sequence lengths of the rps11, rbcLa, and trnL–trnF loci in the studied Lamiaceae species were 417, 573, and 375 base pairs, respectively. The overall PCR amplification success rates were 97% for rps11, 85% for rbcLa, and 75% for trnL–trnF. The obtained sequences were validated against the BOLD and GenBank databases for species identification. Nucleotide BLAST analysis revealed 73% sequence similarity with the corresponding species, whereas 73–100% with the corresponding genera**.** In our study comparatively rbcLa provided high species-level discrimination (73%) and proposed as the most effective DNA barcode among the three evaluated loci for species-level identification of Lamiaceae medicinal plants. To investigate the phylogenetic relationships of the studied Lamiaceae species, phylogenetic tree was constructed using the obtained DNA barcode sequences. The phylogenetic tree improves the placement of species in clades. This study would be useful to understand the phylogenetic relationships and confirming the taxonomic identity of medicinal plant species within the family Lamiaceae.

Key words: DNA barcoding, Lamiaceae, rbcLa, rps11, trnL-trnF, Poonch valley

Introduction

Correct identification of plant taxa is crucial as the importance of biodiversity is growing every day in the domains of agriculture, ecology, conservation biology, and other sciences. Expert taxonomists, systematic biologists, and trained practitioners have traditionally employed morphological traits to identify species (Erić *et al.,* 2019). Taxonomists can identify the range of species that are well known to them. According to the rough estimate there will be a need for a group of 15,000 taxonomists to identify life, however, A rough estimate suggests that 15,000 taxonomists will be needed to identify life. As biodiversity continues to increase, identifying species accurately and comprehensively has become increasingly important (Hawksworth, 1995). DNA barcoding resolves the problem of taxonomists for species identification and lets them focus on delimiting taxa, identifying and discovering new species. DNA barcoding has considerable capacity for the identification and ranking of the organism. It is believed that DNA databases restore biodiversity and increase the rate of species discovery and recognition (Gregory, 2005).

DNA barcoding became a reality in last few years (Frezal & Leblois, 2008). Many DNA regions either coding or non-coding are being used as molecular markers, confirming the universality and high resolution of DNA barcoding. This approach is suggested as an integrated, comprehensive approach alongside traditional taxonomy for species identification, validation and discrimination (Sahare & Srinivasu, 2012; Newmaster *et al*., 2013 and Godfrray, 2007). Further analyzed that a few years ago simple taxonomy was the only consideration to identify the species which was cost-effective, but now the solution has been found by molecular experimentation, which is a more effective and precise method. Flowering plants also have satisfactory gene sequences for DNA barcoding (Kress *et al*., 2005).

Nowadays researchers have looked into the possibility of using short DNA sequences to identify species quickly and easily as well as to define species boundaries (Erić *et al.,* 2019; Sevindik, 2019). DNA barcoding is considered as a crucial technique for plant identification and delimitation (Bayat *et al.,* 2018; Galović *et al*., 2018; Wang *et al*., 2018). It is a novel method to identify species quickly, and accurately (Akinro *et al.,* 2019; Pati *et al.,* 2019). The key objective of the DNA barcoding is to assign a valid name to unidentified species and improve identification, particularly in cases where the organisms are cryptic or have complex morphological characteristics (Hebert *et al.,* 2003). The current research is also designed to correctly identified the species of Lamiaceae from Poonch Valley, Azad Jammu, and Kashmir, Pakistan.

Received: 07-05-2026 | Revised: 28-09-2026 | Accepted: 05-10-2026 | Online: 05-11-2026

In Poonch Valley, Azad Jammu, and Kashmir 50-70% of local inhabitants depends on plant - based remedies; as plants are used as ancient medicines, culinary herbs, spices, and essential oil sources (Khan *et al.,* 2011). However, due to the morphological complexity of the Lamiaceae family, it is frequently difficult to distinguish closely related groups, resulting in numerous taxonomic issues. Species included in this study may be confused with closely related taxa because of their similar vegetative morphology, floral characteristics, and overlapping distributions in the Poonch Valley. DNA barcoding therefore provides a reliable supplementary tool for accurate species identification where traditional morphology alone is insufficient. To date, a considerable amount of work has been done for the taxonomy and morphology of Lamiaceae from the Poonch region but to the best of our knowledge, no earlier reports are yet available regarding the DNA barcoding. Therefore, the current study was designed to perform DNA barcoding and phylogenetic analysis of Lamiaceae species from Poonch Valley, Azad Jammu, and Kashmir by using three barcoding loci (rps11, rbcLa, trnL-trnF). This is the first reference DNA sequence-based study of Lamiaceae from Poonch Valley, Azad Jammu and Kashmir.

Material and Methods

Study area: The study area included Poonch valley which is confined to District Poonch, Sudhnoti, and the adjacent parts of Bagh, Azad Jammu and Kashmir, Pakistan (Fig. 1). Poonch Valley is roughly 8500 hectares in size and is located between an altitude of 1750 and 2500 meters above sea level.

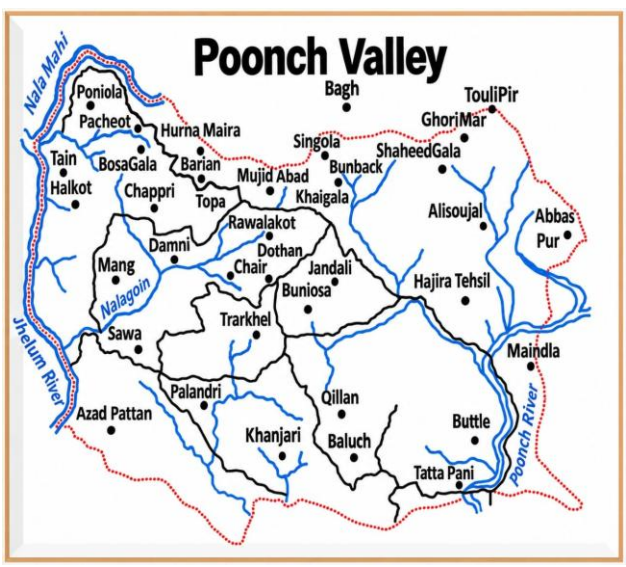


Fig. 1. Map of Poonch Valley, Azad Jammu and Kashmir. The location of the study area is indicated with dots, roads are indicated with black lines, ravines are represented with blue lines, and Poonch and Jehlum rivers are identified with double lines.

Plant material collection and storage: Frequent trips were planned and plant collection was carried out at intervals of years 2019-2021. The total number of sampled taxa was 36, collected from various locations of study area. The Plants were identified by experts in taxonomy, and stored at -80°C for DNA extraction. The details of specimen are presented in Table 1.

Extraction and quantification of chloroplast DNA: DNA was extracted from preserved leaf material using the cetyltrimethylammonium bromide (CTAB) method with minor modifications following Doyle & Doyle (1987) and Cullings (1992). DNA concentration was quantified using the Invitrogen™ Qubit™ dsDNA BR Assay Kit.

DNA cleaning: Most species of Lamiaceae are well known for having secondary metabolites. Therefore, extracted DNA was cleaned by using Wizard®SV Gel, PCR Clean-Up System by following three steps (Processing, Washing, and Elution).

Primer designing: The primer sequences used for the amplification of rps11, trnL-trnF intergeneric spacer region, and rbcLa regions are as follows:

rps11: F:5′TGGCAAAAGCTATACCGAAAA3′R:5′TTCGGAGGTCTACAGCCATT3′
trnL-trnF: F:5′ GGTTCAAGTCCCTCTATCCC3′ R:3′ATTTGAACTGGTGACACGAG5′
rbcLa:F:5′ATGTCACCACAAACAGAGACTAAAG3′R:5′GTAAAATCAAGTCCACCRCG3′

PCR amplification: The PCR reaction for all three regions was carried out in a total volume of 50 µL containing 10µL (5 X) *Taq* buffer, 5 µL (1.5mM) $MgCl_2$, 5µL (10 pmoles) of forward and reverse primer each, 4 µL (0.2 mM) dNTPs, 0.5 µL (2.5 U) *Taq* DNA polymerase, 1 µL DNA template (10 ng) and 19.5 µL nano pure water. PCR conditions were pre–denaturation at 94°C for five minutes, followed by 40 cycles of denaturation at 94°C for one minute, annealing temperature ranging from 50-60°C for one minute, extension at 72°C for one minute and then the final extension was done at 72°C for twenty minutes.

PCR product confirmation and purification: The amplified products were checked on 1.5% agarose gel with a 100plus bp DNA ladder, then staining of gel was carried out and finally, the gel was visualized in the gel documentation system (Wealth, Dolphin Doc^{Plus}). The amplified products were purified by PCR cleanup with an ExoSAP kit (Thermo Fisher Scientific) to remove any impurities.

DNA sequencing of barcoding loci: The PCR products were sequenced at Eurofins Genomic Company, USA.

Nucleotide blast analysis: Forward and reverse electropherograms were assembled and visually inspected in Geneious 6.1.8 (Kearse *et al.,* 2012). Ambiguous base calls at the 5′ and 3′ ends were trimmed. After the cleaning process, clean sequence data of rps11, trnL-trnF, and rbcLa genes from species of Lamiaceae was submitted to the Genbank to get the accession number. Moreover, consensus sequences were subjected to similarity searches against the GenBank nucleotide database (National Center for Biotechnology Information, NCBI; https://www. ncbi.nlm.nih.gov/) using the NCBI BLASTn algorithm with default parameters (Kearse *et al*., 2012). For each query, the top match and the percentage identity of the closest hit were recorded.

Phylogenetic analysis: The optimal nucleotide substitution models were selected with model finder for each alignment via the Alkaline information criterion (AIC) (Kalyaanamoorthy *et al*., 2017) for constructing the phylogenetic tree. A combined/concatenated dataset of three cpDNA markers was analyzed. Maximum Likelihood (ML) analysis was carried out using IQ-TREE phylogenetic software with 1000 bootstrap replicates

(Nguyen *et al*., 2015), while Bayesian inferences (BI) were carried out using MrBayes software (ver. 3.2) (Ronquist *et al*., 2012). For Bayesian analyses, each matrix underwent two separate Markov chain Monte Carlo (MCMC) calculations. One million generations of each dataset were run. The MrBayes program estimated all compatible trees. Posterior probabilities (PP) were used to calculate the clade support and Figtree 1.4 (https://github.com/ rambaut/ fgtree) was used to edix the phylogenetic trees.

Results

The DNA extracted from the 36 Lamiaceae species exhibited concentrations ranging from 35 to 68 ng/µL, with an average concentration of 49.97 ng/µL. Most samples yielded DNA concentrations between 40 and 60 ng/µL, indicating that the CTAB extraction protocol consistently produced DNA of sufficient quantity for downstream molecular analyses (Table 2).

The highest DNA concentration was obtained from *Mentha arvensis* (68 ng/µL), followed by *Prunella vulgaris* (66 ng/µL) and *Isodon coetsa* (64 ng/µL), suggesting efficient DNA recovery from these species. In contrast, *Teucrium royleanum* and *Nepeta erecta* produced the lowest DNA concentrations (35 ng/µL), although these values were within the range, generally considered suitable for PCR amplification. The relatively narrow variation in DNA concentration (35–68 ng/µL) across the sampled species indicated that the extraction protocol was robust, despite differences in plant morphology and the presence of secondary metabolites, which are common in members of the Lamiaceae. Overall, the DNA yields were adequate for successful amplification of the selected DNA barcode loci (rbcLa, rps11, and trnL–trnF), supporting the reliability of the extracted DNA for subsequent sequencing and species identification.

Polymerase chain reaction: rps11 region was successfully amplified from 35 species by standard primers (rps11F, rps11R). The trnL-trnF region was amplified from 26 species and the rbcLa region was amplified from 29 species. The amplified products of various sizes from distinct barcoding regions were confirmed on a 1.5% agarose gel with a 100 bp ladder and High-quality PCR amplicons of ~ 417 base pairs for rps11, ~ 573 base pairs for rbcLa and ~ 375 base pairs for trnL-trnF were observed (Fig. 2. A, B and C).

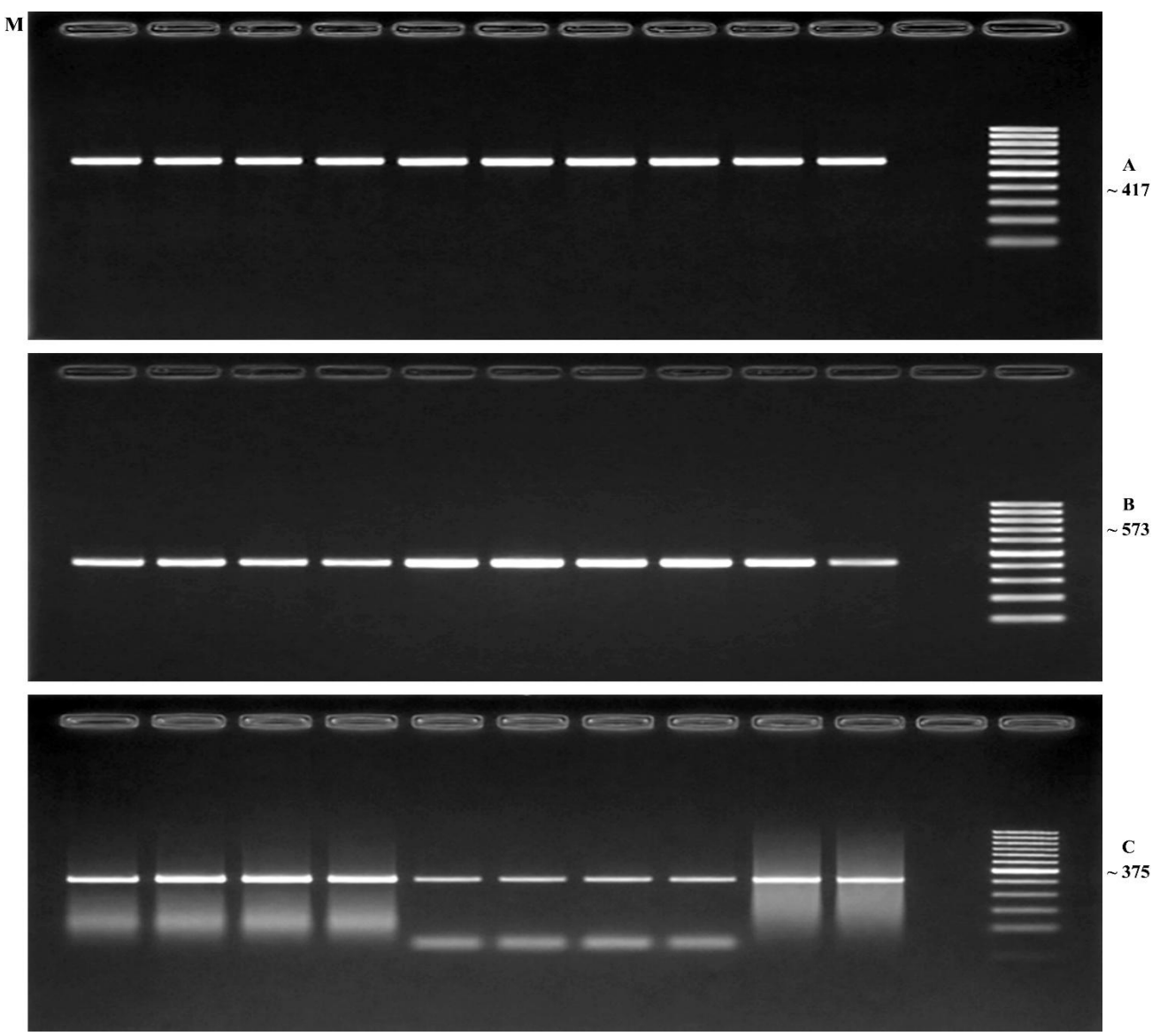


Fig. 2. Visualizations of amplified products from Lamiaceae species. **A**: Amplified product of rps11 gene region. **B**: Amplified product of rbcLa gene region. **C**: Amplified product of the trnL-trnF region. M: 100 bp DNA marker (Fermentas).

Table 1. A list of plants with detail on habit, habitat, collection month, altitude, and locality within the Poonch valley.

Sr. No.	Specimen name	Habit	Habitat	Month of collection	Altitude (ft)	Location (Valley)	Commonly confused with
	Subfamily Ajugoideae						
1.	*Ajuga bracteosa* Wall. ex Benth.	Herb	Moist open places	March-Jun	4000-4500	Hj, Pl, Ch	*Ajuga parviflora* Benth.
2.	*Teucrium royleanum* Wall. ex Benth.	Herb	Silty soil	May-September.	4000-6000	Nar	
	Subfamily Lamiodeae						
3.	*Anisomeles indica* (L.) Kuntze	Herb	Moist open places	April-June	3500-5000	Hj, Pl,	
4.	*Colebrookea oppositifolia* Sm.	Shrub	Dry rocky soil	Apr-June	4000-5000	Hj, Pl, AP	
5.	*Lamium amplexicaule* L.	Herb	Moist shady places	Apr- June	4000-7000	Ch, Jd,KhG	*Lamium album* L.
6.	*Lamium album* L.	Herb	Shady places	Apr- June	4000-5000	Pl	*Lamium amplexicaule* L.
7.	*Phlomis bracteosa* Royle ex Benth.	Herb	Moist shady places	Jul-Sep	6000-8000	Hj	*Phlomis spectabilis* Falc. ex Benth.
8.	*Phlomis spectabilis* Falc. ex Benth.	Herb	Dry rocky Soil	Jul-Sep	6000-8000	Hj	*Phlomis bracteosa* Royle ex Benth.
9.	*Otostegia limbata* (Benth.) Boiss.	Shrub	Dry rocky soil	May-Aug	4000-6000	Ph, Hd	
10.	*Stachys floccosa* Benth.	Herb	Dry rocky soil	June-September	4000-5000	Pl	
	Subfamily Nepetoideae						
11.	*Clinopodium vulgaris* L.	Herb	Dry open places	May-Jul	6000-7000	Hj, Ba, Pl	Clinopodium umbrosum (M. Bieb.) Kuntze
12.	*Clinopodium umbrosum* (M.Bieb.) Kuntze	Herb	Moist shady places	Jun-Aug	5000-6000	Hj, Ba, Pl	*Clinopodium vulgaris* L.
13.	*Clinopodium repens* (D.Don) Benth.	Herb	Moist Open places	Jul-Aug	6000-7000	Tp	*Clinopodium umbrosum* (M. Bieb.) Kuntze
14.	*Elsholtzia fruticosa* (D.Don) Rehder	Shrub	Moist subalpine	Jun-Aug	7000-8000	GM	
15.	*Isodon coetsa* (Buch. -Ham. ex D.Don) Kudô	Herb	Moist shady places	Jul-Aug	5000-6000	Trk, Ch, Hj	*Isodon rugosus* (Wall. ex Benth.) Codd
16.	*Isodon rugosus* (Wall. ex Benth.) Codd	Shrub	Moist Open places	Jul-Aug	6000-7000	Pl	*Isodon coetsa* (Buch. -Ham. ex D.Don) Kudô
17.	*Isodon lophanthoides* (Buch. -Ham. ex D.Don) H. Hara	Herb	Moist Open places	Jul-Sep	6000-7000	DG	*Isodon coetsa* (Buch. -Ham. ex D.Don) Kudô
18.	*Mentha arvensis* L.	Herb	Wet places	Apr-May	4000-7000	Ch,Pl	*Mentha longifolia* (L.) L.
19.	*Mentha longifolia* (L.) L.	Herb	Wet places	Apr-May	4000-7000	Hj, Pl	*Mentha spicata* (L.) L.
20.	*Mentha spicata* (L.) L.	Herb	Wet places	Apr-May	4000-7000	Ch, Pl, Hj	*Mentha longifolia* (L.) L.
21.	*Micromeria biflora* (Buch. -Ham. ex D.Don) Benth.	Herb	Open places	Whole year	3500-5000	Hj, Pl	
22.	*Nepeta govaniana* (Wall. ex Benth.) Benth.	Herb	Dry open places	Aug-Sep	5000-6000	Hj, Dv	*Nepeta erecta* Royle ex Benth
23.	*Nepeta bracteata* Benth.	Herb	Moist places	Jun-Jul	6000-8000	Bj	*Nepata erecta* Royle ex Benth.
24.	*Nepeta erecta* Royle ex Benth.	Herb	Moist places	Jun-Sep	6000-7000	Pl	*Nepeta laevigata* (D.Don) Hand. -Mazz.
25.	*Nepeta laevigata* (D.Don) Hand. -Mazz.	Herb	Dry open places	Jun-Sep	5000-7000	Sr	*Nepeta podostachys* Benth.
26.	*Nepeta podostachys* Benth.	Herb	Dry stony areas	Jun-Sep	6000-7000	MG	*Nepeta laevigata* (D.Don) Hand. -Mazz.
27.	*Origanum vulgare* L.	Herb	Moist grassy field	Jun-Aug	4000-6000	Pl, Ch, Jd	*Mentha longifolia* (L.) L.
28.	*Perilla frutescens* (L.) Britton	Herb	Dry rocky Soil	August-Sep	4000-5000	Ch	
29.	*Prunella vulgaris* L.	Herb	Moist open Places	Apr-Sep	7000-8000	Pl, Ch	
30.	*Salvia lanata* Roxb.	Herb	Dry open places	Mar-June	5000-6000	Ch	*Salvia plebeiax* R.Br.
31.	*Salvia nubicola* Wall. ex Sweet	Herb	Moist Places	Mar-Apr	4000-5000	Bj, Dth, Jd	
32.	*Salvia plebeia* R.Br.	Herb	Drying Pools	Mar-Apr	5000-7000	Gh, Hj, Pl	*Salvia lanata* Roxb.
33.	*Thymus linearis* Benth.	Herb	Moist Places	May-Sep	5000-7000	Dv, Tp	
	Subfamily Scutellarioideae						
34.	*Scutellaria prostrata* Jacq. ex Benth.	Herb	Dry sandy soil	Apr-May	6000-8000	Ch	*Scutellaria discolor* Colebr.
35.	*Scutellaria discolor* Colebr.	Herb	Moist shady places	Apr-May	6000-8000	Pl	*Scutellaria discolor* Colebr.
	Subfamily Viticoideae						
36.	*Vitex negundo* L.	Shrub	Along roadsides	Round the year	5000-7000	Hj	

Key: MG: Mehmood Gali Gh: Ghamir, Hj: Hajira, Ph: Phagwati, B: Bari, Hd: Houd, Tp: Toulipir, KhG: Khaigalla, Jd: Jandali, Bj: Banjosa, Nr: Narwal, Sr: Sarari, Pl: Palandri, Trk: Trarkhel, DG: Devi Gali, AP: Azad Patten, Rwk: Rawalakot, Dth: Dothan, Ch: Chichiri

Table 2. DNA concentration values for Lamiaceae species.

Sr. No.	Specimen name	DNA values (ng/µL)	Sr. No.	Specimen name	DNA values/ (ng/µL)
1.	*Ajuga bracteosa*	44 ng/µL	19.	*Mentha longifolia*	58 ng/µL
2.	*Teucrium royleanum*	35 ng/µL	20.	*Mentha spicata*	54 ng/µL
3.	*Anisomeles indica*	43 ng/µL	21.	*Micromeria biflora*	62 ng/µL
4.	*Colebrookea oppositifolia*	57 ng/µL	22.	*Nepeta govaniana*	45 ng/µL
5.	*Lamium amplexicaule*	56 ng/µL	23.	*Nepeta bracteata*	40 ng/µL
6.	*Lamium album*	53 ng/µL	24.	*Nepeta erecta*	35 ng/µL
7.	*Phlomis bracteosa*	37 ng/µL	25.	*Nepeta laevigata*	50 ng/µL
8.	*Phlomis spectabilis*	46 ng/µL	26.	*Nepeta podostachys*	42 ng/µL
9.	*Otostegia limbata*	48 ng/µL	27.	*Origanum vulgare*	45ng/µL
10.	*Stachys floccosa*	62 ng/µL	28.	*Perilla frutescens*	56ng/µL
11.	*Clinopodium vulgaris*	57ng/µL	29.	*Prunella vulgaris*	66ng/µL
12.	*Clinopodium umbrosum*	48 ng/µL	30.	*Salvia lanata*	51 ng/µL
13.	*Clinopodium repens*	37 ng/µL	31.	*Salvia nubicola*	46 ng/µL
14.	*Elsholtzia fruticosa*	56 ng/µL	32.	*Salvia plebeian*	37 ng/µL
15.	*Isodon coetsa*	64 ng/µL	33.	*Thymus linearis*	57ng/µL
16.	*Isodon rugosus*	51 ng/µL	34.	*Scutellaria prostrata*	53 ng/µL
17.	*Isodon lophanthoides*	49 ng/µL	35.	*Scutellaria discolor*	44 ng/µL
18.	*Mentha arvensis*	68 ng/µL	36.	*Vitex negundo*	38ng/µL
	Average value	**49.97 ng/µ**			

Table 3. Accession numbers and DNA barcode of rps11.

Sr. No.	Species	rps11	rps11ID	FLIS	GLIS	SLIS
1.	*Ajuga bracteosa*	ON987278	*Ajuga lupulina*	✓	✓	×
2.	*Anisomeles indica*	OQ304470	*Anisomeles indica*	✓	✓	✓
3.	*Clinopodium vulgaris*	ON987274	*Clinopodium chinense*	✓	✓	×
4.	*Clinopodium umbrosum*	ON987275	*Clinopodium chinense*	✓	✓	×
5.	*Clinopodium repens*	OP245096	*Clinopodium chinense*	✓	✓	×
6.	*Colebrookea oppositifolia*	OP046423	*Colquhounia coccinea*	✓	×	×
7.	*Elsholtzia fruticosa*	OQ304471	*Elsholtzia fruticose*	✓	✓	✓
8.	*Isodon coetsa*	OP046427	*Isodon coetsa*	✓	✓	✓
9.	*Isodon rugosus*	OP245097	*Isodon rugosus*	✓	✓	✓
10.	*Isodon lophanthoides*	OP245098	*Isodon serra*	✓	✓	×
11.	*Lamium amplexicule*	ON987280	*Lamium amplexicule*	✓	✓	✓
12.	*Lamium album*	OP245099	*Lamium album*	✓	✓	✓
13.	*Mentha arvensis*	KR072659	*Mentha arvensis*	✓	✓	✓
14.	*Mentha longifolia*	KR265167	*Mentha longifolia*	✓	✓	✓
15.	*Mentha spicata*	KR265168	*Mentha spicata*	✓	✓	✓
16.	*Micromeria biflora*	OP245100	*Mentha X piperita*	✓	×	×
17.	*Nepeta govaniana*	OQ304472	*Nepeta cataria*	✓	✓	×
18.	*Nepata bracteata*	OP245101	*Mentha X piperita*	✓	✓	×
19.	*Nepeta levigate*	OQ304473	*Nepeta cataria*	✓	✓	×
20.	*Nepeta podostachys*	OQ304474	*Nepeta cataria*	✓	✓	×
21.	*Origanum vulgare*	OP245102	*Origanum vulgare*	✓	✓	✓
22.	*Perilla frutescens*	OQ304475	*Perilla frutescens*	✓	✓	✓
23.	*Phlomis bracteosa*	OP046422	*Phlomis alpina*	✓	✓	×
24.	*Phlomis spectabilis*	ON987284	*Phlomis alpina*	✓	✓	×
25.	*Prunella vulgaris*	OP046426	*Prunella vulgaris*	✓	✓	✓
26.	*Otostegia limbata*	OP046424	*Eriophyton wallichii*	✓	×	×
27.	*Scutellaria prostrata*	OP046429	*Scutellaria altaica*	✓	✓	×
28.	*Scutellaria discolor*	OP245103	*Scutellaria altaica*	✓	✓	×
29.	*Salvia lanata*	ON987276	*Salvia officinalis*	✓	✓	×
30.	*Salvia nubicola*	OQ304476	*Salvia nubicola*	✓	✓	×
31.	*Salvia plebeain*	ON987277	*Salvia plebeian*	✓	✓	✓
32.	*Stachys floccosa*	ON987281	*Stachys byzantine*	✓	✓	×
33.	*Teucrium royleanum*	OP046425	*Teucrium simplex*	✓	✓	×
34.	*Thymus linearis*	OP046426	*Thymus linearis*	✓	✓	✓
35.	*Vitex negundo*	OQ304477	*Vitex negundo*	✓	✓	✓

Key: FLIS= Family-level identification status, GLIS= Genus-Level identification status, GLIS= Species-level identification status

Accession numbers: The unique accession numbers were assigned to sequences submitted in the GenBank database (Tables 3-5).

rps11 Blast analysis: Through Blast analysis it was revealed that rps11 marker exhibited the highest amplification success (97%) and excellent identification at the family (100%) and generic (91%) levels (Table 3). However, its species-level identification success was comparatively low (42%), indicating limited sequence divergence among closely related Lamiaceae species. It showed that although rps11 was a reliable marker for higher taxonomic identification, its ability to distinguish species was relatively poor when used alone.

rbcLa Blast analysis: MEGABLAST analysis was performed for 30 sequences excluding five species (*Isodon lophanthoides*, *Nepeta erecta*, *Nepeta laevigata*, *Nepeta podostachys*, and *Salvia lanata*), for which the rbcLa region could not be successfully amplified. All 30 rbcLa sequences (100%) were correctly matched to the expected generic level while 73% species were identified at the species level (Table 4).

trnL-trnF Blast analysis: In the case of trnL-trnF, 05 species (*Anisomeles indica, Elsholtzia fruticosa, Nepeta laevigata, Nepeta podostachys,* and *Salvia plebeia*) failed to be amplified. BLAST was performed for 31 sequences and sequences of four species (*Nepeta bracteata, Salvia plebian, Micromeria biflora, Stachys flocoosa*) hits multiple genera and were recorded as ambiguous and excluded from the analysis. From the 27 sequences the 87% showed the 99-100 percent identity matched with the expected genera while 44% sequences matched with the expected species (Table 5).

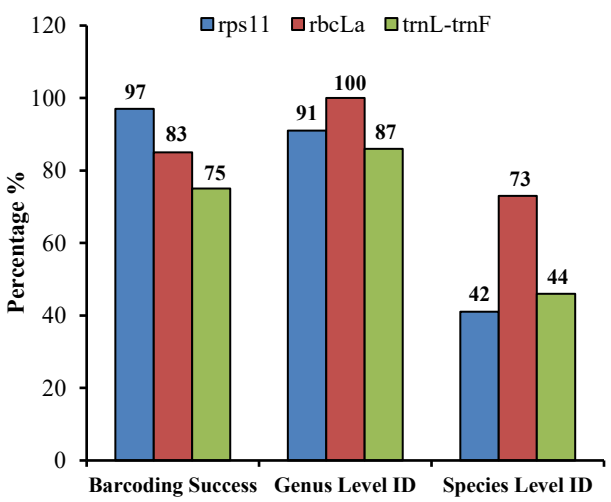


Fig. 3. The ssuccess rate of DNA barcoding loci. rps11 showed the highest barcoding success, but the lowest species-level identification. rbcLa showed the highest Genus-Level and Species Level Identification. and trnL-trnF showed the lowest barcoding success.

Evaluation efficiency of DNA barcoding loci: The comparative amplification and identification success of the 3 loci at the generic and species levels is shown in (Fig. 3). The rbcLa locus although demonstrated lower amplification success (83%) than that of rps11 (97%), it achieved complete identification at generic levels (100%) and the highest at species-level (73%). These results suggested that rbcLa provided an appropriate balance between sequence conservation and nucleotide variation, enabling reliable amplification while retaining sufficient polymorphism for accurate species discrimination. Therefore, among the 3 loci evaluated, rbcLa is proposed as the most suitable DNA barcode for the identification of Lamiaceae species included in the present study.

The amplification and identification success rate among 5 subfamilies of Lamiaceae was also shown in Table 6. The amplification success rate was 100% across all selected species in the 5 Lamiaceae subfamilies. However, species-level identification success varied among subfamilies. The highest species identification success rate (100%**)** was achieved for Ajugoideae and Viticoideae, followed by Lamioideae (75%**)** and Nepetoideae (73.91%**).** In contrast, no species-level identification was achieved for Scutellarioideae (0%**)** (Table 6.).

Chi-square analyses: The Chi-square test was performed for comparative interpretation of three DNA barcodes. The three DNA barcode loci exhibited significant differences in their taxonomic identification efficiency. Chi-square analysis confirmed that the identification success varied significantly among the family, genus, and species taxonomic levels for rps11 ($\chi^2 = 38.86$, df = 2, $p<0.001$), rbcLa ($\chi^2 = 16.42$, df = 2, $p<0.001$), and trnL-trnF ($\chi^2 = 12.28$, df = 2, $p= 0.002$). The Chi-square Analyses summary of 3 barcodes is given in Table 7.

Overall, the comparative evaluation demonstrates that all three loci are suitable for identifying taxa at the family and generic levels, but their performance differs markedly at the species level. Among the evaluated markers, rbcLa consistently provided the highest species discrimination and the best overall identification accuracy, whereas rps11 was superior in amplification efficiency and trnL-trnF exhibited the lowest overall performance. These results support the recommendation of rbcLa as the most suitable single-locus DNA barcode for the molecular identification of Lamiaceae species from the Poonch Valley of Azad Jammu and Kashmir, Pakistan.

Concatenated phylogenetic analysis of Lamiaceae species: The current study examined the combined phylogeny of three chloroplast markers, and the resolution obtained indicated that the combined data set resulted in a higher level of resolution within Lamiaceae (Fig. 4). According to the phylogenetic tree, three primary clades: I, II, and III were recovered within Lamiaceae. The clade I comprised the Viticoideae which was represented by only one species with good support values (MLBS = 87%, BIPP = 0.99).

The clade II of Lamiaceae comprised three subfamilies: Ajugoideae, Scutellarioideae, and Lamioideae, (MLBS = 100%, BIPP = 1). Ajugoideae with support values (MLBS= 86%, BIPP= 0.99) were comprised of two tribes (Teucrieae and Ajugeae) and come in one cluster with high branch support (100%, 1). The Lamioideae has a sister relationship with Clade III that comprised only one family Nepetoideae.

Within Nepetoideae the monophyly of Elsholtzieae, Ocimeae, and Mentheae was robustly supported (97%, 1). Elsholtzieae, and Ocimeae both supported Mentheae as a sister tribe. In the current analysis, the tribe mentheae was the largest tribe having eight genera (*Prunella*, *Nepeta,* *Salvia*, *Micromeria*, *Mentha*, *Origanum*, *Thymus,* and *Clinopodium*) and the maximum number of species. The genus *Prunella* was at the base of mentheae while genus *Clinopodium* was at the top and remained unresolved in current study.

According to the phylogenetic analysis, the species in each cluster showed genetic differences, and species boundaries in the studied taxa were easily identified. The phylogenetic tree distinguished the study species from the outgroup, indicating that all species descended from a common ancestor. Previously, no such study from Poonch Valley, AJK, Pakistan was reported. Therefore, our research provides some basic information based on the barcoding loci to delimit these selected species in the family Lamiaceae from Poonch Valley, Pakistan.

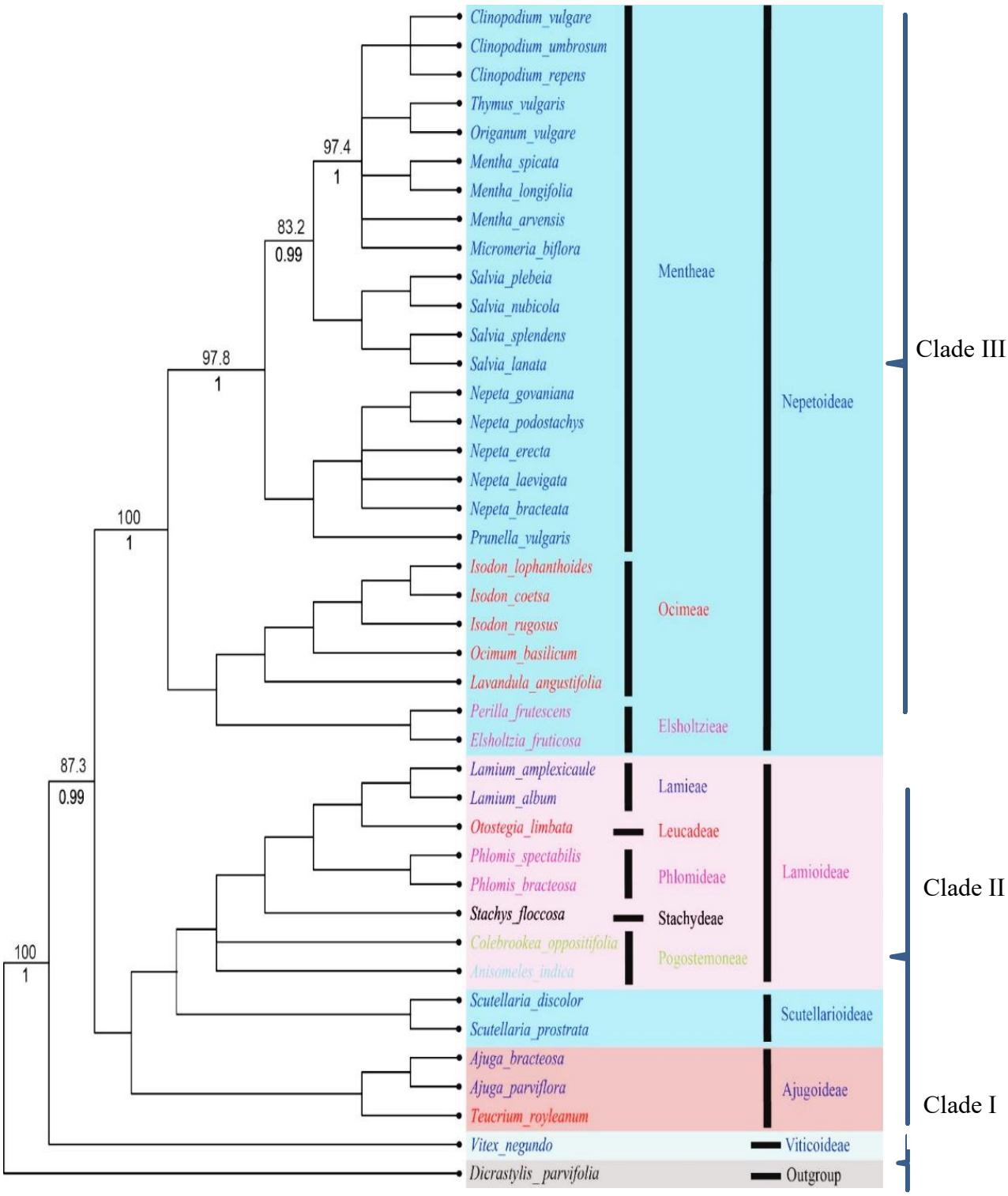


Fig. 4. Lamiaceae phylogeny based on a combined plastid coding regions dataset. Bayesian inference posterior probability (BIPP) and Maximum likelihood bootstrap support (MLBS) is shown below and above and the branches, respectively.

Table 4. Accession numbers and DNA barcode of rbcLa.

Sr. No.	Species	RbcLa	rbcLa ID	FLIS	GLIS	SLIS
1.	*Ajuga bracteosa*	ON947737	*Ajuga bracteosa*	✓	✓	✓
2.	*Anisomeles indica*	OQ304478	*Anisomeles indica*	✓	✓	✓
3.	*Clinopodium vulgaris*	ON947744	*Clinopodium vulgaris*	✓	✓	✓
4.	*Clinopodium umbrosum*	OP245105	*Clinopodium vulgaris*	✓	✓	×
5.	*Clinopodium repens*	OP245106	*Clinopodium repens*	✓	✓	✓
6.	*Colebrookea oppositifolia*	ON947734	*Colebrookea oppositifolia*	✓	✓	✓
7.	*Elsholtzia fruticose*	OQ304479	*Elsholtzia fruticose*	✓	✓	✓
8.	*Isodon coetsa*	ON947753	*Plectranthus zuluensis*	✓	✓	✓
9.	*Isodon rugosus*	OP245107	*Isodon rugosus*	✓	✓	✓
10.	*Lamium album*	OQ304480	*Lamium album*	✓	✓	✓
11.	*Lamium amplexicule*	ON947740	*Lamium amplexicule*	✓	✓	✓
12.	*Mentha arvensis*	ON947747	*Mentha X piperita*	✓	✓	✓
13.	*Mentha longifolia*	ON947748	*Mentha longifolia*	✓	✓	✓
14.	*Mentha spicata*	ON947749	*Mentha spicata*	✓	✓	✓
15.	*Micromeria biflora*	ON947746	*Micromeria biflora*	✓	✓	✓
16.	*Nepeta bracteate*	OQ304481	*Nepeta bracteate*	✓	✓	✓
17.	*Nepeta govaniana*	ON947752	*Nepeta cataria*	✓	✓	×
18.	*Origanum vulgare*	ON947750	*Origanum vulgare*	✓	✓	✓
19.	*Perilla frutescens*	OQ304482	*Perillia frutescens*	✓	✓	✓
20.	*Phlomis bracteosa*	ON947741	*Phlomoides younghusbandii*	✓	✓	×
21.	*Phlomis spectabilis*	ON947742	*Phlomoides younghusbandii*	✓	✓	×
22.	*Prunella vulgaris*	ON947735	*Prunella vulgaris*	✓	✓	✓
23.	*Otostegia limbate*	ON947736	*Rydingia limbate*	✓	✓	✓
24.	*Scutellaria prostrata*	ON947739	*Scutellaria altaica*	✓	✓	×
25.	*Scutellaria discolor*	OP245109	*Scutellaria altaica*	✓	✓	×
26.	*Salvia plebeian*	OQ304483	*Salvia plebian*	✓	✓	✓
27.	*Salvia nubicola*	OQ304484	*Salvia nubicola*	✓	✓	✓
28.	*Stachys floccose*	ON947751	*Stachys Pilosa*	✓	✓	×
29.	*Teucrium royleanum*	ON947754	*Teucrium ornatum*	✓	✓	×
30.	*Vitex negundo*	OQ304485	*Vitex negundo*	✓	✓	✓

Key: FLIS= Family-level identification status, GLIS= Genus-Level identification status, GLIS= Species-level identification status

Discussion

Due to the complexity of Lamiaceae taxonomy, identification based on morphological features alone is difficult, thus the DNA barcode study is an ideal case for solving the family's taxonomic problem. DNA barcoding is an advanced and powerful tool for taxonomic identificaton and drawing phylogenetic relationship using a short genomic region (Hebert *et al*., 2003). The rps11 and the trnL-trnF markers are widely used as a barcode (Mes *et al*., 2000; Kress & Erickson, 2008; Jabeen *et al*., 2012; Ibrahim *et al*., 2014; Mahmood *et al.,* 2015), rbcL has been proposed as the best barcode fragment because of its universality, ease of amplification, and comparability (Ibrahim *et al.,* 2014). Based on our findings, rbcLa was also suggested as the best plant barcode among the potential plant markers because of its effective discrimination power. It was discovered that rbcLa had better resolution (Maloukh *et al.,* 2017), and could be a useful tool for species identification, in situations where other techniques are unfeasible (Kesanakurti *et al.,* 2011). In this study, the overall success rate for barcoding the Lamiaceae is 100% at the generic level, however, at the species level, it reduces to 73% due to a lack of available reference sequence data in GenBank.

Several Lamiaceae species used in the study lacked reference sequence data in GenBank, which posed a significant challenge to our species-level identification of *Clinopodium umbrosum*, *Isodon lophanthoides, Nepata bracteata, Nepata erecta, Nepeta govaniana*, *Nepeta levigata, Nepeta podostachys Phlomis bracteosa*, *Phlomis spectabilis*, *Salvia lanata, Salvia nubicola, Scutellaria prostrata*, *Scutellaria discolor, Stachys floccosa* and *Teucrium royleanum*. In NCBI GenBank only two rbcLa reference sequences were available for *Ototesgia limbata* while *Micromeria biflora* had only one. Another exception was *Phlomis bracteosa*, for which no reference data for any of our chosen markers could be found. With such limited data, there is always a chance of misidentification. Our approach is consistent with the findings of Hollingsworth *et al*., (2016), who observed that more sampling is required to confirm species-level identification. For these previously mentioned species, our sequences data submitted to NCBI GenBank are the first-ever sequences. Another potential issue was that GenBank reference data did not always include the complete gene region. A short sequence from a gene's less variable region may yield a high match rate, but this does not imply that the query sequence was correctly identified. A 99% match with shorter sequences barcode may be more precise than a 99% match with long sequences barcode (Schori & Showalter, 2011).

Table 5. Accession numbers and DNA barcode of trnL-trnF.

Sr. No.	Species	trnL-trnF	trnL-rnF ID	FLIS	GLIS	SLIS
1.	*Ajuga bracteosa*	ON947758	*Ajuga forrestii*	✓	✓	×
2.	*Clinopodium Vulgaris*	ON947756	*Clinopodium vulgaris*	✓	✓	✓
3.	*Clinopodium umbrosum*	OP245110	*Clinopodium chinense*	✓	✓	×
4.	*Clinopodium repens*	OP245111	*Clinopodium chinense*	✓	✓	×
5.	*Colebrookea oppositifolia*	ON964482	*Colebrookea oppositifolia*	✓	✓	✓
6.	*Isodon coetsa*	ON964481	*Isodon coetsa*	✓	✓	✓
7.	*Isodon rugosus*	OP245112	*Isodon serra*	✓	✓	×
8.	*Isodon lophanthoides*	OP245113	*Isodon coetsa*	✓	✓	×
9.	*Lamium album*	OP245122	*Lamium album*	✓	✓	✓
10.	*Lamium amplexicula*	ON947761	*Lamium amplexicula*	✓	✓	✓
11.	*Mentha arvensis*	ON934185	*Mentha X piperita*	✓	✓	×
12.	*Mentha longifolia*	ON947755	*Mentha spicata*	✓	✓	×
13.	*Mentha spicata*	ON934186	*Mentha spicata*	✓	✓	✓
14.	*Nepeta govaniana*	ON947764	*Nepeta menthoides*	✓	✓	×
15.	*Nepata erecta*	OP245114	*Nepeta cataria*	✓	✓	×
16.	*Origanum vulgare*	ON947760	*Origanum vulgare*	✓	✓	✓
17.	*Perilla frutescens*	ON964486	*Perilla frutescens*	✓	✓	✓
18.	*Phlomis bracteosa*	ON964479	*Phlomis younghusbandi*	✓	✓	×
19.	*Phlomis spectabilis*	ON964478	*Phlomis younghusbandi*	✓	✓	×
20.	*Prunella vulgaris*	ON964483	*Prunella vulgaris*	✓	✓	✓
21.	*Otostegia limbata*	ON964484	*Rydingia limbate*	✓	✓	✓
22.	*Scutellaria prostrata*	ON964480	*Scutellaria chamaedrifolia*	✓	✓	×
23.	*Scutellaria discolor*	OP245115	*Scutellaria chamaedrifolia*	✓	✓	×
24.	*Salvia lanata*	ON947757	*Salvia daghestanica*	✓	✓	×
25.	*Teucrium royleanum*	ON964485	*Teucrium simplex*	✓	✓	×
26.	*Thymus linearis*	ON964487	*Thymus linearis*	✓	✓	✓
27.	*Vitex negundo*	ON964488	*Vitex negundo*	✓	✓	✓

Key: FLIS= Family-level identification status, GLIS= Genus-Level identification status, GLIS= Species-level identification status

Table 6. Summary of amplification success (%) and Species Identification (%) across subfamilies.

Subfamilies	No. of species	PCR Amplification (%)	Species identification (%)
Ajugoideae	2	100	100
Lamioideae	8	100	75
Nepetoideae	23	100	73.91
Scutellarioideae	2	100	0
Viticoideae	1	100	100

Table 7. Summary of Chi-square Analyses of three barcodes.

Parameters	rps11	rbcLa	trnL-trnF
Amplification success	97%	83%	75%
Family-level identification	100%	100%	87%
Species-level identification	42%	73%	44%
Chi-square	38.86	16.42	12.28
P-value	<0.001	<0.001	0.002
Overall ranking	**2nd**	**1st**	**3rd**

Several species in the present study could only be identified at the generic level because corresponding reference sequences were unavailable or poorly represented in the GenBank and BOLD databases. This limitation reduces the discriminatory power of DNA barcoding and may lead to ambiguous species assignments among closely related taxa. In practical applications, incomplete reference databases may hinder the accurate authentication of medicinal plants, increasing the risk of species substitution or adulteration, which can affect the quality, safety, and efficacy of herbal medicines. Likewise, generic-level identification may limit the precision of biodiversity assessments and conservation planning, particularly for rare, endemic, or threatened species that require accurate species-level identification for effective management. These findings emphasize the need to expand high-quality reference barcode libraries to improve the accuracy and practical application of DNA barcoding for medicinal plant authentication, conservation, and biodiversity monitoring for Lamiaceae, especially by incorporating sequences from geographically under represented regions such as Azad Jammu and Kashmir, Pakistan.

Phylogenetic analysis improves the placement of all Lamiaceae species from Poonch Valley, Azad Jammu and Kashmir, Pakistan in clades. Several authors recommended that a combination of different barcoding loci improve plant group resolution (Anon., 2009; Gogoi *et al*., 2020) while others suggested that single barcoding loci can be used to resolve diverse plant species (Unsal *et al*., 2019; Saha *et al*., 2020; Pham *et al*., 2021). The current study also proved that the combined data set resulted in a higher level of resolution within Lamiaceae. The current study is also consistent with the Harley *et al*., (2004) finding that largest subfamily Nepetoideae of Lamiaceae, is splitted into 3 tribes Ocimeae, Mentheae, and Elsholtzieae. It is considered a most clearly defined monophyletic subfamily (Erdtman, 1945; Cantino *et al*., 1986) and consistently supported as monophyletic in successive molecular

analysis (Kaufmann & Wink 1994; Wagstaff *et al*., 1995; Chen *et al*., 2016; Li *et al*., 2017) The current study also supports the monophyly of Nepetoideae (Fig. 4). Ocimeae has a sister relationship with Elsholtzieae - Mentheae tribe (Wagstaff, Olmstead & Cantino, 1995). One recent study revealed, Elsholtzieae was a sister to Ocimeae but not a sister to Mentheae (Zhao *et al*., 2021). Our study is also in agreement that Elsholtzieae is a sister to Ocimeae but sister to Mentheae (Fig. 4).

The Lamioideae is the 2nd largest subfamily of Lamiaceae consists 62 genera and above 1260 species (Roy & Lindqvist, 2015). Cantino (1992) suggested the Pogostemonoideae subfamily to accommodate *Anisomeles*, *Colebrookea*, *Leucosceptrum*, *Comanthosphace*, *Rostrinucula*, *Eurysolen* and *Pogostemon*. Our result recovers the tribe Pogostemoneae within Lamioideae as a sister to a clade containing other members of Lamioideae. Previous molecular studies have also confirmed that Pogostemonoideae is a sister to Lamioideae and has been classified as part of the Lamioideae subfamily (Scheen *et al*., 2010; Zhao *et al.,* 2021).

Our findings suggest that the subfamily Scutellarioideae is related to the Lamioideae, which is consistent with prior research (Chen *et al*., 2016). In current analysis Ajuga *bracteosa* and *Teucrium royleanum* of subfamily Ajugoideae collected from Poonch Valley, placed in one clade confirming Ajugoideae as monophyletic. A recent phylogenetic analysis based on four cpDNA markers (matK, rbcL, trnL-trnF, and rps16) also strongly supported Ajugoideae monophyly (Xiang *et al*., 2018). *Vitex negundo* collected from Poonch valley was at the base indicating that all species descended from a common ancestor" with "reflecting their shared evolutionary ancestry despite genetic divergence among the studied species."

Conclusion

This study represents the first assessment of DNA barcode markers for the identification of Lamiaceae species collected from the Poonch Valley of Azad Jammu and Kashmir, Pakistan. Therefore, the current study gives an updated account of Lamiaceae by using DNA sequence data from Poonch Valley in support with NCBI GenBank data. The identities of the analyzed specimens were further confirmed through comparisons with authenticated reference sequences in the GenBank and BOLD databases with 99–100% sequence similarity, enabling reliable species identification. Here it is concluded that this study establishes a foundation for future research on the identification of Lamiaceae and may also assist in the development and evaluation of DNA barcodes for other medicinal plant species.

Acknowledgements

The authors extend their gratitude to Prof. Dr. Pamela Soltis, Prof. Dr. Douglas Soltis and Dr. Evgeny Mavrodiev for assistance with the research and providing the laboratory facilities at the Laboratory of Molecular Systematics & Evolutionary Genetics, University of Florida, Florida, USA where the whole experimental work was performed. The authors thank the Higher Education Commission of Pakistan for the IRSIP scholarships.

Author's Contributions: All authors contributed to the study conception and design. Supervision and Data collection was performed by (RK) and (MSK) respectively. TMT, my co-author and fellow researcher, has made valuable contributions to my research work throughout my PhD studies. Her support and assistance have been particularly helpful in the present study, especially in the application of various bioinformatics tools and techniques for DNA sequence analysis and interpretation. Her technical input and collaborative support have significantly contributed to the bioinformatics component of this research. The molecular work and first draft of the manuscript was done by (NA). All authors read and approved the final manuscript.

Data Availability: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest: The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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