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# GENETIC DIVERSITY AND PHYLOGENETIC STUDY OF GENUS *MENTHA* FROM HAZARA REGION, KHYBER PAKHTUNKHWA, PAKISTAN

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**Abstract**
The *Mentha* genus is a member of the commercially significant and medicinally useful Mentheae tribe, as well as the mint family Lamiaceae, subfamily Nepetoideae. As a result, precise *Mentha* species identification requires molecular identification. Because of the complexity and ambiguity of the classification of the genus *Mentha*, this research is the first time carried out in the Hazara region in Khyber Pakhtunkhwa, Pakistan. The goal of the most recent study was to verify the accuracy of the morphological and molecular parameters of *M. arvensis*, *M. royleana*, *M. spicata*, *M. piperita*, and *M. longfolia*. Applying the molecular markers *matK*, genetic variations in five specimens of Mentha were examined. Gene-specific primers were utilized throughout the DNA amplification process. Consensus sequences were produced from each Mentha specimen using Bio Edit software by meticulously sequencing the PCR data. By BLAST examination of consensus nucleotide sequences for each *Mentha* species and every query sample indicated similarity to gene bank sequences. The determined species of the genus *Mentha* are organically different according to the NCBI analysis, nucleotide diversity, nucleotide discrimination, haplotype diversity, and phylogenic analysis through MEGA software. All *Mentha* species have a total haplotype diversity of 0.9989 with a nucleotide diversity of 0.01379. Nucleotide sequences of *Mentha* species were correlated for phylogenetic analysis, and the neighbor-joining tree and Maximum Parsimony were constructed. Neighbor-joining trees and maximum parsimony verified that these species are authentic. Additionally, this investigation solved a slight problem in the identification of flora in the field of botany.

**Keys words**: Genetic diversity, *Mentha*, Phylogeny, *matK*, and Hazara Region.

## INTRODUCTION

According to Wagstaff et al. (1995), every species of Mentha belongs to the tribe Mentheae, subfamily Nepetoideae, and mint family Lamiaceae (Labiatae A. L. de Jussieu). The vast majority of *Mentha* species are found in Asia and Europe and are extensively distributed. Additionally, only *M. cunninghamii* is found in New Zealand, whereas three other species *M. diemenica, M. satureioides, and M. australisare* found in Australia. Specifically, Sardinia, the island of Monte Cristo Island (Italy), but Corsica (France) are home to the indigenous *Mentha requienii. Mentha gattefossei* can only be identified in or close to Morocco. Furthermore, according to Bunsawat *et al.*

(2004), The only species endemic to the new continent is M. canadensis, which is found in North America. The taxonomy of *Mentha* is still unknown because of continued interspecies hybridization and polyploidy in both wild and cultivated populations (Heylen *et al.,* 2021). Throughout the past 200 years, the genus Mentha has expanded across several taxonomic levels and has been linked to morphological alterations. Due to the process of hybridization, Mentha species showed composite variability across the wilder populations (Xiao *et al.,* 2021. Jakovljevic *et al.,* (2013) state that the antifebrile, carminative, and antiseptic properties of carvone, linalol, and limonene are associated with their respective therapeutic properties. Due to its therapeutic qualities, mint is often used to treat a wide range of conditions, including fever, bronchitis, anorexia, and flatulence. Their essential oils are employed as a scent-enhancing ingredient in mouthwashes, medicines, confections, oral hygiene products, and herbicides (Shaikh *et al.,* 2014). Mentha tea has been used for millennia to cure minor ailments such as fevers, headaches, and digestive problems (Ship and Chavez, 2002). These days, many gastrointestinal disorders are treated using Mentha species. For example, the antioxidant and anti-inflammatory qualities of Mentha longifolia (L.) methanolic extract, which has a high eucalyptol content, showed antiulcer effectiveness against acetic acid-induced colitis in rats (Gilani *et al.,* 2004). A clinical study demonstrated the challenging-provocative abilities of mint important oils for example, utilizing *M. spicata* essential oil may help people with osteoarthritis feel less uncomfortable. These analgesic properties are mainly caused by the main ingredients of *M. spicata* essential oil, which include limonene, menthol, and carvone (Toker *et al.,* 2004). To examine the genetic diversity and phylogeny of the genus Mentha utilizing the chloroplast gene *matK* from the Hazara Region of Khyber Pakhtunkhwa, Pakistan, therefore a comprehensive investigation was organized.

## METHOD AND MATERIALS

### Study Area

The Hazara area is located (Figure 1) (Sarim and Kouser, 2007) extending from 33º-44′ to 35º-35′ north latitude and from 72º-33′ to 74º-05′ east longitude.

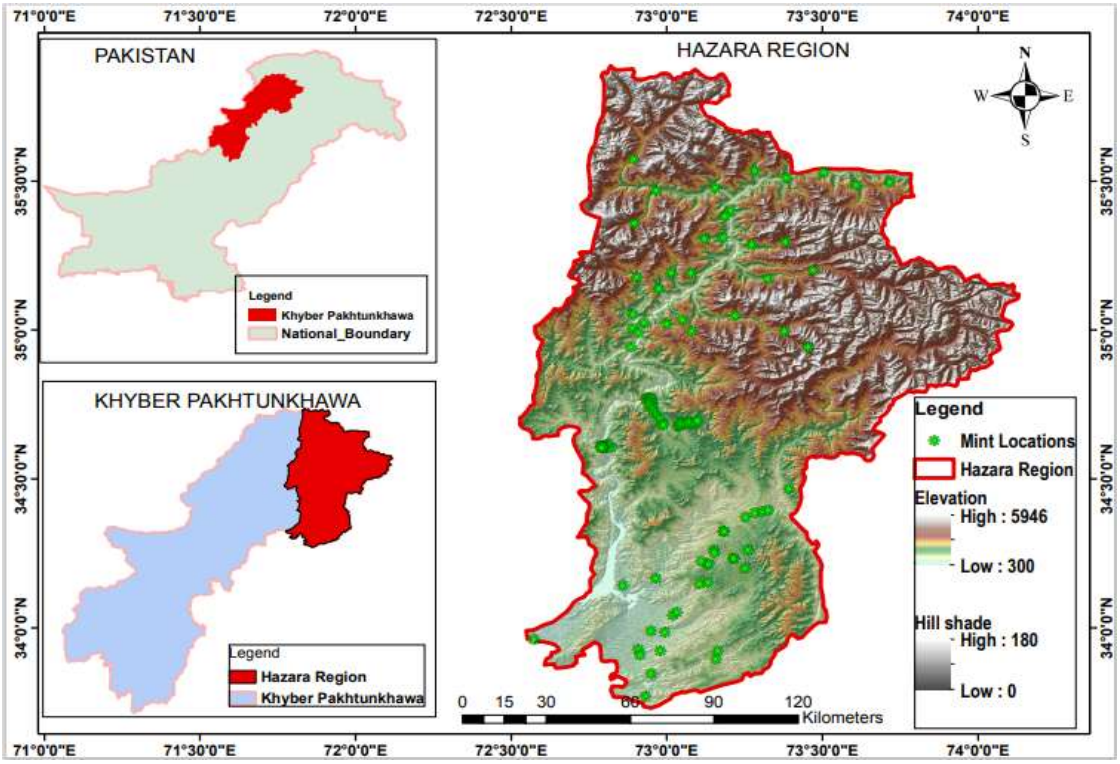


**Figure** 1. Map of Hazara area, Khyber Pakhtunkhwa, Pakistan; five districts of Hazara Division: Kohistan, Battagram, Mansehra, Abbottabad, and Haripur**.**

**Collection of *Mentha* Species**

Every specimen was collected, dried, cleaned, and adhered to a typical herbarium sheet. At Hazara University's Department of Botany, voucher samples (also known as specimen numbers) were conserved and dried with standard herbarium techniques.

**Identification of *Mentha* Species**

Malik and Ghafoor (1988) conducted a thorough examination of the morphological features within accessible literature. The species were identified by comparison with the Pakistani Flora. On prepared herbarium sheets, samples with voucher names were sent to the botany department's herbarium. Anatomical investigation was conducted using air-dried, finely powdered samples and cross-sections of the fresh stem and leaves stored in 70% ethyl alcohol with 5% glycerin. Pakistan's flora and the literature that was accessible were used to identify these samples. The identified specimens were given to the Herbarium Hazara University Mansehra for future use.

**Genomic Extraction of DNA and DNA amplification**

**DNA isolation**

The genomic DNA was extracted using enhanced Doyle and Doyle techniques (Lodhi *et al.*, 1994; Ye *et al.*, 1966).

**Amplification of DNA**

Utilizing Gel electrophoresis, the quantity and quality of the recovered DNA were assessed, and the DNA was diluted appropriately. When necessary, the PCR process's concentrated DNA was diluted two or three times. Each sample had a 12.5 µl PCR pre-mix produced and put on ice. 0.25 µl of each dNTP (10 mM), 0.5 µl of genomic DNA, 0.5 µl of primer, 1 µl of 10X buffer, 1 µl of MgCl2, and 0.5 µl of Taq Polymerase were combined to make the pre-mix for PCR in 0.5 ml PCR tubes. The capacity of the tube was raised to 12.5 µl by adding 8 µl of water. The PCR technique experiment was carried out using an Applied Biosystems heat cycler 2720. The DNA amplification process made use of eleven RAPD primers. DNA was extracted from dried leaves using the CTAB method (Ye et al., 1966). One µl of primers, one-half µl of master mix, and one µl of anhydrous nuclease make up the amplifying mixture utilized throughout the Amplification reaction. The total volume of reaction used in the Amplification process is 12.5 µl. PCR is conducted in a thermal cycler with the following temperatures: 52 °C to 60 °C for one minute of annealing, 72 °C for two minutes of elongation, and 94 °C for one minute of initial denaturation.

**Phylogenetic Analysis**

The sequence alignment was done using BLAST software after each species was identified utilizing the *matK* gene. MEGA4 software was used to generate a phylogenetic tree for a variety of species (Lodhi *et al.*, 1994; Tamura *et al.*, 2007).

**Statistical Analysis**

Most of the alterations were determined to be significant at the p-value criterion of less than five. A 95% confidence interval was employed for each test. Version 18.0 of the Statistical Software Package in Sciences (SPSS) software was used for all statistical testing. (Lodhi *et al.*, 1994; Kirkwood *et al.*, 2012). The amounts of menthol and the content of essential oils were compared between species using One-way Analysis of Variance (ANOVA).

**RESULTS**

A total of 200 specimens were collected from the study area and classified phenotypically and the five kinds of *Mentha* (*M. longfolia, M. spicata, M. piperita, M. arvensis, and M. royleana*) were identified from the Hazara region of KP, Pakistan, throughout the current investigation.

**Analysis of NCBI**

By applying the software Bio Edit, the query samples for every species of Mentha were successfully sequenced, and consensus sequences were produced from each sample. The consensus DNA strand Each query sample sequence was subjected to blast analysis, and the results demonstrated a range of 97 to 100% sequence matching the Gene Bank sequences, suggesting that they are authentic species.

**The Phylogenetic study of genus *Mentha***

The most recent investigation utilized chloroplast *matK* genes to elucidate the evolutionary status of *Mentha* species in Pakistan's Khyber Pakhtunkhwa, Hazara region. Twelve more (*matK*) sequences were obtained from NCBI data, and 125 sequences from the genus Mentha species were Blasted for the phylogenetic analysis. Twelve (*matK*) GenBank sequences and 125 in all, sequences belonging to the Mentha Genus were analyzed from the collection site. A total of 137 nucleotide sequences for the Mentha species were aligned, and phylogenetic trees were built utilizing the Neighbor-Joining Method. With maximum composite likelihood methods, the evolutionary relationships and phylogenetic distances were determined for each Mentha group independently. These are legitimate species of the genus *Mentha*, as evidenced by the similarity between all 137 nucleotide analysis query samples of Mentha species that belong to the same cluster (Figure 2).

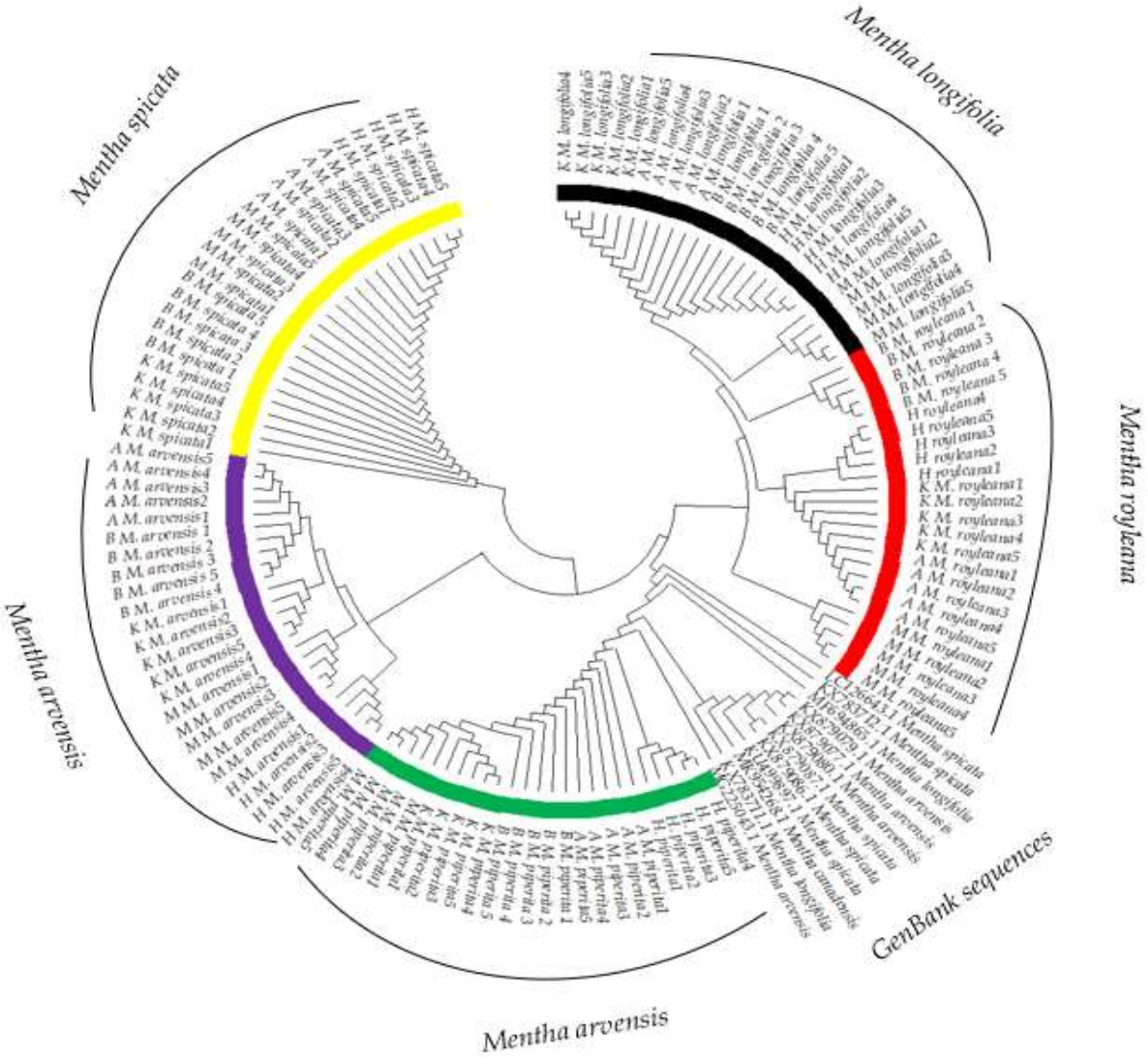


**Figure 2.** The neighbor-joining tree created using MEGA software To determine the evolutionary rank of 125 matK sequences of all query samples of *Mentha* species (*M. longfolia, M. spicata, M. arvensis, M. royleana,* and *M. piperita*) in the Hazara area of Pakistan**.**

**Study Genetic Diversity of Genus *Mentha***

*M. longfolia* (0.004734) had the lowest nucleotide diversity while *M. piperita* (0.007721) from the Hazara area had the most. The nucleotide diversity in the Genus *Mentha* was 0.00754 for all species combined. *M. spicata* had the lowest haplotype diversity (0.5341), whereas M. piperita had the highest haplotype diversity (0.8621). The total nucleotide diversity in the Genus *Mentha* (all species included) was 0.7979. For every species in the genus *Mentha*, the overall Tajima's D value was 0.30379. Positive selection, also known as selective sweeps, is acceptable for a negative Tajima's D in a population in which there are no demographic shifts, such as migration, population growth, or reduction (Table 1).

**Table 1. The genetic diversity determined through the DNAsp 5.10 software of all query samples of *Mentha* species (M. *spicata, M. piperita, M. arvensis, M. longfolia, and M. royleana*) in the Hazara region of KP, Pakistan.**

| Genetic Values | *M. spicata* | *M. royleana* | *M. piperita* | *M. longfolia* | *M. arvensis* | Total |
|---|---|---|---|---|---|---|
| **No of Sequences** | 25 | 25 | 25 | 25 | 25 | 125 |
| **No of haplotypes** | 19 | 18 | 21 | 20 | 22 | 100 |
| **Haplotype diversity** | 0.5341 | 0.6123 | 0.8621 | 0.7123 | 0.5432 | 0.7979 |
| **Nucleotide diversity** | 0.00645 | 0.00583 | 0.007721 | 0.004734 | 0.004843 | 0.00754 |
| **Theta** | 0.005063 | 0.004321 | 0.0070431 | 0.005421 | 0.006521 | 0.00680 |
| **Average no of nucleotide difference K** | 4.1911 | 6.55112 | 4.78426 | 8.98421 | 6.2123 | 3.89961 |
| **Tajima's D test** | -0.54321 | -0.15922 | -0.45541 | - 0.03233 | - 0.01411 | 0.30379 |

The data analysis DNAsp 5.10 software.

**Phylogenetic study of single genotype sample**

The most recent study employed chloroplast matK genes to elucidate The evolutionary status of the mentha species in Pakistan's KP Hazara area. 25 sequences from the genus Mentha species were BLASTed for the phylogenetic study, and 23 additional (*matK*) sequences were retrieved from the Genbank database. Twenty-five sequences from the Mentha Genus and twenty-three (*matK*) GenBank sequences were analyzed. A total of 48 nucleotide sequences from the entire Mentha species were aligned, and the Neighbor-Joining Method was used to create phylogenetic trees for each Mentha species. The evolutionary relationship and phylogenetic distances were established for each Mentha group independently using maximum composite likelihood approaches. Samples of Mentha species from Clade I that were analyzed and utilizing 48 nucleotide sequences show similarity to the species in this clade (Figure 3).

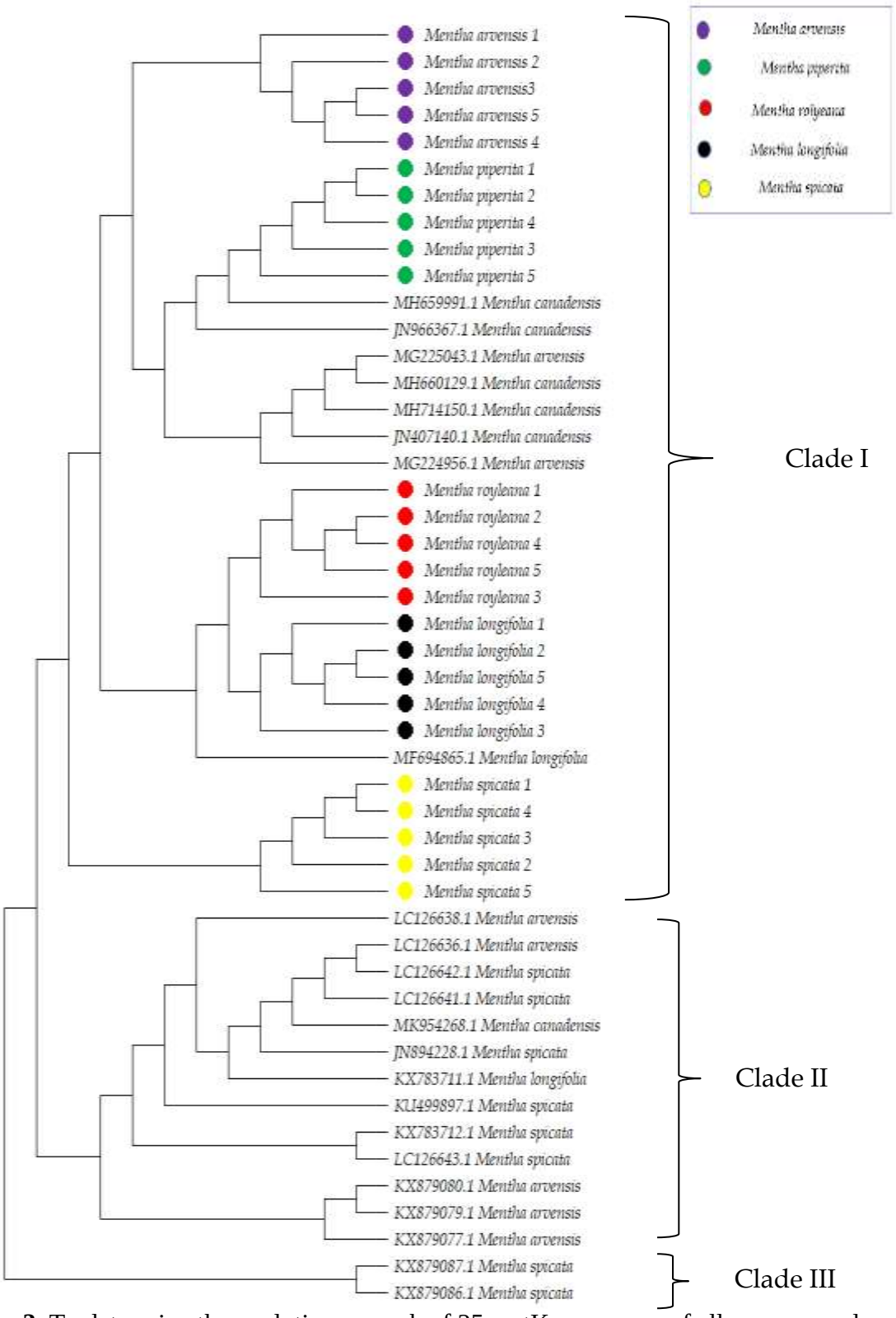


**Figure 3.** To determine the evolutionary rank of 25 matK sequences of all query samples of *Mentha* species ( *M. arvensis, M. longfolia, M. royleana, M. Piperita, and M. spicata*) in the Hazara area of Pakistan, a neighbor-joining phylogenetic tree was created using MEGA software.

## DISCUSSION

A genus of fragrant perennial herbs in the Lamiaceae family is called *Mentha*. Mints have a complex taxonomy because of their significant morphological variety, and potential to hybridize, and domesticated (Behrouz et al., 2004). Mentha's categorization is ambiguous because of persistent polyploidy and interspecies hybridization in both cultivated and wild populations (Gobert *et al.*,

2002). Plant varieties with a range of morphometric traits may be effectively recognized using the DNA barcoding approach (Hebert *et al.,* 2004). A review of the state of our understanding of mint highlights the continued need for a thorough examination of its scent and a deeper comprehension of the olfactory variety of several mint species among them. Utilizing traditional sensory assessment and contemporary molecular technology, this study has chosen five mint species (*Mentha spicata L., Mentha suaveolens L., Mentha citrata L.,* and *Mentha piperita* L. as representative samples for investigating the distinctive features of mint scent. (Zhang *et al.,* 2022). This technique is also applied in the discovery of intra- and inter-specific differences. Initially, a few metazoan species were classified using DNA barcoding and gene sequences. To classify flowering plants, *ITS, trnH-psbA, matK,* and other subsequent barcode regions were used. Consequently, the current study's objective was to measure the genetic diversity among *Mentha* species in the Hazara region of KP, Pakistan, using molecular markers. In the current investigation, five Mentha specimens were examined using molecular markers matK. Because morphological identification is not more exact and needs a high level of taxonomic competence, molecular identification is used as an alternative and more accurate approach for the identification of Mentha species. There are several landrace mints in Pakistan, although it is unknown how diverse their genetic makeup *M. arvensis, M. piperita,* M. royleana, *M. longifolia,* and *M. spicata* were the five *Mentha* species evaluated in the current study. Molecular markers *matK* were employed to examine genetic variation among the five *Mentha* specimens. Successful DNA extraction from *Mentha* species allowed for successful sequencing and BioEdit software was used to construct consensus sequences from each *Mentha* specimen. The consensus strand of DNA in each query sample revealed a similarity of 97–100% to gene bank sequences when the sequences of each *Mentha* species were compared using blast, confirming that these are authentic species. The nucleotide diversity, and haplotype diversity in five species of genus *Mentha* (*M spicata, M arvensis, M longfolia, M royleana, and Mentha piperita*). respectively, were discovered using an analytical investigation of genetic diversity within the 25 chloroplast gene (matK) sequences analyzed using DNAsp 5.10 software. All Mentha species have an overall haplotype diversity of 0.9989 and nucleotide diversity of 0.01379. There are nucleotide base composition sequences in five of the *Mentha* genera. Phylogenetic analysis using molecular methods was also used to illustrate the relative relationships of plant species. Because they enable more accurate assessments of genetic diversity and genotype identification of the organism's developmental stage, molecular markers are essential tools for phylogenetic studies (Salama *et al.,* 2019). Nucleotide sequences of Mentha species were aligned for phylogenetic analyses. The Maximum Parsimony and Neighbor-Joining trees were developed. All neighbor-joining trees and maximum parsimony proved that these taxa are genuine species. These genetic primers were employed to confirm the genus *Mentha* by amplifying the *matK* gene. Using BLAST, the sequence of each Mentha species was identified molecularly. The similarity between ALL of the blast sequences and the GenBank sequences varied from 97 to 100%. It has been determined that these are legitimate members of the *Mentha* genus. In the most recent study, BLAST was looked for and examined using several computational tools. The neighbor-joining, maxim likelihood, UPGMA, test minimum evolution, and maximum parsimony Method were used in the phylogenetic study. These species are distinct, according to the evolutionary tree. Substantial portions of DNA markers for species identification have been studied in molecular identification. Some scientists ask forthright questions of researchers, while others are reasonable by nature. Herbert's 2003 paper generated debate on the usefulness of taxonomists and whether DNA barcoding will eventually supplant conventional morphological techniques for species identification (Pennisi, 2003). Additionally, this study fixes a small issue that will affect botany's ability to identify plants in the future.

## CONCLUSION

Genetic diversity is a comparatively fresh method for identifying species just at the molecular level, which usually uses a short fragment of the chloroplast gene of *matK*. Alternative to focusing exclusively on morphological methods, which need a significant degree of fieldwork and taxonomic knowledge. A framework for the rapid and accurate identification of cryptic species of the genus

Mentha in the Hazara area was developed using DNA sequencing. Molecular identification through chloroplast gene *matK* is a promising taxonomic tool for the identification of the genetic diversity of Mentha species. This method is superior to the traditional method of identifying species by their morphology. Five different species of the genus Mentha, including *M. royleana, M. piperita, M. arvensis, M. spicata, and M. longfolia* were identified in the present study. In the future, precise identification of Pakistani flora will depend on the genetic diversity and phylogenetics of the *Mentha* genus.

**Disclaimer: None**
**Conflict of Interest: None**

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**Contributions of the author**
Hafza Siddique, Muhammad Azhar Khan, Ghulam Mujhtaba Shah, Alia Gul, and Manzoor Hussain designed the project and collected the plant specimens. Hafza Siddique, Nazia Shaheen, Muhammad Fiaz, Nazia Shaheen, and Muhammad Azhar Khan performed molecular biology work and sequence analysis. Hafza Siddique, Omer Dad, and Muhammad Sajid performed the phylogenetic and genetic analysis. Hafza Siddique and Omer Dad wrote the manuscript.

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