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DNA BARCODE FOR PHYLOGENETIC ANALYSIS OF GENUS *MORUS* SPECIES FROM AZAD JAMMU AND KASHMIR

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Abstract

The identity and interrelationship of *Morus* species were confirmed and authenticated in the current research effort using DNA barcode analysis as a molecular technique. Chloroplast DNA of Specimens were evaluated through PCR, Sequence homology and Neighbor-Joining (NJ) clustering. Sequence recoveries of the *rbc*L and *mat*K were 91.66 & 88.88% respectively. All the samples with *matK* depict BLAST similarity more than 96% with sequence cover higher than 75%, whereas in case of *rbc*L BLAST similarity was greater than 97% along with more than 74% sequence coverage. The *mat*K phylogenetic tree diagram; revealed five main divergent groupings and a few subgroups. The first three groups comprised *M. alba* varieties. Group four consists of one *M. alba* and two *M. macroura* variants. *M. alba* V1 is present on a separate node. Varieties of *M. macroura* resemble one another more than sibling taxa. Group five is the largest group comprising four subgroups and five varieties of two species. Variants of *M. nigra* belong to various subgroups and are spread across various intra-species evolutionary nodes. Variants of *M. serrata* are connected. The hierarchical clustering of *rbc*L observed to consist of five main groups and several smaller ones. *M. macroura* and *M. serrata* varieties are included in group one. *M. serrata* species were closely resembling, whereas *M. macroura* species were located on distinct nodes, indicating small differences. 2nd and 3rd groups represent variants of *M. alba*. The fourth and 5th group includes *M. nigra* varieties, with V1 and V2 showed close relationship. The barcoding method divided our subject strains into different groups, which strengthened the identification process. *rbc*L genes had a maximum rate of conservation than *mat*K. According to the *rbc*L alignment all 12 sequences had at least 91.6% identity at the 91.4% of sequence coverage. The results of *mat*K were somewhat diverged sharing a minimum of 69.3% identity and 67% sequence coverage. The findings show that *rbc*L and *mat*K markers can efficiently distinguish between species. Additionally, our research could be useful for identifying other species of *Morus* and contribute to the taxonomy of the genus.

Key words: *rbc*L, *mat*K, Molecular phylogeny, DNA barcode, *Morus*, Azad Jammu and Kashmir.

Introduction

The (mulberry) family Moraceae contains 37 genera and about 1,100 species that are found in temperate, tropical, and subtropical regions of the world in both wild and cultivated forms (Clement & Weiblen, 2009). The genus *Morus* species are found in Southern Europe, the South of North America, the Northwest of South America, and some regions of Africa. They are also known as mulberries. *Morus* species are highly adaptable to a variety of ecological conditions (Clement & Weiblen, 2009; Ercisli & Orhan, 2007). According to the APG IV classification system (APG IV, 2016) the Family Moraceae is a member of the Order Rosales. Milky latex, a stipule, anatropous ovules, apical placentation, fruits (achenes or syconous), and a cystolith are the diagnostic features of *Morus* species (Pramanick, 2017).

Due to their use in sericulture and traditional human medicine, particularly in China and India, these species have economic importance in the majority of nations (Ozgen & Kaya, 2009; Ramesh & Yogananda, 2014). Flavonoids, anthocyanin, and alkaloids present in various parts of the mulberries guarantee several pharmacological activities such as antidiabetic, antioxidant, anti-inflammatory, antimutagenic, hostile to cancer-causing, and hepatoprotective properties (Ozgen & Kaya, 2009; Deniz *et al*., 2018). In Turkey, one of the regions with great diversity, they have been grown for more than 400 years for food. Mulberries produce edible fruits in countries like Turkey and Greece. *Morus* fruits are eaten fresh and additionally used to produce syrup, pulp, ice cream, jam, vinegar, and natural colors (Ercisli & Orhan, 2007; Zafar *et al*., 2013; Eyduran *et al*., 2015).

The four species of this genus, *Morus alba* L., *Morus nigra* L., *Morus serrata* Roxb and *Morus macroura* Miq are present in the flora of Pakistan. Because of its intriguing breeding systems, interspecies hybridization, extensive distribution, naturalization under various conditions, invasiveness of some taxa, and taxonomic confusion in the genus, gets attracted many researchers (Burgess *et al*., 2008). The taxonomy of *Morus* has been unstable as a result of different species numbers being determined by taxonomists. First, Linnaeus identified seven species that belong to this genus. Later, (Burea, 1873; Koidzumi, 1917) identified five and twenty-four species, respectively. Zeng reported 14 species of *Morus* spread over the globe (Zeng *et al*., 2015). Numerous taxonomic studies and revisions have been done on *Morus*. However, in the genus taxonomic, problems still exist (Browicz, 1982; Rao & Jarvis, 1986; Zhou & Gilbert, 2003). The current study aims to identify and compare the *Morus* species at a molecular level found in AJK.

The preservation and use of biodiversity depend on identification of species, yet this process is frequently hindered by a lack of expertise in taxonomic knowledge (Chase & Fay, 2009). There are growing concerns from human activities to the most sensitive floras (Janzen, 1988). Regrettably, conventional morphological taxonomy is tardy and dependent on taxonomic knowledge and established classifications (Costion *et al*., 2011). In addition, the absence of reproductive organs during field surveys, which are required to discriminate between morphologically

identical species, makes it difficult for experts to identify tropical trees (Gonzalez *et al*., 2009).

Rapid identification techniques are urgently required for tropical plant species in order to devise suitable conservation measures (Brooks *et al*., 2006). In the last few years, various DNA barcode publications provide a novel system for the identification and authentication of the species and open a new horizon in plant taxonomy (Noshad *et al*., 2021). Many genetic strategies have been used to address this, but (Hebert *et al*., 2003) demonstrated that DNA barcoding is a key tool that may be used to quickly and accurately identify species without the use of specialized taxonomic knowledge. A good barcode should have conserved sections, be capable of rapid evolution to distinguish across species, and serve as universal primer binding site for PCR (Kress *et al*., 2005). For plants, a combination of two or more loci is typically used, as a single locus is insufficient. The substitution rate spectrum, which describes genes that develop quickly or slowly, includes several genes that are utilized in plant taxonomy. The level of phylogenetic analysis carried out by the researchers typically dictates which genes to be used. A gene has distinct strengths and weaknesses.

rbcL (ribulose-1, 5-bisphosphate carboxylase oxygenase large subunit) has high-quality sequences, but because they are widely conserved across plant species, they show little power of species differentiation (Duan *et al*., 2019). *rbcL* is the best locus for PCR amplification and sequencing (Roy *et al*., 2010). The *mat*K (maturase K) gene, whose sequence is more variable than other genes and has a high degree of substitution, has sequences with a high degree of species differentiation. Because of quick evolution, it has enough power to evaluate lower taxa (Barthet, 2006; Holligsworth, 2011). The *mat*K sequences were highly conserved and had low variable sites (Pham *et al*., 2021). However, recently a consensus has emerged for using the plastid genes *matK* and *rbcL* locus for barcoding of plants (Kress & Erickson, 2008; Anon., 2009; Ismail *et al*., 2020). These two genes are essential for reconstructing the phylogeny of land plants (Kuzmina *et al*., 2012).

The aim of project is to (1) Create a DNA barcode library for *Morus* species, especially in Azad Jammu and Kashmir. (2) Assess the effectiveness of the *mat*K and *rbc*L sequence diversity in identifying species. (3) The molecular differentiation and relationships of *Morus* species using the barcoding approach.

Materials and Methods

Plant sampling: Plant samples were collected from different locations including *Morus* species that are native to Azad Jammu and Kashmir. Voucher specimens of *Morus* species were confirmed and deposited in Herbarium at Mirpur University of Science and Technology's Botany Department. Samples of leaves were collected during April to July. To lower the moisture level, silica gel was added to the zipper-sealed bags containing the leaf samples. The *matK* and *rbcL* core barcode sections are the entire focus of this article, for which experimental work was done at the Biotech Lab. of Mirpur University of Science and Technology AJ&K.

DNA extraction: DNA was extracted from specimens by crushing the tissue in liquid nitrogen, applying modified CTAB method (Doyle & Doyle, 1987). The acquired pellet was cleaned with 70% ethanol and permitted to dry at room temperature. DNA pellet suspended in T.E. buffer was quantified by using a UV Spectrophotometer with A260/280 ranges from 1.62-1.78.

Primers: PCR amplification *matK* gene primers. *matK*3 F ***5***'GTACAGTACTTTTGTGTTTACGAG3', matK1 R ***5***'CCCAGTCCATCTGGAAATCTTGGTTC3' (Hollingsworth *et al*., 2009) and *rbcL*; *rbcL* F ***5***'TGTCACCACAAACAGAGACTAAAGC3' (Levin *et al*., 2003) *rbcL* R ***5***'GTAA_AATCAAGTCCACCRCG3' (Kress & Erickson, 2007) were applied.

PCR Amplification: A PCR reaction was carried out in 0.2 ml PCR tubes by using 25µl total volume to amplify the desired markers. The valuable loci were amplified by using a robust PCR kit (Applied Bio systems). For the *matK*, the cycling conditions were 95°C for 10 minutes, 30 cycles at 95°C for the 30s, 55°C for 45s, 72°C for 1min; and the final elongation at 72°C for 4min. The *rbcL* primers were cycled at 95°C for 10 min, 30 cycles at 58°C for the 30s, 72°C for 1 min, and finally for 4 min, at 72°C for final elongation. PCR products were separated were visualized on 1% agarose gel and purified through a DNA purification kit (Thermo Scientific).

Sequencing: Sequencing was performed by using an ABI DNA sequencer employing standard protocol, the samples were examined in triplicate.

Statistical analysis

BLAST: Using a basic local alignment tool (BLAST), the sequence homology of the *Morus* species were examined.

Sequence alignment: In MEGA X Clustal W algorithm (Larkin *et al*., 2007) was used to align nucleotide sequences.

Phylogenetic analysis: Phylogenetic trees were created for *rbcL* and *matK* nucleotide sequences by Neighbor-Joining (NJ) (Saitu & Nei, 1987) method in MEGA X (Kumar *et al*., 2018).

Results and Discussion

In the current research, a total of 12 phenotypes of *Morus* species from various divisions of Azad Jammu and Kashmir were examined by utilizing DNA barcode data for identification and classification (Fig. 1). DNA isolation and amplification followed the same general procedure, but in some situations, the PCR conditions were modified (Figs. 2-6). For the family Moraceae, significant improvements were made in DNA isolation and PCR annealing temperature. Nucleotide sequences of *matK* and *rbcL* found in good order were selected for further use in phylogenetic analysis (Figs. 7 & 8).

Sequences recovery: The samples, respectively, represent four species and eight varieties. For the *rbcL* and *matK* genes, the sequence size distribution was found to range

between 476-562 and 324-595bp. In contrast, in *rbcL M. nigra* V1 had the longest sequence (562bp) and *M. alba* had the shortest sequence (476bp). The longer *matK* sequence in *M. macroura* V1 was 595bp and the shorter one in *M. serrata* V1 was 324bp (Tables 2 & 3). Sequence recoveries for the two genes *rbcL* and *matK* varied among species from 216 specimens, but in our investigation *rbcL* had a higher success rate of 91.66% compared with *matK*, which reflects a lower percentage of 88.88% and supports the finding that *matK* frequently had lower amplification success (Kress & Ericson, 2007) (Table 1).

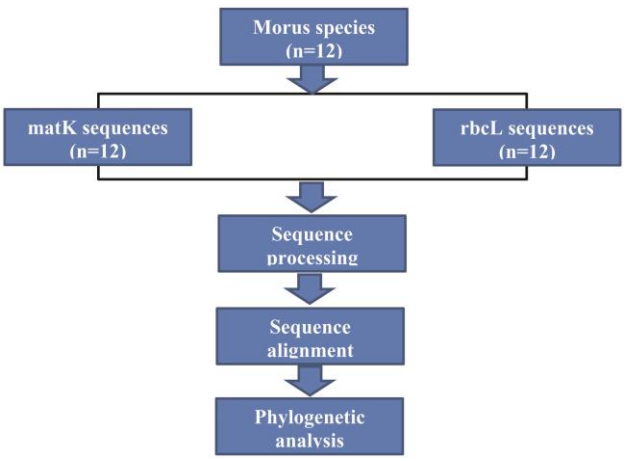

Fig. 1. is an illustration of the research work's technique?
The plant samples' DNA was extracted, and it was examined for post-sequence research and nucleotide sequencing.

Sequence homology: Using the Basic Local Alignment Tool, the biological homology of the specimen sequences, including *M. alba, M. nigra, M. macroura, M. serrata* and their variants was investigated (BLAST). The lengths of the *matK* sequence were 553, 486, 516, 502, 479, 532, 413, 325, 389, 595, 329, and 324 nucleotides respectively (Table 2). The sequence lengths for *rbcL* were 476, 557, 559, 556, 552, 514, 562, 535, 558, 561, 559, and 560 nucleotides for each species respectively (Table 3). *matK* marker revealed that the biological homology of *M. alba* and its variations ranged from 96.44 to 99.17%, that of *M. nigra* from 98.40 to 99.47%, and that of *M. macroura* from 96.13 to 97.64% and that of *M. serrata* from 97.91 to 100 percent (Table 2). Similar to this, the *rbcL* homology values for the four species and their varieties were 98.56–100% for *M. alba*, 97.73–99.80% for *M. nigra*, 99.63–100% for *M. macroura*, and 99.76–100% for *M. serrata*, respectively (Table 3).

Phylogenetic tree analysis: The *matK* gene was selected as one of the two molecular markers for estimating DNA barcodes for *Morus* plants. The phylogenetic tree diagram was constructed with 12 nucleotide sequences of *matK* revealing five major divergent groups and a few sub-groups. The first major group comprised two varieties of *M. alba*, *M. alba* V2 and V4 have a close relationship. *M. alba* V3 forms a distinct node a single species representing the second group. The third group consisted of *M. alba* only. Both species of *M. macroura* and *M. alba* V1 varieties construct 4th group. *M. macroura* varieties show more homology than *M. alba* V1. *M. alba* variants are found in four different groups presenting a lot of intra-species variations. According to the results, *M. macroura* species form a closer link with *M. alba* V1 than other *M. alba* varieties. The phylograms 5th group comprised of *M.* nigra and *M. serrata* species. This group makes four subgroups. 1st three subgroups consist of *M. nigra* variants making different nodes close but showed variations. *M. serrata* and *M. serrata* V1 represent a closer link. *mat*K genetic maker remained useful in distinguishing species as well as depicting intra-species variations in the genus *Morus*. Here the DNA barcoding approach successfully identified and classified the 12 strains in their respective groups (Fig. 7).

The *rbcL* gene is considered to be the second biological marker for identifying *Morus* species. The analysis involved 12 variant sequences. The phylogenetic tree diagram shows plant strains represented by their voucher number, strain name and variants at the nodes. The branch lengths of each node are mentioned on their above. The hierarchical clustering of the sequence was observed to be made into five distinct groups and many sub-groups. According to the diagram, the first group comprises *M. macroura* and *M. serrata* strains. This group comprises three subgroups. *M. macroura* varieties make different nodes indicating intra-species variations, whereas *M. serrata* species are more closely related and present in the same subgroup. *M. alba*V3 independently present in the 2nd group show wide variations with other variants of *M. alba*. 3rd group consists of all four variants of *M. alba* in three subgroups. *M. alba*V2 and V4 are distantly related to *M. alba* and *M. alba* V1 which formed sister taxa. *M. nigra* species are included in the 4th & 5th groups. *M. nigra* V1 and V2 closely resemble each other. *rbcL* marker indicating intra-species variation among *M. nigra* varieties. This marker proved beneficial in indicating interspecies and intra-species variations. The barcoding approach has classified our subject strains into various groups, thus making the identification method more robust (Fig. 8).

The discrimination ability of a barcode is its power to recognize a species in view of interspecies variations along with DNA sequences. A species is recognized as identified if its members form a particular monophyletic branch. The outcome showed that NJ is more helpful in classifying species. Computers make it simple and rapid to complete NJ, which is commonly employed in phylogenetic research. The *COI* sequence which is frequently used as a key barcode region in animal identification is similar to the sequence of the *matK* locus in plastids and is recognized as having undergone the most rapid evolution.

Sequence alignment: The multiple sequence alignment of various sequences of *matK* and *rbcL* genes revealed a region of higher similarity in the midst of the sequences, while the flanking regions have a higher proportion of variation. The results of *matK* somewhat diverged. Except for the three variants of *M. alba* V2, V3, and V4, all other variants shared at least 69.3% identity at 67% sequence coverage (Fig. 9). *rbcL* genes had a higher rate of conservation as compared to *matK*. The alignment of *rbcL* illustrated that at 91.4% of query coverage, all 12 sequences had at least 91.6% identity among them (Fig. 10).

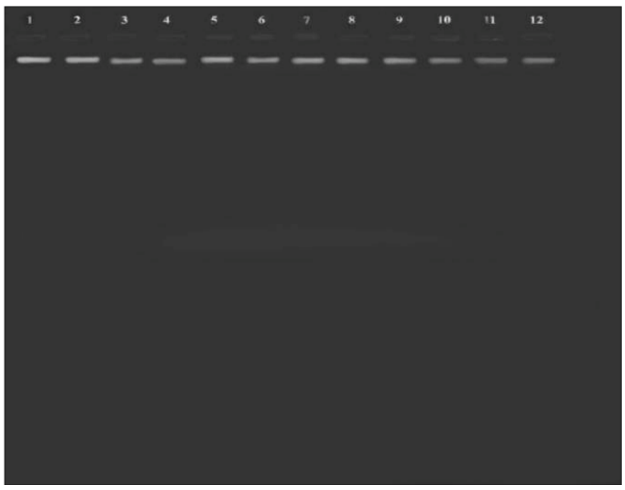

Fig. 2. DNA of different *Morus* species.

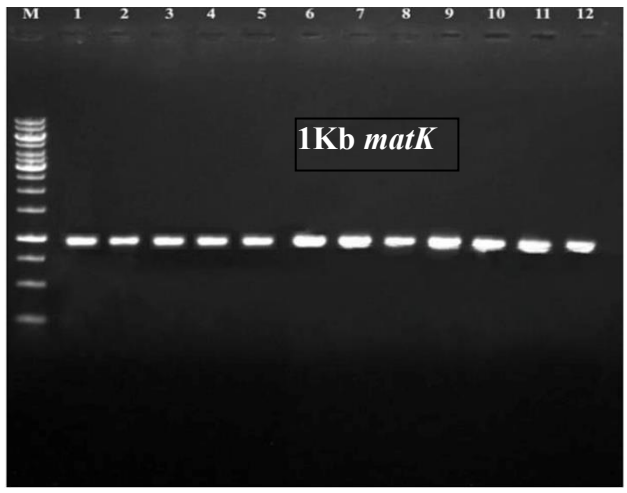

Fig. 3. PCR amplification of *mat*K.

1 *M. a* 2 *M. a*V1, 3 *M. a*V2, 4 *M. a*V3, 5 *M. a*V4, 6 *M. n*, 7 *M. n*V18 *M. n*V2, 9 *M. m*, 10 *M. m*V1, 11 *M. s* and 12 *M. s*V1. (*M= morus a= alba, n=nigra, m=macroura, s=serrata*) (Gene Ruler DNA Ladder 1KB Catalog number: SM0311)

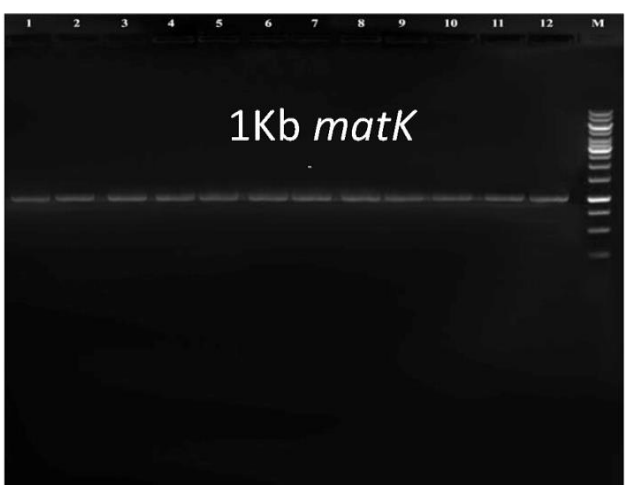

Fig. 4. Gel purified PCR products of *mat*K.

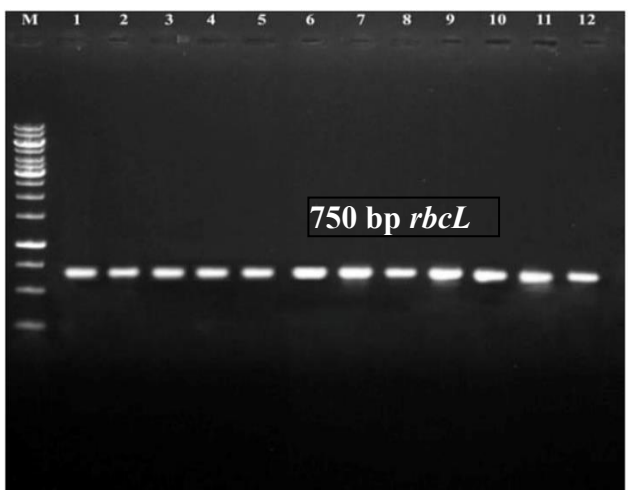

Fig. 5. PCR amplification of *rbc*L.

1 *M. a*, 2 *M. a*V1, 3 *M. a*V2, 4 *M. a*V3, 5 *M. a*V4, 6 *M. n*, 7 *M. n*V1, 8 *M. n*V2, 9 *M. m*, 10 *M. m*V1, 11 *M. s* and 12 *M. s*V1.

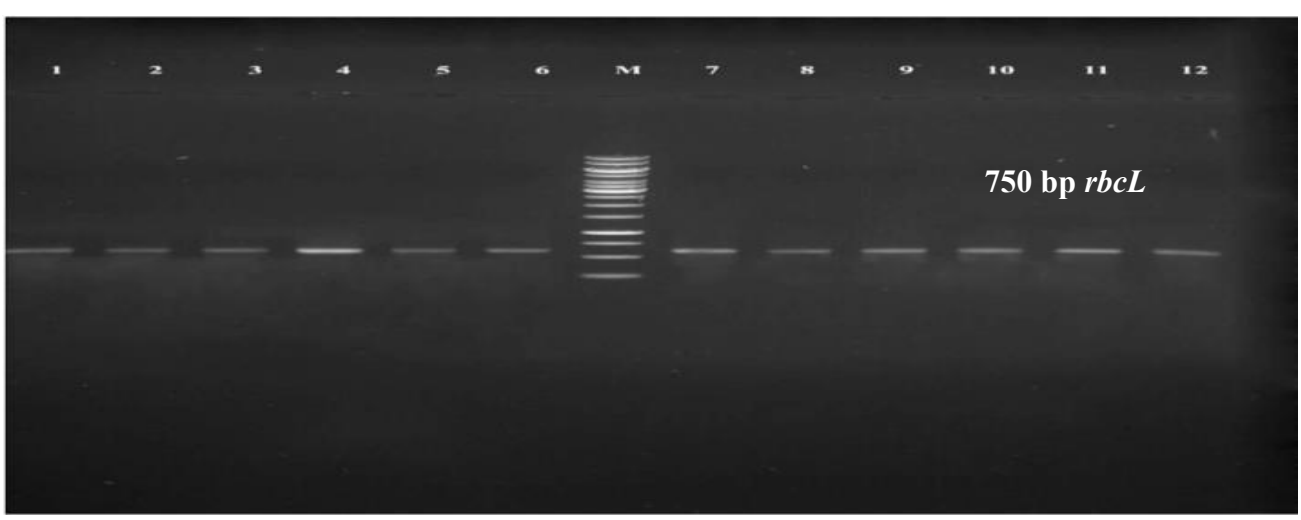

Fig. 6. Gel purified PCR products of *rbc*L in different Morus species.
1 M. a, 2 M. aV1, 3 M. aV2, 4 M. aV3, 5 M. aV4, 6 M. n, 7 M. nV1, 8 M. nV2, 9 M. m, 10 M. mV1, 11 M. s and 12 M. sV1.
(Gene Ruler DNA Ladder1KB Catalog number: SM0311)

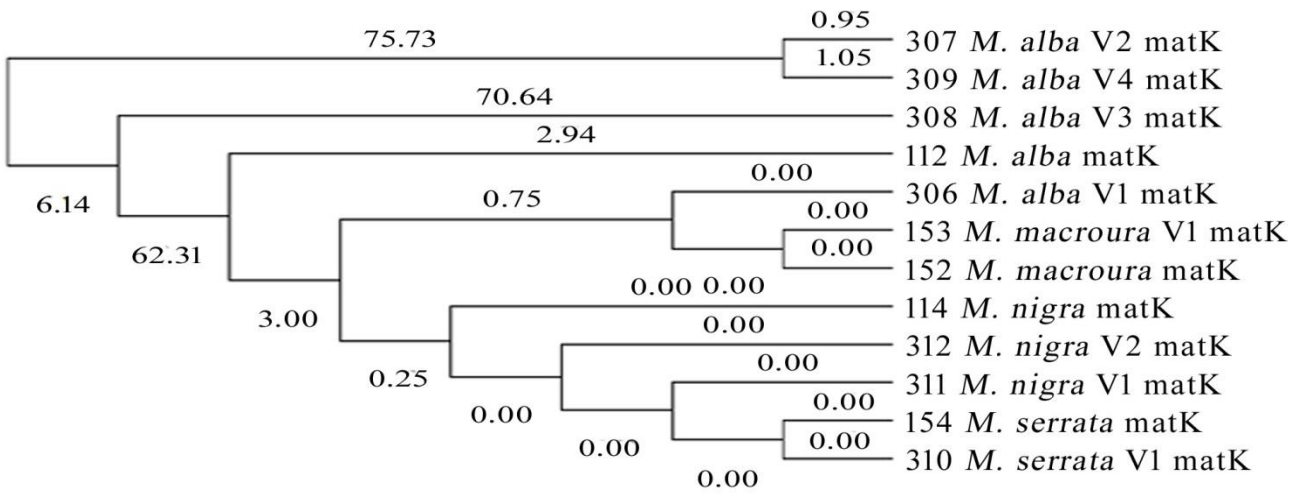

Fig. 7. Phylogenetic evaluation of various *Morus* species utilizing *matK* gene (n=12).

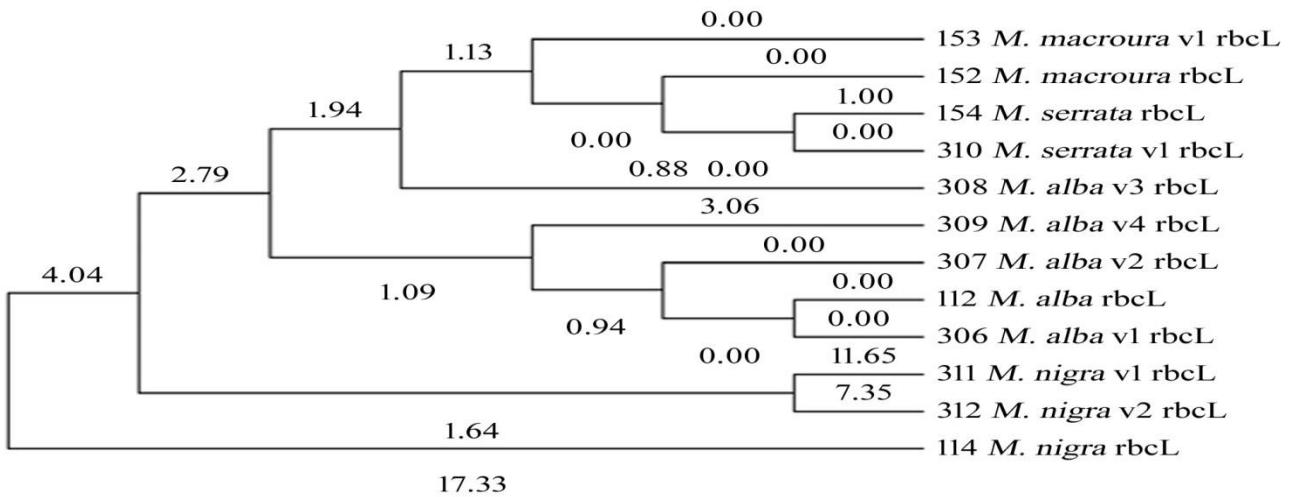

Fig. 8. Phylogenetic evaluation of various *Morus* species utilizing *rbc*L gene (n=12).

Table 1. Success rate of PCR amplification and sequencing.

Localities	Sum of individuals	Amplification success rate		Sequencing success rate	
Sites	**Specimens**	***Rbc*L**	***mat*K**	***rbc*L**	***mat*K**
Kotli	36	97.22%	94.44%	94.44%	88.88%
Rawalakot	36	97.22%	91.66%	88.88%	91.66%
Neelum	36	94.44%	94.44%	91.66%	86.11%
Total	108	96.29%	93.50%	91.66%	88.88%

Table 2. Statistical modeling of *Morus* species' BLAST sequence homology using *mat*K.

Species	Genomic region	Sequence length	Maximum score	BLAST similarity	Sequence cover	E value BLAST	Accession
Morus alba	*matK*	553	752	98.82%	88%	0.00	MK520331.1
Morus alba V1	*matK*	486	778	96.44%	96%	0.00	MH187248.1
Morus alba V2	*matK*	516	926	98.85%	100%	0.00	MK520331.1
Morus alba V3	*matK*	502	835	98.53%	93%	0.00	MK520331.1
Morus alba V4	*matK*	479	867	99.17%	100%	0.00	MF694867.1
Morus nigra	*matK*	532	874	98.40%	93%	0.00	JX495737.1
Morus nigra V1	*matK*	413	680	99.47%	90%	0.00	JX495737.1
Morus nigra V2	*matK*	325	575	98.48%	99%	1e_166	JX495737.1
Morus macroura	*matK*	389	505	97.64%	100%	6e_146	KR531251.1
Morus macroura V1	*matK*	595	708	96.13%	93%	0.00	KR531251.1
Morus serrata	*matK*	329	494	97.91%	86%	2e_143	GU145563.1
Morus serrata V1	*matK*	324	451	100%	75%	1e_130	GU145563.1

Table 3. Statistical modeling of *Morus* species' BLAST sequence homology using *rbc*L primers.

Species	Genomic region	Sequence length	Maximum score	BLAST similarity	Sequence cover	E value BLAST	Accession
Morus alba	*rbcL*	476	869	100%	98%	0.00	KU981119.1
Morus alba V1	*rbcL*	557	990	98.92%	99%	0.00	KF031063.1
Morus alba V2	*rbcL*	559	1000	99.64%	97%	0.00	KF031063.1
Morus alba V3	*rbcL*	556	974	98.56%	99%	0.00	KF031063.1
Morus alba V4	*rbcL*	552	1000	99.82%	98%	0.00	KF031063.1
Morus nigra	*rbcL*	514	826	97.73%	92%	0.00	JX571868.1
Morus nigra V1	*rbcL*	562	896	99.80%	86%	0.00	JX571868.1
Morus nigra V2	*rbcL*	535	885	98.80%	92%	0.00	JX571868.1
Morus macroura	*rbcL*	558	998	99.63%	9 7%	0.00	KR529786.1
Morus macroura V1	*rbcL*	561	1009	100%	97%	0.00	KR529786.1
Morus serrata	*rbcL*	559	779	99.76%	74%	0.00	GU145577.1
Morus serrata V1	*rbcL*	560	776	100%	75%	0.00	GU145577.1

The present work expressed the molecular systematics, genomic diversity and phylogenetic relationship of *Morus* species collected from various sites of Azad Jammu and Kashmir. This study utilized the most advanced and robust form of identifying and classifying the closely related strains of a genus i.e. DNA barcoding approach through evolutionary linkage analysis. Twelve strains belonging to four different species of *Morus* were analyzed and identified as distinct nodes in the phylogenetic tree. Our work is pioneering to barcode the *Morus* genus in the flora of Pakistan. The current investigation resulted in the creation of a molecular data inventory of plants included in the genus *Morus* at AJK with their pictures, barcode sequence data, and other information's. Large-scale applications that depend on species identification will have a local platform.

The findings in the present research determine Sequence recoveries for the two genes, *rbcL* and *matK* varied among species, but in our investigation *rbcL* had a higher success rate of 91.66% compared with *matK*, which reflects a lower percentage of 88.88%. Our results are similar to Wattoo *et al*., (2016) that both primers (*matk+rbcl*) showed good amplification and sequence recoveries. The biological homology of the specimen sequences, including *M. alba, M. nigra, M. macroura*, *M. serrata* and their variants were analyzed. *matK* marker revealed the homology ranged from 96.13 to 100 percent among *Morus* species. Similar to this, the *rbcL* homology values for the four species ranged from 97.73 to 100% respectively. Results of Tran *et al*., (2021) are comparable to our results. Additionally, both barcode markers were found to be accurate and consistent with Venkateswarlu *et al*., findings (2012). The *matK* gene phylogenetic tree was constructed with 12 nucleotide sequences revealing five major divergent groups and a few sub-groups. *mat*K maker remained useful in distinguishing species as well as depicting intra-species variations in the genus. Here the DNA barcoding approach successfully identified and classified the 12 strains in their respective groups. The *rbcL* gene is considered to be the second biological marker for identifying *Morus* species. The hierarchical clustering of the sequence was observed to comprise of five distinct groups and many sub-groups. This marker proved effective in indicating interspecies and intra-species variations. For identification of novel species, phylogenetic analysis of several plant species is useful (Onstein *et al*., 2015). Phylogenetic analysis of *Euphorbia* genus was done by (Yang & Berry 2011) on the basis of chloroplast DNA loci i.e. *matK* along with two other markers and find it effective.

Understanding of the evolutionary link between distinct plant species depends greatly on correctly identifying and analyzing the major plant families. Researchers can use phylogram and sequence alignment techniques to identify similarities and differences between various families. The multiple sequence alignment of various sequences of *matK* and *rbcL* genes revealed a region of higher similarity in the midst of the sequences, while the flanking regions have a higher proportion of variation. The results of *matK* somewhat diverged variants shared at least 69.3% identity at 67% sequence coverage. *rbcL* genes had a higher rate of conservation as compared to *matK*. The alignment of *rbcL* illustrated at least 91.6% identity and 91.4% of query coverage among all 12 sequences. Maloukh *et al*., (2017) findings endorse our results. He described that plastid *matK* region has more nucleotide substitutions, which evolves faster than *rbcL* region among the tested UAE plants.

According to some previous researcher's DNA barcode is a quick, easy, and affordable method to identify and organize various species (Khan *et al*., 2015; Shinwari & Shinwari 2010; Ikram *et al*., 2015). The chloroplast genes *matK* and *rbcL* function as all-inclusive plant barcodes (Group *et al*., 2009). Some genes, including *rbcL, matK, trnH-psbA, trnL-trnF,* and *ITS*, are employed alone or in combination for plant DNA barcode studies (Moylan *et al*., 2004). Zaib Un Nisa *et al*., (2022) describe the efficacy of all three barcode markers used *rbcL, matK* and *trnH-psbA* in discriminating order, Rosales. On the contrary some investigators like (Chase & Fay, 2009 and Zhang *et al*., 2012) found that in genetically complicated plants it is not much reliable or fruitful. Using *ITS* and plastid *ndhF* loci with an overall genetic variability of 0.03 percent (Peirson *et al*., 2014) revealed the systematic relationship of globe leafy species of the genus Euphorbia.

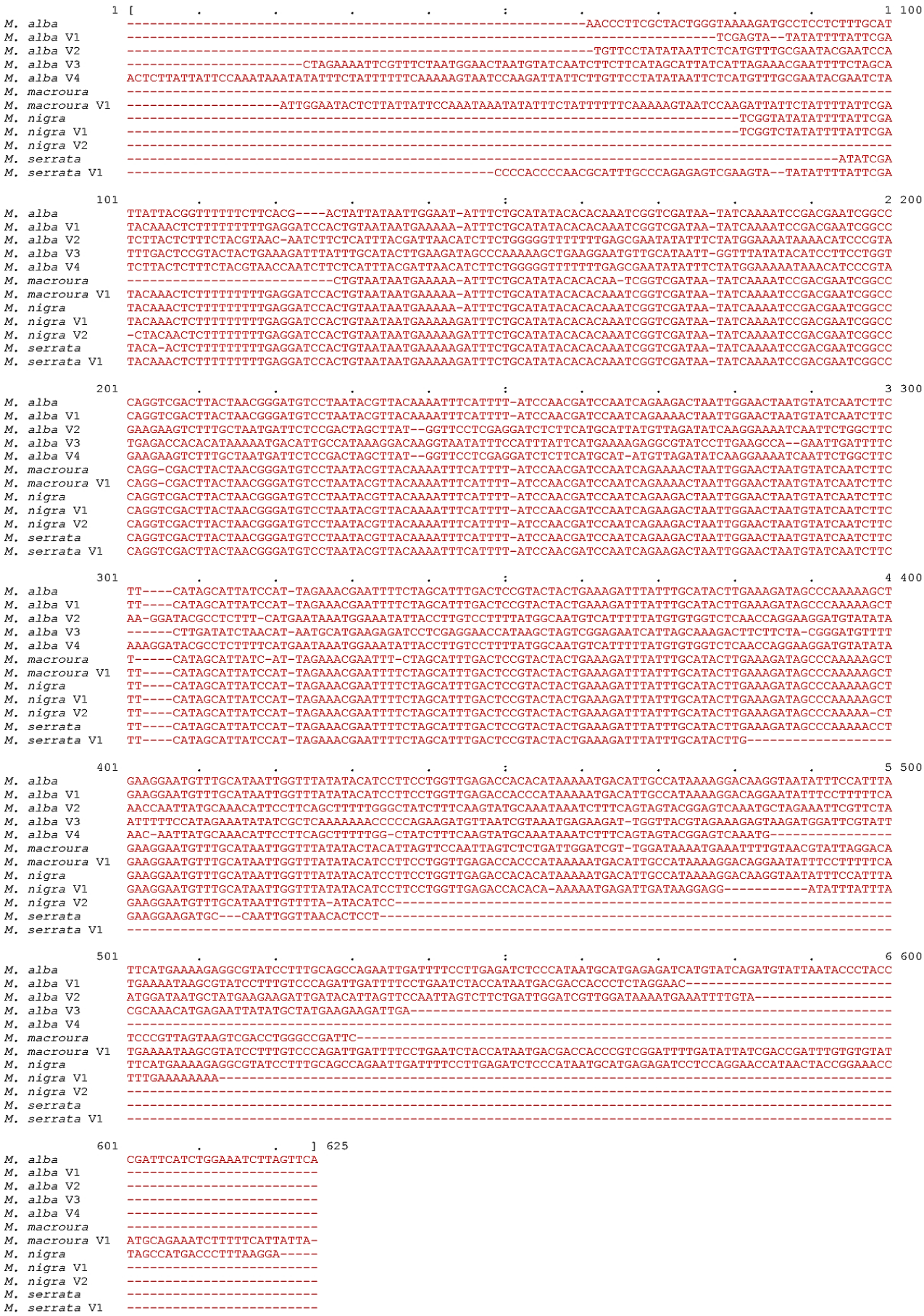

Fig. 9. Sequence alignment of some *Morus* species using *mat*K gene.

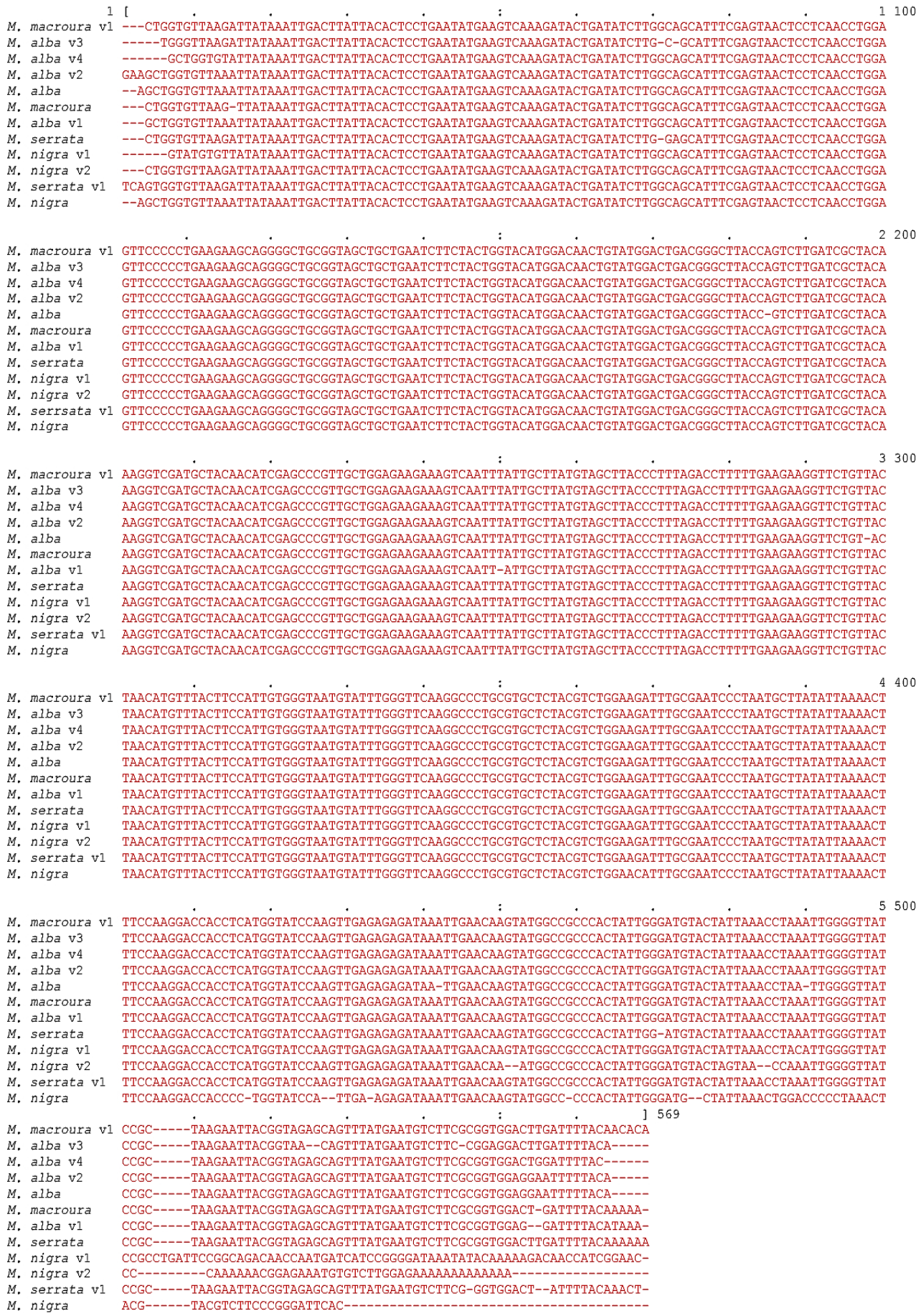

Fig. 10. Sequence alignment of some *Morus* species using *the rbc*L gene.

Conclusion

Both *matK* & *rbcL* genetic markers are useful in species level discrimination. The results showed that the discrimination power of *matK* marker is lower than *rbcL*. Present work is an initial barcoding evaluation of *Morus* species from Azad Jammu and Kashmir. These recently discovered Moraceae family sequences offer vital information for future evolutionary investigations. The role of each locus should be investigated independently but both the *matK* and *rbcL* markers are useful for identifying species.

Recommendation

Future studies along with novel markers are suggested either alone or in combination. For an improved and efficient method of classifying various species and discovering fresh connections, particularly in the genus *Morus*.

References

Anonymous. 2009. CBOL Plant Working Group. A DNA barcode for land plants. *P. Natl. Acad. Sci. USA.,* 106: 12794-12797.

APG, IV. 2016. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants. *Bot. J. Lin. Soc*., 181: 1-20.

Barthet, M.M. 2006. Expression and Function of the Chloroplast-encoded Gene *matK*. Doctoral dissertation, *Virginia Tech.*

Brooks, T.M., R.A. Mittermeier, G.A.B. Da Fonseca, J. Gerlach, M. Hoffmann, J.F. Lamoreux and A.S.L. Rodrigues. 2006. Global biodiversity conservation priorities. *Sci.*, 313(5783): 58-61.

Browicz, K. 1982. Flora of Turkey and the East Aegean Islands. *Eden. Univ. Pres.*, 7: 641-642.

Burea, E. 1873. Moraceae. *Prod. Syst. Nat. Reg. Veg.,* 17: 211-288.

Burgess, K.S., M. Morgan and B.C. Husband. 2008. Interspecific seed discounting and the fertility cost of hybridization in an endangered species. *N. Phytol.*, 177(1): 276-284.

Chase, M.W. and M.F. Fay. 2009. Barcoding of plants and fungi. *Sci.,* 325: 682-683.

Clement, W.L. and G.D. Weiblen. 2009. Morphological evolution in the mulberry family (Moraceae). *Syst. Bot*., 34(3): 530-552.

Costion, C., A. Ford, H. Cross, D. Crayn, M. Harrington and A. Lowe. 2011. Plant DNA barcodes can accurately estimate species richness in poorly known floras. *PLoS ONE*, 6 (11): e26841.

Deniz, G.Y., E. Laloglu, K. Koc, H. Nadaroglu and F. Geyikoglu. 2018. The effect of black mulberry extract on carbon tetrachloride-induced liver damage. *Arch. Biol. Sci.*, 70(2): 371-378.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.,* 19: 11-15.

Duan, H., W. Wang, Y. Zeng, M. Guo and Y. Zhou. 2019. The screening and identification of DNA barcode sequences for Rehmannia. *Sci. Rep.*, 9(1): 1-12.

Ercisli, S. and E. Orhan. 2007. Chemical composition of white (*Morus alba*), red (*Morus rubra*) and black (*Morus nigra*) mulberry fruits. *F. Chem.*, 103(4): 1380-1384.

Eyduran, S.P., S. Ercişli. M. Akın, Ö. Beyhan, M.K. Geçer, E. Eyduran and Y.E. Ertürk. 2015. Organic acids, sugars, vitamin C, antioxidant capacity and phenolic compounds in fruits of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry genotypes. *J. Appl. Bot. F. Qual.,* 88: 134-138.

Gonzalez, M.A., C. Baraloto, J. Engel, S.A. Mori, P. Pétronelli, B. Riéra and J. Chave. 2009. Identification of Amazonian trees with DNA barcodes. *PLoS ONE*, 4(10): e7483.

Group, C.P.W., P.M. Hollingsworth, L.L. Forrest, J.L. Spouge, M. Hajibabaei, S. Ratnasingham and D.P. Little. 2009. A DNA barcode for land plants. *Proc. Nat. Acad. Sci.*, 106(31): 12794-12797.

Hebert, P.D.N., A. Cywinska, S.L. Ball and J.R. de-Waard. 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. Biol. Sci. Ser. B.,* 270: 313-321.

Hollingsworth, P. M., L.L. Forrest, J.L. Spouge, M. Hajibabaei, S. Ratnasingham and D.P. Little. 2009. A DNA barcode for land plants. *Proceed. Nat.l Acad. Sci.,* 106(31): 12794-12797.

Hollingsworth, P.M. 2011. Refining the DNA barcode for land plants. *Proc. Nat. Acad. Sci.*, 108(49): 19451-19452.

Hollingsworth, P.M., S.W. Graham and D.P. Little. 2011. Choosing and using a plant DNA barcode. *PLoS ONE.,* 6(5): e19254.

Ikram, A., N.B. Zahra, Z.K. Shinwari and M. Qaiser. 2015. Ethnomedicinal review of folklore medicinal plants belonging to family Apiaceae of Pakistan. *Pak. J. Bot.,* 47(3): 1007-1014.

Ismail, M., A. Ahmad, M. Nadeem, M.A. Javed, S.H. Khan, I. Khawaish and S. Qamer. 2020. Development of DNA barcodes for selected Acacia species by using *rbcL* and *matK* DNA markers. *S. J. Biol. Sci.*, 27(12): 3735-3742.

Janzen, D.H. 1988. Tropical ecological and bio cultural restoration. *Sci.*, 239: 243-244.

Khan, M.Q., A.T. Khalil, K. Rehman and Z.K. Shinwari. 2015. Searching for DNA Barcodes in Plants. *Global J. Biotechnol. Biochem.,* 10(1): 1-10.

Koidzumi, G. 1917. Taxonomy and phytogeography of the genus *Morus*. *Bul. Seri. Exp. St.*, 3: 1-62.

Kress, W.J. and D.L. Erickson. 2007. A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding trnH-psbA spacer region. *PLoS One,* 2: e508.

Kress, W.J. and D.L. Erickson. 2008. DNA barcodes: genes, genomics, and bioinformatics. *Proc. Nat. Acad. Sci. USA.,* 105: 2761-2762.

Kress, W.J., K.J. Wurdack, E.A. Zimmer, L.A. Weigt and D.H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci. USA,* 102: 8369-8374.

Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.*, 35(6): 1547-1549.

Kuzmina, M.L., K.L. Johnson, H.R. Barron and P.D. N. Hebert. 2012. Identification of the vascular plants of Churchill, Manitoba, using a DNA barcode library. *B.M.C. Ecol.*, 12(1): 1-11.

Larkin, M.A., G. Black shields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam and D.G. Higgin. 2007. Clustal W and Clustal X version 2.0. *Bioinform.*, 23(21): 2947-2948.

Levin, R.A., W.L. Wagner, P.C. Hoch, M. Nepokroeff, J.C. Pires and E.A. Zimmer. 2003. Family-level relationships of Onagraceae based on chloroplast *rbcL* and *ndhF* data. *Am. J. Bot.,* 90(1): 107-115.

Maloukh, L., A. Kumarappan, M. Jarrar, J. Salehi, H. El-wakil and T.V. Rajya Lakshmi. 2017. Discriminatory power of *rbcL* barcode locus for authentication of some of United Arab Emirates (UAE) native plants. *Biotech.,* 7: 144-151.

Moylan, E.C., J.R. Bennett, M.A. Carine, R.G. Olmstead and R.W. Scotland. 2004. Phylogenetic relationships among Strobilanthessl (Acanthaceae): evidence from *ITS nrDNA, trnL-F cpDNA*, and morphology. *Amer. J. Bot.,* 91(5): 724-735.

Noshad, Q., M. Ajaib, A. Kiran, M. Ishtiaq, T. Bashir and M.F. Siddiqui. 2021. Study on genetic diversity of *Cuscuta reflexa* Roxb. and few members of Convolvulaceae on the basis of *RAPD* and SDS-Page. *Pak. J. Bot.,* 53(3): 959-965.

Onstein, R.E., R.J. Carter, Y. Xing, J.E Richardson and H.P Linder. 2015. Do Mediterranean–type ecosystems have a common history? —Insights from the Buckthorn family (Rhamnaceae). *Evvol.,* 69(3): 756-771.

Ozgen, M., S. Serçe and C. Kaya. 2009. Phytochemical and antioxidant properties of anthocyanin-rich *Morus nigra* and *Morus rubra* fruits. *Sci. Hort.*, 119(3): 275-279.

Peirson, J.A., R. Riina, M.H. Mayfield, C.J. Ferguson, L.E. Urbatsch and P.E. Berry. 2014. Phylogenetics and taxonomy of the New World leafy spurges, Euphorbia section Tithymalus (Euphorbiaceae). *Bot. J. Linn. Soc.,* 175 (2): 191-228.

Pham, N.T.T., D.P. Le, K.T.N. Pham, X. Thipphavong and M.H. Chu. 2021. DNA barcode of *matK* combined with *ITS* effectively distinguishes the medicinal plant *Stephania brachyandra* Diels collected in Local, Vietnam. *J. Appl. Biol. Biotech*., 9: 63-70.

Pramanick, D.D. 2017. A Synoptic Account of the Family Moraceae in Uttar Khand. *J. Appl. For. Ecol.*, 5(1): 17-26.

Ramesh, H.L., V. Sivaram and M.V.N. Yogananda. 2014. Antioxidant and medicinal properties of mulberry (*Morus* sp.). A review; *W.J. Pharm. Res.*, 3(6): 320-343.

Rao, C.K. and C.E. Jarvis. 1986. Lecto typification, taxonomy and nomenclature of *Morus alba*, *M. tatarica* and *M. indica* (Moraceae). *Tax.*, 35(4): 705-708.

Roy, S., A. Tyagi, V. Shukla, A. Kumar, U.M Singh, L.B. Chaudhary and R. Tuli. 2010. Universal plant DNA barcode loci may not work in complex groups: a case study with Indian Berberis species. *PloS one*, 5(10): e13674.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* 4: 406-425.

Shinwari, Z.K. and S. Shinwari. 2010. Molecular data and phylogeny of family Smilacaceae. *Pak. J. Bot.,* (SI) 4(2): 111-116.

Tran, T.K.P., T.T.T. Vu and S. Widiarsih. 2021. Comparison of *matK* and *rbcL* DNA barcodes for genetic classification of jewel orchid accessions in Vietnam. *J. Gen. Eng. Biotech.*, 19(1): 1-8.

Venkateswarlu, M., G. Ravikumar, N.B. Vijayaprakash, C.G.P. Rao, C.K. Kamble and A. Tikadar. 2012. Molecular phylogeny of *Morus* species differentiation based on chloroplast *matK* sequences. *Ind. J. Seri.*, 51(1): 16-19.

Wattoo, J.I., M.Z. Saleem, M.S. Shahzad, A. Arif, A. Hameed and M.A. Saleem. 2016. DNA Barcoding: Amplification and sequence analysis of *rbcL* and *matK* genome regions in three divergent plant species. *Advan. L. Sci.*, 4(1): 03-07.

Yang, Y. and P.E. Berry. 2011. Phylogenetics of the Chamaesyce clade (Euphorbia, Euphorbiaceae): Reticulate evolution and long-distance dispersal in a prominent C4 lineage. *Amer. J. Bot.,* 98: 1486-1503.

Zafar, M.S., F. Muhammad, I. Javed, M. Akhtar, T. Khaliq, B. Aslam and H. Zafar. 2013. White mulberry (*Morus alba*): A brief phytochemical and pharmacological evaluation account. *Int. J. Agri. Biol.*, 15(3): 612-620.

Zaib-Un-Nisa, Z.D. Khan, M. Ajaib, S. Ullah, S. Muhammad and M. F. Siddiqui. 2022. Taxonomic reaffirmation of some members of family Cannabaceae, Moraceae, Rhamnaceae, Rosaceae, and Urticaceae of order Rosales using DNA barcoding markers. *Pak. J. Bot*., 54(1): 231-241.

Zeng, Q., H. Chen, C. Zhang, M. Han, T. Li, X. Qi and N. He. 2015. Definition of eight mulberry species in the genus *Morus* by internal transcribed spacer-based phylogeny. *PloS ONE*, 10(8): 0135411.

Zhang, C.Y., F.Y. Wang, H.F. Yan, G. Hao and C.M. Hu. 2012. Testing DNA barcoding in closely related groups of *Lysimachia* L. (Myrsinaceae). *Mol. Ecol. Resour.,* 12: 98-108.

Zhou, Z. and M.G. Gilbert. 2003. Moraceae. Flora of China. Beijing; *Sci. Pres.,* 5: 21-73.

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