J. Plantarum., 7(1): 164-173

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

MOLECULAR AND PHENETIC EVIDENCE FOR TWO DISTINCT MORUS SPECIES (MORUS MACROURA MIQ. AND MORUS CATHAYANA HEMSL.) IN PAKISTAN

HAFEEZA AMNA SALEEM*, ABDUL REHMAN NIAZI

[1]*Institute of Botany, University of the Punjab, Pakistan Lahore*
**Corresponding author's email: amnasaleem884@gmail.com*

Received on: 02-11-24; Reviewed on: 08-04-25; Accepted on: 11-05-2025; Published on: 15-06-2025

Abstract
Morus is a versatile plant genus with significant economic and medicinal value. It contributes to economic development and is used in the production of various important medicines. To fully utilize its medicinal properties, it is essential to understand its taxonomic and phylogenetic relationships. Morphological and phylogenetic studies have identified two monophyletic species, *Morus macroura* and *Morus cathayana*. In Pakistan, *Morus macroura* is characterized by greenish-white infructescences when mature and has largely ovate to orbicular leaves, often lobed with 2–3 sinuses, measuring 7–15 cm in width and 10–20 cm in length. In contrast, Pakistani *Morus cathayana* displays red to dark purple infructescences at maturity and features lanceolate to cordate leaves, sometimes lobed on one side with 2–3 sinuses. Despite these differences, the two species are nearly indistinguishable at first glance due to their many shared morphological traits.

Key words: Systematics, identification, mulberry, taxonomic study, Evolutionary study

INTRODUCTION

Morus is a small genus of mulberries belonging to a family Moraceae, consists of 13 species distributed in eastern temperate North America, Southwest United States of America to Andes, tropical Africa, Southwest Asia eastwards to Japan and Europe (Ghafoor, 1985, Nasir and Ali, 1994).

This genus is economically significant due to its impact in making 90% of the silk worldwide. *Morus* is also vital medicinally as well as ecologically (Zeng *et al*., 2015). Species of this genus can be used to lower the level of glucose in our blood. It also helps our body to get rid of extra fluid. Its medicinal properties showed more miracles in acting as powerful astringent. As a traditional Chinese medicine, leaves had used to treat fever and reduce blood pressure due to their high flavonoid content (Chen *et al.,* 2020).

The primary economic value of *Morus* species lies in their leaves, which work as the main feed for the sericultural industry. Additionally, livestock is also dependent on their leaves while bark is used in making fiber industry as well as in the paper industry.

For centuries, nutritious fruits have also been consumed as food. *Morus* species can be accurately identified at the molecular level using DNA barcoding techniques. Only a limited amount of research has been conducted on its taxonomic and phylogenetic identification. Many species continue to be misidentified, causing challenges in the identification of both new and existing species. Species are accurately and quickly identified using minute amount of tissue sections from any plant at any growing phase while using said method (Marizzi *et al.*, 2018). DNA-based markers facilitate an improved classification of *Morus* species. At present, ITS data has the maximum

better power for determining plant species. This approach is extensively used for taxonomic and molecular analyses. ITS and *mat* K markers were used universally for identification of plants (Jones 2021).

Represented in Pakistan by four species viz; *Morus macroura* Miq., *Morus serrata* Roxb., *Morus nigra* L., *Morus alba* L. (Ghafoor 1985, Butt *et al*. 2008). An inadequate work has been done on the molecular phylogenetics of *Morus* genus, this study will provide baseline data for further correct identification of *Morus* species to fully utilize its ecological, medicinal & economic potential. The proposed study facilitated the accurate identification and documentation of this genus from Pakistan by using morphological and molecular characterization to explore its phylogeny and evolutionary patterns. It will also serve as baseline data for future biochemical studies to assess their pharmacological significance.

The sap of the bark of *M. macroura* Miq. applied to cuts and wounds. The fresh fruit is used as a tonic and to treat throat irritations. Leaves of *M. cathayana* Hemsl. are the main food for silkworms, which is why growing these plants has been crucial for Chinese silk production for thousands of years. In the Flora of China, *M. wittiorum* and *M. liboensis* are recognized as separate species, but they are included under *M. macroura* Miq. (Zhou and Gilbert 2003).

This research work was designed to discover the biodiversity of *Morus* in Pakistan. During this survey of *Morus* species from different regions of Pakistan, eight collections revealed their identification as *M. macroura* and *M. cathayana* Hemsl. were reported as novel records for Pakistan.

METHODOLOGY

Collection and preservation

Out of 100 samples, (8) eight samples of leaves of the *Morus* trees were selected from different areas of Pakistan (Faislabad, Multan, Sharaqpur, Lahore, Gujrat, Chiniot, Kasur and Hafizabad) during survey from March–May of three consecutive years (2020-2022). The leaves were air dried.

DNA extraction and PCR Analysis

Genomic DNA was isolated from leaf tissue following the CTAB protocol outlined by Doyle and Doyle (1987). The amplification of the coding regions (LSU, ITS and SSU regions) was done using by the primers ITS5 (Downie and Katz-Downie 1996) and ITS4 (White et al. 1990). The PCR was carried out in a 45 µl reaction mixture containing 25 mg of genomic DNA. PCR amplification of DNA started with an initial denaturation at 98°C for 7 minutes, followed by the addition of Taq polymerase at 72°C. Then denaturation was done at 90 °C followed by 1 and half minute annealing at 35 °C. Final elongation step was done for 5 minutes at 74 °C.

DNA Sequencing and Alignment

The sequencing protocol involves the bidirectional sequencing with specific thermal cycling conditions, followed by sample processing and outsourcing for analysis. Sequenced reactions were cleaned and sent to China for further analysis.

Phylogenetic analysis

BioEdit software was used to assemble bi-directional ITS region sequences (Hall, 1978). Using Blast analysis, highly similar sequences were edited through with extreme percent identity and query coverage to related taxa recorded. From GenBank

sequences were downloaded and then aligned using the web PRANK multiple alignment tool with default settings (Loytynoja and Goldman, 2010). Sequence ends were trimmed to approximately equal lengths in BioEdit. MEGA X was used to construct the evolutionary tree by selecting model i.e., Kimura 2-parameter model (Kumar et al., 2016).

Morus macroura Miq

Morphological analysis: Dioecious tree reaching up to 7 meters in height. Bark is dark brown. Buds are ovoid, measuring 0.5–1 cm by 0.4 cm, brown and covered with fine hairs, with bud scales featuring white bands. Buds: axillary, dormant, 0.5 cm in length and the leaf stalks measuring 2.5–3.5 cm in length, slightly glabrous. Leaf blades: broadly egg shaped to round, 10–20 cm × 7–15 cm, bases round to cordate. Minutely serrated leaf margin with obtuse to acute leaf apices. Dorsal surfaces rough or pubescent. Ventral surfaces shiny and glabrous. Fruits: 6–7.5 cm greenish to off-white (5GY9/8) at maturity, not dry. Male catkins occur 1 per node, each measuring 4.5– 5.5 cm in length and containing with 55 to 60 flowers with peduncles 1–2.5 cm. Female catkins occur 2 per node, each catkin measuring 3–7 cm long having 80 to 95 flowers containing peduncles 1–1.5 cm. Flowers: male flowers with ovate perianths; stamens globose. Female flowers feature oblong perianths, styles absent having bifid stigmas.

Examined Specimen: Pakistan, Faislabad (31.4504° N-73.1350° E, 186 m a.s.l) Punjab, Pakistan. April 25, 2022, H.A. Saleem.

Distribution: Located in Faislabad, Multan, Lahore and Gujrat. Chiniot, Sharaqpur. Samples were also collected from The University of the Punjab, Lahore.

Economic importance: The bark fiber is utilized in paper production, whereas the bark and leaves are employed for dyeing purposes.

Morus cathayana Hemsl.

Morphological analysis: Either monoecious or dioecious, grows up to 4.5 meters tall. Bark brown and the petioles are light green, slightly glabrous, measuring 1.5–2.6 cm in length. The leaf blades range from lanceolate to cordate, sometimes exhibiting lobes on one side or featuring 2–3 sinuses, measuring 8.0–9.5 centimeters long with bases that are cordate. Lamina of the leaf are lanceolate to heart shaped, occasionally lobed on one side or with 2–3 sinuses, measuring 8.0–9.5 cm by 4.5–5.5 cm, with cordate bases. Closely serrated margins. The lower (abaxial) surfaces are covered with dense white hairs, while the upper (adaxial) surfaces are smooth and hairless. Veins: multi-costate reticulate (divergent). Male catkins occur 1–2 per node, each measuring 2.5–5 cm in length and containing 40–45 flowers with peduncles approximately 1.5 cm long. Female catkins occur 1 per node, each measuring 3.5–5 cm in length 95 to 100 flowers, the peduncles from 1.5 cm. A greater number of male catkins were observed. Male flowers have ovate perianths with four stamens and a small pistilloid present. Female flowers feature obovate perianths, short styles, and bifid stigmas.

Examined specimen: Pakistan, Okara (30° 48' 30.6000" N and 73° 27' 33.8256" E 105 m a.s.l) Punjab, Pakistan. April 25, 2022, H.A. Saleem.

Distribution: Located in Hafizabad, Multan, Lahore and Gujrat. Chiniot, Kasur.

ISSN PRINT 2710-4079
ISSN ONLINE 2710-4087

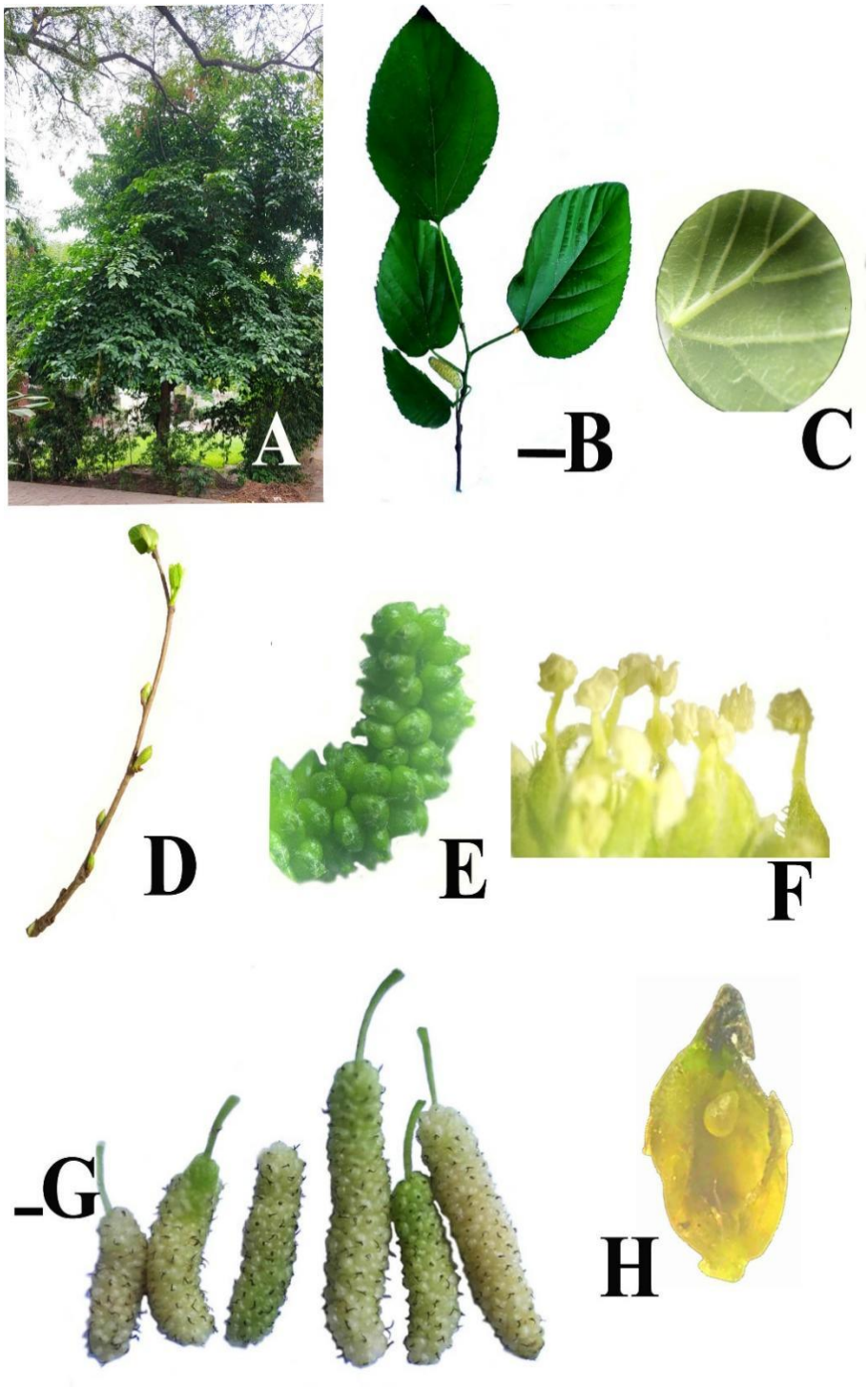

Fig. 1 :A – Tree of *M. macroura* (AB-01), B – Leaves ,C– Adaxial surface under stereo-microscope, D–Bud, E–Female catkin, F– Male catkin, G – Fruit, H– T.S of ovary.

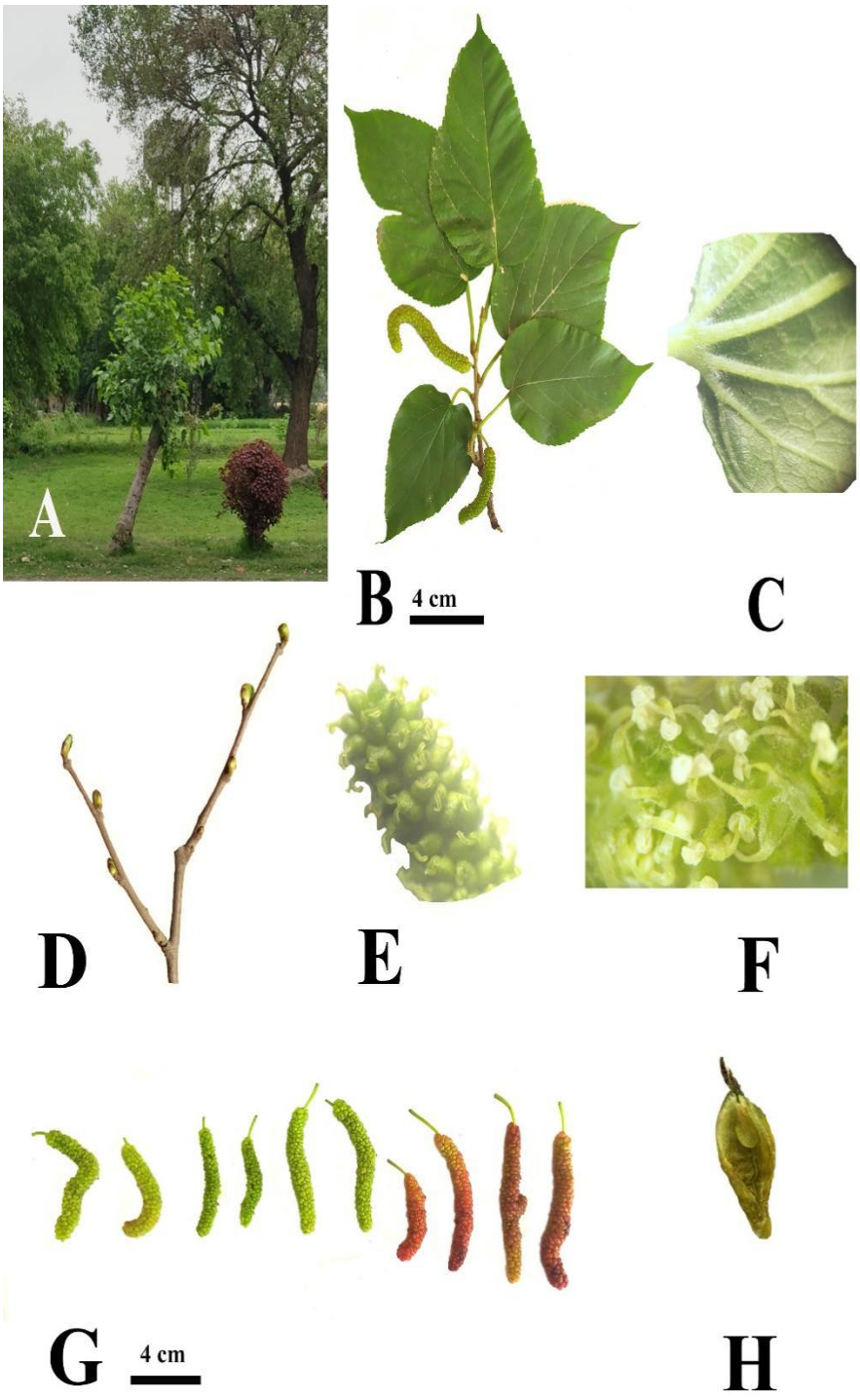

Fig. 2 :A – Tree of *M. cathayana* (AM1F), B – Leaves ,C– Adaxial surface under stereo-microscope, D–Bud, E–Female catkin, F– Male catkin, G – Fruit, H– T.S of ovary.

Table 1- showing similarities and differences with *M. cathayana* & *M.macroura* (Nepal and Purintun 2021).

Characteristics	**Pakistani *Morus cathayana***	**Pakistani *Morus macroura***	***M. cathayana* (Nepal and Purintun 2021)**	***M. macroura* (Nepal and Purintun 2021)**
Presence of sex organs	Monoecious or dioecious, 4m.	Dioecious, wild trees to 5 m.	Monoecious /Dioecious.	Dioecious trees to 20 m.
Petiole size	1.5–2.6 centimeters	2.5–3.5 centimeters	1–3.5 centimeters	1 centimeter
Leaf base	Heart shaped ovate	Bases round to cordate.	Bases cordate to truncate.	Bases rounded, rarely cordate.
Leaf margins	regular, closely serrated	Margins minutely serrate.	Mostly serrate-serrate-crenate	closely serrate - almost entire.
Apices of leaf blade	Aristate- caudate.	Leaf apices obtuse -acute	apices acute-acuminate.	Acute-shortly acuminate.
Leaf lower surface	pubescent with dense white hairs.	Pubescent. with dense white hairs	Pubescent with dense white hairs.	Abaxial surfaces subglabrous with sparse hairs along the veins.
Leaf upper surface	glabrous	Adaxial surfaces glabrous	The upper surfaces are rough while the areas between the veins are covered with fine hairs	The upper surfaces range from smooth to slightly rough.
Male catkin	40–45 flowers, 2.5–5 cm long. 1–2 per node	1 per node measuring 4.5– 5.5 cm long	1–2 per node, measuring 3–6 cm in length and containing 35–40 flowers	Male catkins 2 per node, 4–16 cm long
Female catkin	1 per node	Pistillate catkins 2 per node	Pistillate catkins 1 per node	Pistillate catkins 2 per node
Fruit colour	red, dark purple, 4 cm	greenish white at maturity. 6-8.5 cm not dry	dark purple fruit or sometimes reddish black having size of 2.5-4 cm	Light yellowish white fruit, often dry and loosely arranged having size of 6-16 cm.

Phylogenetic analysis of AB-01:

Data Matrices—Sequences of the ITS region in *Morus* ranged from 620–640 base pairs (bp),

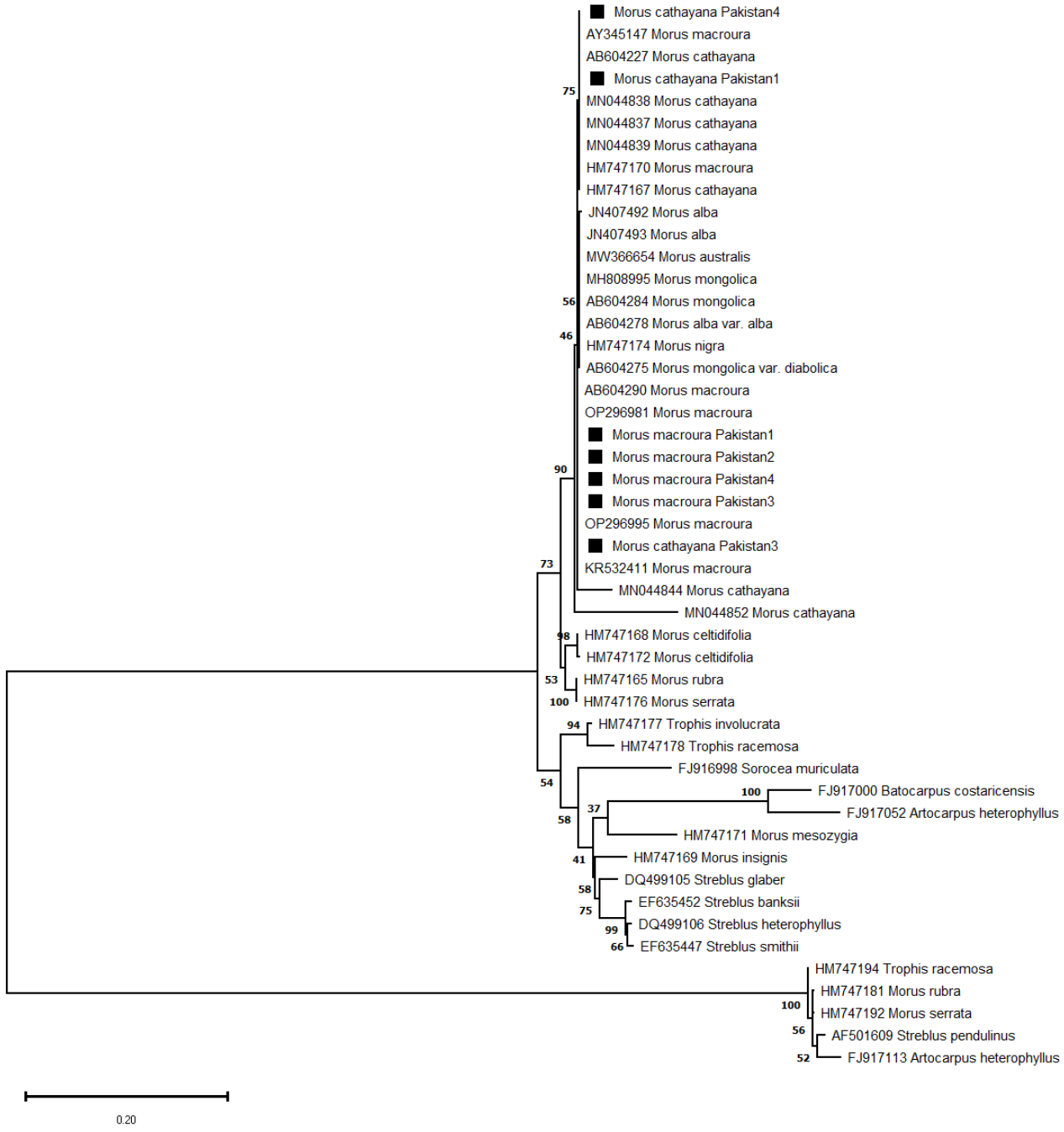

Fig. 1. Combine tree of *M. macroura* and *M. cathayana*. Maximum Likelihood consensus trees for ITS resulting from analyses. The numbers displayed above the branches represent ML bootstrap values derived from 1,000 replicates.

DISCUSSION

Most *Morus* species constitute a well-supported monophyletic clade that aligns with *Morus* subgenus *Morus* as described by Leroy (1949). In this study, all Asian *Morus* species are closely situated in given phylogenetic tree (including *M. alba, M. nigra, M. cathayana* etc) while North American species (including *M. celtidifolia*) form a separate clade. Phylogenetic analyses conducted using these data, whether individually or combined, demonstrate that the genus *Morus* forms a monophyletic group (Weiguo *et al*., 2005).

M. macroura shows resemblance with *M. macroura* (HM747170) in having dioecious, glabrous leaf texture, broadly ovate leaves, margins minutely serrate *M. macroura* differs in having petiole size 2.5–3.5 cm long while *M. macroura* (HM747170) has petiole size 1 cm and pistillate catkins 2 per node. *M. macroura* also differs in having, sometimes lobed with 2–3 sinuses but *M. macroura* (HM747170) has unlobed blades *M. macroura* differs from *M. macroura* (HM747170) in that it has bases that are round to cordate, whereas *M. macroura* (HM747170) typically has rounded bases, with cordate bases occurring only rarely. M. *M. macroura* also differs from *M. macroura* (HM747170) by possessing leaf apices that range from obtuse to acute. It also differs in having the lower (abaxial) surfaces covered with dense white hairs, giving them a pubescent texture, lateral veins reaching across the entire length of the leaf, the secondary veins form an angle of 55–60º with the midrib. Each node bears one staminate catkin, containing 55 to 100 flowers, fruit greenish to white at maturity. 6-8.5 cm, not dry but in compact catkin. *M. macroura* (HM747170) fruit dry and sparsely arranged, 6-16 cm.

M. macroura shows resemblance with *M. cathayana* (HM747167), in having round bases, pubescent lower surface of leaf with dense white hairs, staminate catkins were almost same size per node in both. *M. macroura* also shows some differences in morphology with *M. cathayana* (HM747167) as the later one may have truncate bases, margins serrate to crenate and sometimes serrations may space out. *M. macroura* also varies in having only a dioecious nature than *M. cathayana* (HM747167).

Some different features in *M. macroura.* was also observed from *cathayana* (HM747167) and *M. macroura* (HM747170) in having greenish to off white fruit at maturity while *cathayana* (HM747167) have red, dark purple or white fruit and *M. macroura* (HM747170) have yellowish white fruit at maturity. Lateral veins reaching across the entire length of the leaf in *M. macroura* while *cathayana* (HM747167) has lateral veins reaching up to half of the lamina and *M. macroura* (HM747170) lateral veins reaches from 1/2 to 2/3 of the lamina.

Our species AB-01A, AB-1B, AB-02A, AB-02B nested with *M. macroura (*KR532411 reported from China), *M. macroura* (OP296995, OP296981 reported from Indonesia) in same clade with a supportive bootstrap value (Huang *et al*., 2015; Nuratika, 2020). From this study, we conclude that some species are morphologically similar but due to environmental factors they are not similar genetically such species are called cryptic species. There is one difference between Nepal's *Morus* list that climate also vary in America and Pakistan.

CONCLUSION

M. macroura Miq. was reported first approach time from Pakistan on the basis of molecular level. *Morus cathayana* Hemsl. was new record from

Pakistan. The phylogenetic studies revealed that AB-01A, AB-1B, AB-02A, AB-02B were phylogentically identified as *M. macroura* Miq. While AMF1, AMF4, AMF3.We can use this information for further research work like developed countries in papermaking, dyes and for giving best feed to silkworms of sericulture industry.

FUNDING STATEMENT

This study was supported by all authors.

AUTHOR'S CONTRIBUTION

A. R. Niazi designed the paper. H.A. Saleem performed the morphological as well as molecular analysis and wrote the manuscript with the support of A. R. Niazi. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

All the data is primary.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Prof. Dr. Nasir Khalid in identification of the species and Dr. Aneeqa Ghafoor for her help in the preparation of the manuscript.

CONFLICT OF INTEREST

The authors declared that the present study was performed in absence of any conflict of interest.

REFERENCES

Butt, M. S., Nazir, A., Sultan,M. T., and Schroën. K. 2008. *Morus alba* L. nature's functional tonic. *Trends in Food Sciences Technol*ogy, 19(10): 505-512.

Chen, C. Y., Kao, C. L., Yeh, H. C., Song, P. L., Lin, R. J., Li, H. T. 2020. Chemical constituents of *Morus alba*. *Chemistry of Natural Compounds,* 56(5): 904-905.

Ghafoor, A. 1985. *Flora of Pakistan*. *Department of Botany, University of Karachi and National Herbarium.* Stewart. Islamabad, Pakistan, 171: 67-68.

Huang, X. C., Ci, X. Q., Conran, J. G., and Li, J. 2015. Application of DNA barcodes in Asian tropical trees–a case study from Xishuangbanna Nature Reserve, Southwest China. *PLoS one*, 10(6): e0129295.

Jones, L., Twyford, A. D., Ford, C. R., Rich, T. C., Davies, H., Forrest, L. L., Hart, M. L., McHaffie, H., Brown, M. R., Hollingsworth, P. M., De Vere, N. 2021. Barcode UK: A complete DNA barcoding resource for the flowering plants and conifers of the United Kingdom. *Molecular Ecology Resources,* 21(6): 2050-2062.

Leroy, J. F. 1949. Les Muriers sauvages et cultives. La sericiculture sous les tropiques. *Journal d'agriculture traditionnelle et de botanique appliquée*, 29(323): 481-496.

Marizzi, C., Florio, A., Lee, M., Khalfan, M., Ghiban, C., Nash, B., Dorey, J., McKenzie, S., Mazza, C., Cellini, F., Baria, C. 2018. DNA barcoding Brooklyn (New York): A first assessment of biodiversity in Marine Park by citizen scientists. *Plos one* 13(7): e0199015.

Nuratika, E., Aseny, N., Syamsuardi, N., Fitmawati, F. 2020. Clarification of sumatran mulberry (*Morus macroura* var. macroura, Moraceae) from West Sumatra, Indonesia using nucleus ribosomal its (Internal Transcribed Spacer) gene. *Indian Journal Of Agricultural Research*, 54: 635-640.

Weiguo, Z., Yile, P., Zhifang, Z., Shihai, J., Xuexia, M., Yongping, H. 2005. Phylogeny of the genus Morus (Urticales: Moraceae) inferred from ITS and trnL-F sequences. *African Journal of Biotechnology,* 4(6): 563-569.

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

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

Downie, R., Katz-Downie, D. S. 1996. A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *Am. J. Bot*., 83(2): 234-251.

Nasir, E., Ali, S. I., "Flora of Pakistan," National Herbarium, NARC, Islamabad, Department of Botany, University of Karachi, Karachi, (Fascicles), 1972-1994.

Nepal, M. P., Purintun, J. M. 2021. Systematics of the genus *Morus* L. (Moraceae) taxonomy, phylogeny and potential responses to climate change. In *Mulberry: Genetic Improvement in Context of Climate Change*; Razdan, M.K., Thomas, D.T., Eds.; The CRC Press: Boca Raton, FL, USA, pp. 2–20

Kumar, S., Stecher, G., Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.,* 33(7): 1870–1874.

Hall, I. R. 1978. A key to the Endogonaceae. *Trans. Br. Mycol. Soc*. 73: 261- 270.

Löytynoja, A., Goldman, N. 2005. An algorithm for progressive multiple alignment of sequences with insertions. PNAS, 102(30): 10557–10562.

Zhou, Z. K., Gilbert, M. G. 2003. Moraceae. Flora of China. Beijing, China & Saint Louis, Missouri: *Ann. Mo. Bot. Gard.* 5(1): 22–26.