

MORPHOLOGICAL AND PHYLOGENETIC ANALYSES OF NEW SPECIES OF *MORUS* L. (MULBERRY) AND A NEW VARIETY INFERRED FROM DNA BARCODING TECHNIQUE

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Abstract

Morus L. is a small genus including a number of different significant varieties and species. This genus attracts scientists to study its important medicinal properties like antimicrobial, antibacterial and even anti-viral. It also contributes economically to raise good quality of food for silkworms. In Pakistan, four species of *Morus* have been reported so far on the basis of morphology since 1985 in Flora of Pakistan. During this research work, we have reported one new species *Morus pakistanica* Amna & Niazi and one new variety *Morus alba* var. *macroura* Amna and Niazi on the basis of morphological and phylogenetic analyses. This study will provide a valuable addition in the flora of Pakistan. It is notable that we are going to submit molecular data of *Morus* spp. for the first time from Pakistan.

Key words: Flora, Mulberry, Variety, Phylogenetic study, Taxonomic study, Authentic identification.

Introduction

The family Moraceae. This family has mostly trees with milky latex like *Ficus* and *Morus* etc., (Ahmad *et al.*, 1985). This family displays diverse inflorescence ranging from simple to complex and densed (Leite *et al.*, 2021).

In Pakistan, only 5 genera of this family viz., *Ficus*, *Broussonetia*, *Maclura*, *Artocarpus* and *Morus* have been reported (Ghafoor *et al.*, 1985). One of the important genus is *Morus* L., only 4 species of this genus have been reported so far from Pakistan compared to 13 known species across the world. *Morus* is commonly known as Mulberry. This genus is characterized by inflorescence, staminate flowers with imbricate perianth, inflexed stamens, pistillate flowers with valvate perianth and equally branched stigmas, fleshy perianth in the fruit, and a berry-like syncarp. Morphological characters including of the leaf, winter bud, bark, pistil and syncarp have generally been employed in the delimitation of species. Mostly, the chromosome number in *Morus* are 14 but the highest number of chromosome was observed in *M. nigra* as $2n=308$ (Nepal & Ferguson 2012).

Morus has drawn more and more attention in recent years. In the light of the published articles over the last few decades, flavonoids and benzofurans have been reported to be the main bioactive constituents of *Morus* (Yan *et al.*, 2020).

DNA barcoding technique is significant tool for accurate identification. This technique allows for quick and accurate species identification using only minimal amounts of tissue samples taken from any organism at any developmental phase (Marizzi *et al.*, 2018). Phylogenetic analysis has become essential tool for genome comparisons. (Brady & Salzberg 2011). ITS region has been used universally for identification of plants (Jones, 2021). The present research is conducted to explore diversity of this genus in Pakistan.

It is important that DNA data should be applied for the classification and phylogeny of the plants (Jansen & Kim, 1996). Sequences of the internal transcribed spacers (ITS) region of rRNA has proven to be of great significance in

understanding the phylogeny of angiosperms at the specific and generic levels. It has been widely used for determining phylogenetic relationships of plant groups at lower taxonomic level (Baldwin *et al.*, 1995; Tian & Li, 2002).

Morus diversity from Pakistan is poorly understood. Four species were previously reported from Pakistan on a morphological basis. Our study is the first attempt at a detailed morphological and phylogenetic analysis of *Morus* species of Pakistan. Eight collections of *Morus* were made representing one new species and one new variety and are here described and illustrated based on morpho-anatomical and phylogenetic data. These findings increase the number of reported *Morus* species from four to five and a variety in Pakistan. No work has been done on the *Morus* at molecular level in Pakistan, hence this research study will be a pioneer in the field.

Material and Methods

Plant samples of the genus *Morus* were preserved in the Fungal Biotechnology and Biosystematics Laboratory, Institute of Botany, University of the Punjab. From 30 samples 8 species were selected to study detailed morphology and phylogenetic analysis.

Morphological analysis: For morphology following parameters like tree height, bark colour, leaf shape, leaf size, leaf tip, leaf base, pistillate and staminate catkins, number of pistillate and staminate catkins per node, size petiole and peduncle size, size and colour of a fruit were studied. Flowering and fruiting season period was also noted.

Molecular analysis: CTAB method was used for the extraction of DNA from leaf of *Morus* (Doyle, 1991). Quality of DNA was checked on 1% agarose gel. ITS regions of the extracted DNA were amplified using the mulberry specific primers combination (Schmitz-Linneweber *et al.*, 2001).

390F=5'-CGATCTATTTCATTCAATATTTTC-3'
1326R=5'-TCTAGCACACGAAAGTCAAGT3'

After amplification, the same pair of amplification primers were used for sequencing in both directions. The sequence was searched in an online tool using BLAST and then more sequences were also downloaded from Genbank. Phylogenetic trees were constructed by using Maximum Likelihood (ML) analysis. It was done using Molecular Evolutionary Genetic Analysis (MEGA 11.0) with default settings of program i.e., Kimura 2-parameter model (Tamura *et al.*, 2013). 1000 bootstrapping replicates were implemented for analysis. The phylogenetic position of some species were confirmed by making Maximum Parsimony Tree with 1000 bootstrapping replicates (Swofford 2002).

Results

Morus pakistanica Amna and Niazi

Description: Monoecious, 5–6 m in height. Bark: light brown. Buds: axillary, oblong, 3.5–4.0 cm × 0.4 cm, pubescent, bud scales with light green bands, prominent leaf scars on stem. Leaves: exstipulate, petioles 4.0–4.5 cm long, glabrous. Blades broadly deltoid, lobed with 2–3 sinuses, 12–14 cm × 10–11 cm, bases cordate to round, margins regular, minutely serrate. Leaf apices obtuse to acute. Abaxial surfaces pubescent. Adaxial surfaces glabrous. Lateral veins extending up to whole of the leaf, the secondary veins at 60° to the mid-rib. Venation: multicostate reticulate. Inflorescence: Pistillate catkins 5–7 per node, each with 180 to 185 flowers, 4.0–5.0 cm long, the peduncles 1 cm long. Staminate catkins 2 per node, each with 55 to 60 flower, 2.0–2.2 cm long, the peduncles 0.5 cm long. Flowers: staminate flowers with obovate perianths; stamens globose. Pistillate flowers with oblong perianths, styles curved with bifid stigmas. Fruits: infructescences 9–9.5 cm greenish yellow (2.5GY8/10) at maturity, loose and moist. Flowering period: December, fruiting: January (Fig. 1).

Diagnostic characters: Petiole size not much longer like other big sized lobed leaves of *Morus*. Leaves are thick and leathery. Leaf blade: deltoid, darker adaxial surface than abaxial. Less number of male catkins. Short male catkins. More hairy pistillate catkins. Fruit moist, juicy and delicate as compared to other species of *Morus*

Holotype: Faisalabad (31.4504° N, 73.1350° E, 186 m a.s.l.) Punjab, Pakistan. Saline soil, January 25, 2022, H.A. Saleem FR-01 (LAH0250622; Genbank PP532838).

Additional material examined: Lahore, at 217m asl., February 20, 2019., LH-02 (LAH20219; Genbank PP532839)., Multan, at 122m asl., January 9, 2020., ML-07 (LAH190121; Genbank PP532840), Punjab, Kot Addu, at 133m asl., April 15, 2021 Gilgit Baltistan. Gilgit, at 1500m asl., January 19, 2021., AM18F (LAH150421; Genbank PP532841) H.A. Saleem and A.R. Niazi.

Distribution: Located in Faisalabad, Lahore, Multan and Gilgit.

Phylogenetic analysis: ITS sequences of our taxon *M. pakistanica* Amna and Niazi (FR-01) shown clear distinction from all other species of *Morus* by making a separate clade in a phylogenetic tree. Most closed species of this new species was *M. mesozygia* (HM747171) from United States with only 76.6% similarity with our new taxon. The final data set of ITS consisted of 46 sequences including *Artocarpus heterophyllus* (FJ917052) as outgroup. After removing and editing the ambiguous letters from aligned dataset, the aligned partial 18S rRNA, ITS1, 5.8S, rRNA, ITS2, and the partial 28S RNA region comprised 925 sites, of which 317 were conserved and 578 variable, 528 sites were parsimony-informative and 47 were singleton.

Phylogenetic tree was constructed in Mega X software with default settings. The phylogenetic tree showed that the newly generated sequences formed a clear separate clade from already reported *Morus mongolica* with high bootstrap support 100. It showed a clear distinction from *Morus mesozygia* (HM747171). A phylogenetic tree is constructed with the Maximum Likelihood (ML) algorithm. Phylogeny was performed by a bootstrap value of 1000 replicates and bootstrap values > 50% are cited in the tree (Fig. 2).

Morus alba var. *macroura* Amna and Niazi

Description: Dioecious, cultivated tree to 6 m. Bark: light brown. Buds: Dominant, scaly, 0.2 cm. Leaves: exstipulate. Petioles 3.0 cm long, light green slightly glabrous. Blades: deltoid unlobed, 8 cm × 5–6 cm, bases truncate. Margins irregular closely minute serrated. Leaf apices obtuse to cuspidate. Abaxial surfaces glabrous. Adaxial surfaces pubescent. Veins: multicostate reticulate (divergent). Lateral veins extending through up to half of the leaf, the secondary veins at 45° to the mid-rib. Inflorescence: Pistillate catkins 2 per node, each with 120 to 125 flower, 2.0–3.2 cm long, the peduncles from 1 cm. Pistillate flowers with ovate perianths, no style with bifid stigmas. Fruits: infructescences: 4.5–6.5 cm, unripened green. Riped: Yellow to purple at maturity. Flowering: start of March. Fruiting: April (Fig. 3).

Diagnostic characters: Blade unlobed. Leaf apex: obtuse to cuspidate, irregular serrated margins. Fruits: Prominent stigma (hair like stigma on head of each). Style absent. Short peduncle. Our new variety of *Morus* differs from type variety *M. alba* in having long catkin size as well as fruit size more than 6 cm.

Holotype: Lahore, Pakistan, (31.5204° N, 74.3587° E, 236 m a.s.l.) Punjab, Pakistan. Saline soil, January 25, 2022, H.A. Saleem. JH-03 (LAH250122); Genbank PP532842).

Additional material examined: Kot Addu, at 133 m asl., April 15, 2021., AB-04 (LAH150421; Genbank PP532843).

Distribution: Located in Kot Adu, Sibbi and Karachi

Economic importance: It can be used in sericulture as a sole food for silkworms.

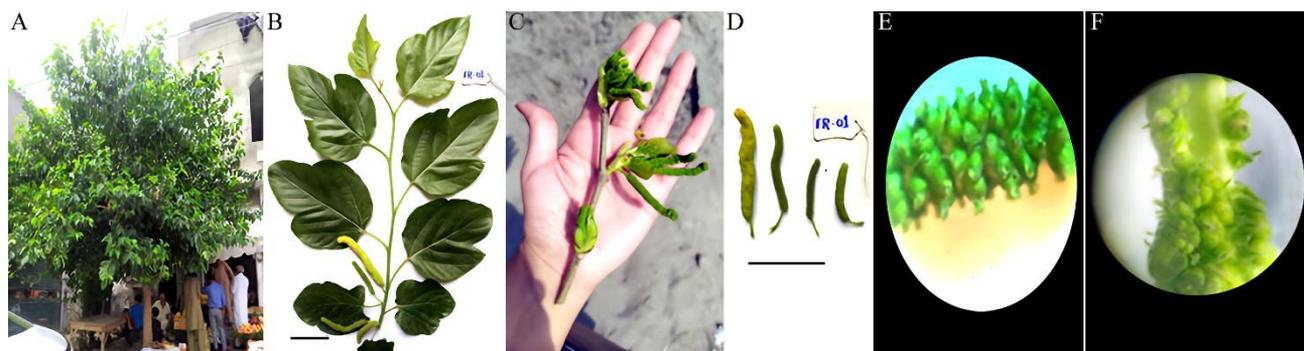


Fig. 1. A. Morphological view of *M. pakistanica* Amna & Niazi (FR-01), B. Lobed Leaves, C. Inflorescence, D. Fruit, E. Stereomicrophotographs of Female catkin F. Stereomicrophotographs of Male catkin (Scale bar=3.9 cm).



Fig. 3. A. Morphological view of *M. alba* var. *macrourea*, B. Leaves, C. Stereomicrophotographs of Female catkin, D. Fruit (Scale bar=3.9 cm).

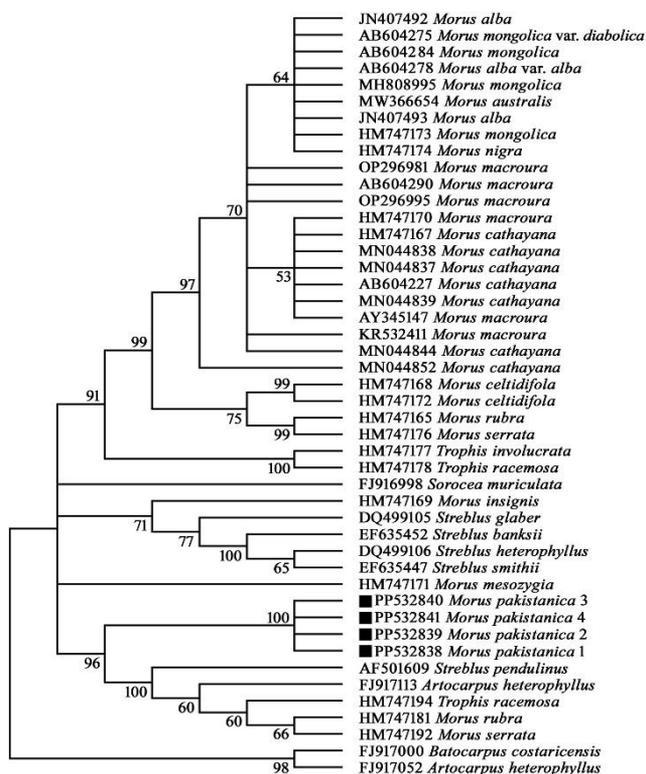


Fig. 2. Phylogenetic tree of *M. pakistanica* Amna & Niazi. Maximum Likelihood consensus trees for ITS resulting from analyses. The numbers above the branches are ML bootstrap values based on 1000 replicates. Sequences representing the taxa from Pakistan are in bullets.

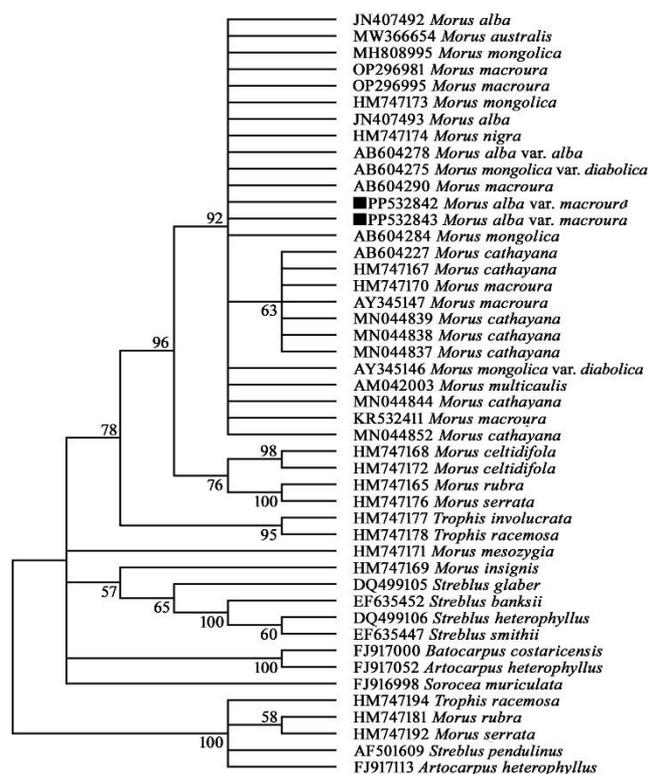


Fig. 4. Phylogenetic tree of *M. alba* var. *macrourea*. Maximum Likelihood consensus trees for ITS resulting from analyses. The numbers above the branches are ML bootstrap values based on 1000 replicates. Sequences representing the taxa from Pakistan are in bullets.

Phylogenetic analysis: ITS sequences of our taxon *M. alba* var. *macroura* Amna and Niazi made a sister clade with *M. alba* (JN40744) with 99.9% similarity while *M. macroura* (AB604290) with 99.8% similarity. The final data set of ITS consisted of 46 sequences including *Streblus pendulinus* (AF501609) as outgroup. After removing and editing the ambiguous letters from aligned dataset, the aligned partial 18S rRNA, ITS1, 5.8S, rRNA, ITS2, and the partial 28S RNA region comprised 889 sites, of which 431 were conserved and 414 variable, 334 sites were parsimony-informative and 78 were singleton.

Phylogenetic tree was constructed in Mega X software with default settings. The phylogenetic tree showed that the newly generated sequences aligned with *M. alba* var. *alba* (AB604278), *M. alba* (JN407493) and *M. multicaulis* (AM042003) with high bootstrap support 93. A phylogenetic tree was constructed with the Maximum Likelihood (ML) algorithm. Phylogeny was performed by a bootstrap value of 1000 replicates and bootstrap values > 50% are cited in the tree (Fig. 4).

Discussion

Morus L., is considered to be of substantial economic importance as it benefits our society in different ways. This genus is impacting positively the environment by reducing pollutants from soil as well as air. It is widely cultivated worldwide; its taxonomy is complex and has been doubtful. In past years, researchers have been working on its taxonomy on the basis of its morphological features.

M. pakistanica form distinct clade with *M. mesozygia* (HM747171) previously reported from U.S. It resembles morphologically *M. mesozygia* (HM747171) in having minutely serrate leaf margins. *M. mesozygia* (HM747171) differs from *M. pakistanica* in having ovate to orbicular leaf blades, with less prominent secondary venation (from the mid-rib) and peduncle is longer than inflorescence (Nepal & Ferguson, 2012).

M. pakistanica also forms distinct clade with *Morus mesozygia* (HM747171) previously reported from United States. This new taxon showed a clear distinction from all other species of *Morus* by making a separate clade in a phylogenetic tree with 100 bootstrap value. Most close species was *M. mesozygia* revealed in a phylogenetic tree with only 76% similarity. Morphological characters showed that this species also shares some characters from its ancestor group i.e., *Streblus pendulinus* (AF501609) due to long catkins but differs in having different shapes of leaf blades, and leaf margins, these characters reveals that our new taxon might be evolve from *Streblus*. It is also differ in flowering season (Sytsma *et al.*, 2002). *M. pakistanica* resembles to *S. smithii* (EF635447) previously reported from New Zealand in having glabrous adaxial leaf surface and having unisexual flower on different plants but differs from *S. smithii* (EF635447) in having different leaf parameters (Moyers *et al.*, 2018).

M. alba var. *macroura* shown distinct features from all other species of *Morus* in having unlobed leaf blade, obtuse to cuspidate leaf apices, irregular serrated margins,

prominent stigma (hair like stigma on head of each), lack of style and short peduncle. This taxon possess many morphological features resembles with both *M. alba* and *M. macroura*. Our new variety of *Morus* differs from type variety *M. alba* in having long catkin size as well as fruit size more than 6 cm. It also shown resemblance with both *M. alba* and *M. macroura* in a phylogenetic tree.

M. alba var. *macroura* made a sister clade with *M. alba* (JN40744) with 99.9% similarity while *M. macroura* (AB604290) with 99.8% similarity. *M. alba* (JN40744) shown similarity with our new variety in having same leaf shape but different fruit size, fruit colour, leaf apex and leaf base (Chen *et al.*, 2011). Molecular data generated from ITS region of DNA of the above presented Pakistani specimens support the morphological data to represent as new species and new variety.

Conclusion

M. pakistanica is a new species of *Morus* from Pakistan as well as worldwide confirmed morphologically and phylogenetically. It was observed that many sequences were submitted wrongly in Genbank which need to be revised. This species can be used in future for further biochemical characterization as well as in nanotechnology. *M. alba* var. *macroura* is also confirmed morphologically and phylogenetically as a new variety worldwide.

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