

Reporting Mitochondrial Genome of North American Native *Morus rubra* L. (Red Mulberry)

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Abstract

Morus rubra L. (Family: Moraceae), native to Eastern North America, is distributed in the heart of pristine riparian forests and holds important ethnobotanical and ecological values. The species is severely threatened by introgressive hybridization with the introduced congener *Morus alba*, which is native to Asia. Insights into the mitogenome of *M. rubra* along with comparative analyses against *M. alba* could potentially bridge the current knowledge gaps in understanding the genome architecture and hybridization patterns. The objectives of this study were to 1) sequence and assemble the draft mitochondrial genome of *M. rubra*, and 2) perform a comparative mitogenomic phylogenetic analysis with the other 36 angiosperm taxa. *M. rubra* sampled from Waubonsie State Park, Fremont County, Iowa, was used for mitochondrial genome sequencing, as part of the whole genome sequencing project. The mitogenome of *M. rubra* was 359,221 base pairs (bp) long with 45.8% GC content, comprising 57 genes: 32 protein-coding, 21 transfer, and four ribosomal RNAs. The chloroplast-to-mitogenome DNA transfer analysis revealed genes being synchronized with 17 homologous fragments from the chloroplast, accounting for 3.63% of the mitogenome. A total of 372 C to U RNA editing sites were detected in the mitochondrial protein-coding genes (PCGs) – responsible for the preprocessing of *rpl16* and *rps4* by adding a start codon, while postprocessing *atp9*, *ccmFN*, and *sdh4* by introducing a stop codon. The phylogenetic analysis of 37 species based on 23 shared mitochondrial PCGs revealed a tree topology identical to that proposed by the Angiosperm Phylogeny Group (APG) IV. This study is the first to report on the mitochondrial genome of *M. rubra*, elucidating the mitogenome-based phylogeny and providing insights into the population genetics and evolution of mulberries. The publicly available *M. rubra* mitogenome enriches genomic resources for Moraceae and highlights the roles of SSRs, RNA editing, and inter-organellar DNA transfer in shaping mitochondrial genome architecture.

Key message

Detailed analysis of the *Morus rubra* mitogenome provided insights into the genome with significant fragments inserted from the chloroplast and requirements for a few genes undergoing RNA pre- and post-processing.

Introduction

Morus rubra L. (Family: Moraceae), the North American red mulberry, is one of the 13 recognized *Morus* species and an endemic flora that maintains the biodiversity of the pristine riparian forests of North America (Adhikari et al. 2025; Burgess 2004; Nepal and Wichern 2013; Nepal 2008; Nepal and Purintun 2021). The species' habitat ranges from the eastern margin of the Great Plains, extending north to southern Ontario, Canada, and distributed as far south as southern Florida (Nepal and Wichern 2013; Nepal and Purintun 2021; Parks 2011). The palatable fruit of this North American native plant is not only valued as a source of food, but Native Americans also use the plant's sap for the treatment of ringworm; tea made from leaves to treat and cure difficulty urinating, dysentery, and weakness (Carlson and Jones 1939; Foster and Duke 1990; Hamel and Chiltoskey 1975; Moerman 1998). Conservation assessments now flag mounting concern: Michigan lists red mulberry as threatened; Massachusetts lists red mulberry as endangered under the Massachusetts Endangered Species Act (SWAP 2025), Ontario lists it as endangered under provincial law (Ontario 2014), and NatureServe's 2025 review notes ongoing threats despite a global G5 rank—chiefly genetic swamping and habitat pressures (NatureServe 2025).

Morus rubra's species integrity has been threatened by introgressive hybridization with the introduced Asian congener *Morus alba* (Burgess and Husband 2006; Burgess and Husband 2004; Burgess et al. 2005; Burgess et al. 2008; Salah 2006). *M. alba* was introduced to North America in the early colonial period (ca. 1600 AD) to establish a silk industry (Klose 1963). Despite the then government's efforts through perks and bounties, the attempts to establish the silk industry failed (Brockett 1876; Hatch 1957; Matsui 1927). Nevertheless, this did not prevent the spread of white mulberries, which escaped the cultivated range, encroached and naturalized in the native red mulberries' habitats (Nepal and Purintun 2021; Wunderlin 1997). The invasive congener soon started interweaving with the native population through hybridization, introducing fertile progeny, advancing to asymmetrical introgression with chloroplast capture and a nuclear bias toward *M. alba* alleles in sympatry (Burgess and Husband 2006; Burgess and Husband 2004; Burgess et al. 2005; Burgess et al. 2008). This has not only challenged the conservation of *M. rubra*, but has also generated taxonomic confusions, even among experts, land managers, and other stakeholders (Nepal and Purintun 2021). This emphasizes the need for molecular markers to distinguish species and hybrids. Recent chloroplast genome analyses of *M. rubra* revealed multiple haplotypes and highly variable regions, providing valuable tools for species identification, hybrid diagnosis, and conservation planning (Adhikari et al. 2025). Understanding the underlying genetics of these two hybridizing species is essential for establishing conservation strategies for red mulberry. While chloroplast genome data have advanced our capacity to identify species and diagnose hybrids, the mitochondrial genome remains unexplored in *M. rubra* and offers an additional layer of information for studying phylogenetics, organelle inheritance, and species integrity.

Mitochondria, also referred to as the “powerhouse of the cell,” predominantly provide cells with energy through oxidative phosphorylation occurring during the tricarboxylic acid (TCA) cycle (Anderson et al. 2019). They are double-membraned semi-autonomous organelles found across eukaryotes, comprising their own genome (Mahler 1973; Saccone et al. 2000; Sjöstrand 1956). The endosymbiotic theory explains that once free-living prokaryotes evolved to form the mitochondria as an autonomous eukaryotic cell organelle (Wallin 1927; Zimorski et al. 2014). Unlike in animals, where the mitochondrial (mt) genome is circular and ranges from 15-17 kilobases (kb), the mt genome size in plants varies

greatly between similar, as well as within species (Allen et al. 2007; Gualberto et al. 2014; Kubo and Newton 2008). The smallest known size of a plant mitogenome is 66 kb (Skippington et al. 2015) in a parasitic plant – *Viscum scurruloideum*, whereas the largest size of 11.7 megabases (Mb) (Putintseva et al. 2020) is reported in a gymnosperm - Siberian larch (*Larix sibirica* Ledeb.). Despite the size of the mt genome, it does not harbor a substantial number of genes (Zardoya 2020). The expanded size of the plant mt genome is primarily due to the accumulation of repetitive sequences from the different organellar genomes: nuclear and chloroplast (Chu et al. 2024; Greiner and Bock 2013; Petit et al. 2005; Tan et al. 2022). Moreover, gene transfer across mitochondria and different organellar genomes has been well-documented (Adams and Palmer 2003; Xie et al. 2024). Within *Morus*, mitogenome resources are emerging. Liangliang et al. (2021) reported the mitogenome of *M. alba* var. *atropurpurea* and *multicaulis* with a circular genome of 361,546 and 395,412 bp, respectively, accounting for ca. 7.80% cp to mt gene transfer. Building on our recent chloroplast genome of *M. rubra* (Adhikari et al. 2025), we are now first to report *M. rubra* mitochondrial genome, assembled from pair-ended short reads obtained from Illumina sequencing technology. These findings offer invaluable insights into the genetic, structural, and phylogenetic aspects of *M. rubra*, along with providing a baseline for investigating conservation approaches for the species.

Materials and Methods

Sample Collection and Sequencing—The sample (Ames 35887 - #172-13) obtained through the U.S. National Plant Germplasm System (USDA 2023) used in this study was collected from Waubonsie State Park, Fremont County, Iowa [Coordinates: 40.674843N, 95.689384W] on July 12, 2022. The plant identification was carried out following Nepal and Purinton (2021) and also confirmed by Dr. Gary Larson – former curator of South Dakota State University's C.A. Taylor Herbarium (SDC) and Dr. Madhav Nepal, a *Morus* expert. The specimen voucher has been deposited at SDC with voucher number #172-13. DNA extraction and sequencing was carried out as described in Adhikari et al. (2025). **Supplementary File 1** shows the leaf, fruits, and voucher of the specimen used.

Mitogenome Assembly and Annotation—The raw reads were subjected to quality control using FastQC (Andrews 2023) version 0.11.9, adapter trimming using Trimmomatic (Bolger et al. 2014) version 0.39, and final quality assurance visualization using MultiQC (Ewels et al. 2016) version 1.13. The curated reads were subjected to *de novo* mitogenome assembly using GetOrganelle (Jin et al. 2020) version 1.7.7.1. For annotation of protein-coding genes (PCGs) in the mitogenome using Geseq (Tillich et al. 2017), we selected *Arabidopsis thaliana* (NC037304), *Cannabis sativa* (NC029855), and *Morus notabilis* (NC041177) as reference genomes. Annotation of tRNA and rRNA-coding genes within the mitogenome was accomplished using tRNAscan-SE (Chan et al. 2021) version 2.0. BLAT (Kent 2002) search with protein, rRNA, tRNA, and DNA search identity of 50 was also employed. Annotation errors in the genome were manually corrected using Artemis (Carver et al. 2011) version 18.2.0, and OrganellarGenomeDRAW (OGDRAW) (Greiner et al. 2019) was used for visualization of the genome.

Repeated Sequences and Codon Usage Analysis—The PCGs were subjected to relative synonymous codon usage (RSCU) analysis through the PhyloSuite (Xiang et al. 2023) program, while graphical visualization was carried out in Microsoft Excel.

The mono-, tri-, tetra-, penta-, hexa-, and hepta- nucleotide repeats were detected using the MicroSatellite Identification Tool (MISA) (Beier et al. 2017) version 2.1. The parameters were set to ten for mono-, five for di-, four for tri-, and three for tetra-, penta-, hexa-, and hepta-nucleotide repeats. The maximum length of a sequence between two simple sequence repeats (SSRs) to register as a compound SSR was set to 50 nucleotides. For the detection of forward (f), reverse (r), complement (c), and palindromic (p) repeats, a web-based REPuter (Kurtz et al. 2001) with default parameters to detect the repeats, ranging from 20 to 100 bases, was employed. The results were plotted in Microsoft Excel.

Tandem Repeats Finder (Benson 1999) with the criterion set to Basic: Use default parameters, was used to identify the Tandem Repeats.

Prediction of RNA Editing Sites—The C- to -U RNA editing sites in the mitochondrial PCGs were detected using Deepred-Mt (Edera et al. 2021) with default parameters. Only results exceeding the probability value of 0.9 were considered. Microsoft Excel's graphing function was used to visualize the results.

Chloroplast to mitochondrial DNA transformation—The chloroplast genome of the same sample we assembled earlier (NCBI GenBank Accession: PQ309081) (Adhikari et al. 2025) was employed for the chloroplast-to-mitochondrial DNA transfer analysis. NCBI-blast (Altschul et al. 1990) version 2.14.1+, accessed through the High-Performance Computing cluster at South Dakota State University, was used to compare *M. rubra*'s organellar genomes. The chloroplast genome was used as a query, whereas the mitogenome was used as a database, and the remaining parameters were set as default. The Advanced Circos program in TBtools-II (Toolbox for Biologists) (Chen et al. 2023) version 2.330 software was used to visualize regions of DNA transformation.

Syntenic Analysis—Reciprocal BLASTp of *M. rubra*'s mitochondrial proteome was carried out against other *Morus* mitogenomes, and the results were subjected to collinearity analysis using the Multiple Collinearity Scan toolkit (MCScanX) (Wang et al. 2024). The block size was defined as 5, with other parameters set as: match score 50, gap penalty -1, overlap window 5, e-value 1e-5, and maximum gaps 25. The collinear genes were visualized using the Multiple Synteny Plot tool built in TBtools-II.

Phylogenetic Inference—The mitogenomes of 37 species were retrieved from the NCBI GenBank (Sayers et al. 2024), while the *M. rubra* and scaffold-level *M. alba* mt genomes were assembled as part of this study. The mitogenomes of the NCBI downloaded species were reannotated

using GeSeq (Tillich et al. 2017), and the PCGs were extracted from PhyloSuite (Xiang et al. 2023). The common 23 mitogenome genes, including *atp1*, *atp4*, *atp6*, *atp8*, *atp9*, *ccmB*, *ccmC*, *ccmFC*, *ccmFN*, *cob*, *cox1*, *cox2*, *cox3*, *matR*, *mttB*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *nad7*, and *nad9*, were concatenated before multiple sequence alignment (MSA) carried through Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh and Standley 2013) version 7.525. The MSA output was subjected to Maximum Likelihood (ML) phylogenetic analysis with 1000 bootstrap replicates in IQTREE2 (Minh et al. 2020) version 2.3.1 selecting GTR + F + R4 as the best evolutionary model as determined by ModelFinder (Kalyaanamoorthy et al. 2017). The ML tree file was converted to a Newick (.nwk) file in Interactive Tree of Life (iTOL) (Letunic and Bork 2024) version 7.2.1, while visualization was done in Molecular Evolutionary Genetic Analysis (MEGA) version 12 (Kumar et al. 2024).

Results

Characteristics of the Mitochondrial Genome of *M. rubra*—As shown in **Figure 1**, the draft mitochondrial genome of *M. rubra* displayed a circular structure. The genome size was 359,221 bp with a GC content of 45.8%. We identified a total of 57 genes, including 32 PCGs, 17 unique tRNA genes – present in multiple copies (a total of 21 tRNAs), and four rRNA genes. The core genes consist of five ATP synthase (*atp1*, *atp4*, *atp6*, *atp8*, and *atp9*), nine NADH dehydrogenase (*nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *nad7*, and *nad9*), four cytochrome C biogenesis (*ccmB*, *ccmC*, *ccmFC*, and *ccmFN*), three cytochrome C oxidase (*cox1*, *cox2*, and *cox3*), one protein transport subunit (*mttB*), one maturase (*matR*), and one ubiquinol-cytochrome C reductase (*cob*). The *nad1* and *nad5* genes were found to be trans-splicing genes. Additionally, the non-core genes include one ribosomal large subunit gene (*rpl16*) and six ribosomal small subunit genes (*rps3*, *rps4*, *rps7*, *rps12*, *rps13*, and *rps19*). There is also one succinate dehydrogenase gene (*sdh4*). **Table 1** provides complete gene inventory, including copy numbers and intron information in the *M. rubra*'s mitogenome.

Table 1. Annotated genes in the *Morus rubra* mitochondrial genome. The symbols ‘*’ and ‘x’ followed by a number, indicate intron-containing genes and number of gene copies, respectively.

Gene group	Gene names
ATP synthase	<i>atp1</i> , <i>atp4</i> , <i>atp6</i> , <i>atp8</i> , <i>atp9</i>
NADH dehydrogenase	<i>nad1</i> , <i>nad2</i> *, <i>nad3</i> , <i>nad4</i> *, <i>nad4L</i> , <i>nad5</i> *, <i>nad6</i> , <i>nad7</i> *, <i>nad9</i>
Cytochrome b	<i>cob</i>
Cytochrome c oxidase	<i>cox1</i> , <i>cox2</i> *, <i>cox3</i>
Cytochrome c biogenesis	<i>ccmB</i> , <i>ccmC</i> , <i>ccmFN</i> , <i>ccmFc</i> *
Maturases	<i>matR</i>
Protein transport subunit	<i>mttB</i>
Ribosomal protein large subunit	<i>rpl16</i>
Ribosomal protein small subunit	<i>rps3</i> , <i>rps4</i> , <i>rps7</i> , <i>rps12</i> , <i>rps13</i> , <i>rps19</i>
Succinate dehydrogenase	<i>sdh4</i>
Ribosomal RNA	<i>rrn4.5</i> , <i>rrn5</i> , <i>rrn18</i> , <i>rrn26</i>
Transfer RNA	<i>trnA</i> -UGC, <i>trnC</i> -GCA, <i>trnD</i> -GUC, <i>trnE</i> -UUC, <i>trnF</i> -AAA*, <i>trnF</i> -GAA, <i>trnG</i> -GCC, <i>trnI</i> -GAU, <i>trnK</i> -UUU, <i>trnM</i> -CAU (x4), <i>trnN</i> -GUU, <i>trnP</i> -UGG (x2), <i>trnQ</i> -UUG, <i>trnR</i> -ACG, <i>trnS</i> -UGA, <i>trnW</i> -CCA, <i>trnY</i> -GUA

Repeat Sequences in Mitogenome—We identified diverse repeat motifs within the mitogenome, including mono-, di-, tri-, tetra-, penta-, and hexa-nucleotide repeats as 41, 13, 12, 27, 1, and 1, respectively (see **Figure A**). The most frequent mono-, di-, tri-, and tetra-nucleotide repeats (number in parentheses represents total # repeats) were A/T (37), AG/CT (11), AAG/CTT (6), and AAAC/GTTT (5), while TTAGG and GAAAGA were the penta- and hexa-nucleotides each with a single occurrence (See **Supplementary File 2**).

In addition, we found 178 repeats longer than 20 nucleotides, including 84 forward, 4 reverse, and 90 palindromic repeats. The pie chart in **Figure 2B** illustrates the percentage distribution of the repeat types. No complement repeats were detected in the genome. A total of 17 tandem repeats were identified in the mt genome, with the percentage match of repeats ranging from 79 to 100. The consensus size of the repeats ranged from 13 to 128 bp (See **Supplementary File 3**).

Codon Usage in Mitogenome—Codon usage analysis of 32 PCGs resulted in 9,084 codons, including 243 start and 52 stop codons (some subjected to RNA editing). The most frequently used codon was UUU (Phenylalanine, 330 occurrence), while the least frequently used stop codon

was UGA (14 occurrences). Cysteine was the least frequent amino acid (141 codons, 1.56%), and Leucine was the most abundant (999 codons, 11.07%). The codon usage for each amino acid is presented in **Supplementary File 4**. With an RSCU value of 0.51, GAG encoding Glutamine was the least preferred codon, while GCU encoding Alanine was the preferred codon with an RSCU value of 1.65. Both AUG and UGG, coding for Methionine and Tryptophan, respectively, had an RSCU value of 1.0. **Figure 3** shows the summary RSCU values for the used codon in the mitogenome of *M. rubra*. Additionally, the overall GC content of the PCGs was 37.63%.

RNA Editing Sites in the Mitogenome— We predicted a total of 372 potential C-to-U (Cysteine-Uracil) RNA editing sites across 27 of 32 unique PCGs (cutoff ≥ 0.9). The highest number was detected in *nad7* (36 sites), followed by *mttB* (34), and *ccmB* (33). Also, *cox1* and *sdh4*, each had a single editing site. The *rps4* and *rpl16* genes lacking the start codon were subjected to modification through RNA editing. Similarly, the stop codons were added to the *atp9*, *ccmFN*, and *sdh4* genes. **Figure 4** shows the distribution of the C-to-U RNA editing sites in the genes of *M. rubra* mitogenome. See **Supplementary File 5** for details.

Chloroplast to Mitochondrial Gene Transfer—Through sequence similarity analysis, we identified 17 homologous fragments between the mitogenome and chloroplast genome, totaling 13,043 bp (**Table 2**). These fragments accounted for 3.63% of the entire mitogenome. When including inverted repeats from the cp genome, the transfer accounted for 23,780 bp (6.62%). Most of these fragments migrated from chloroplast DNA (cpDNA) to mitochondrial DNA (mtDNA), except for a few tRNA genes that exhibited high sequence similarity, making it challenging to determine the direction of migration. The longest-spanning gene fragment was 5046 bp, while the smallest fragment was 43 bp long. The complete PCGs transferred between cp and mt genomes included *rrn4.5* and *rrn5*, while most other genes are transfer RNAs, including *trnA-UGC*, *trnI-GAU*, *trnM-CAU*, *trnW-CCA*, *trnN-GUU*, *trmP-UGG*, and *trnD-GUC*, whereas the transferred fragmented genes include *rbcl*, *psbA*, *psbC*, *rrn16*, *rpl2*, *rpl23*, *pafI*, *ndhB*, and *ycf2* (see **Table 2**). This finding suggests a notable sequence migration between the cpDNA and mtDNA of *M. rubra*, which was accompanied by gene transfer, a topic discussed in detail later. **Figure 5** schematically illustrates the cp–mt gene transfers (i.e., homologs shared between the cp and mt genomes).

Table 2. The homologous sequences interchanged in the chloroplast and mitochondrial genomes of *M. rubra*.

S.N.	Percent Identity	Length (bp)	Mismatch	Gapopen	Cp start	Cp end	Mt start	Mt end	E-value	Bitscore	Associated gene(s)
1	99.881	5052	0	1	106831	111882	238972	233927	0	9291	<i>trnA-UGC/rn23-frag/rn4.5/rn5/trnR-ACG-frag</i>
2	100	1717	0	0	140859	142575	43200	41484	0	3171	<i>trnI-GAU</i>
3	100	1691	0	0	88646	90336	58478	56788	0	3123	<i>rpl2-frag/rpl23-frag/trnM-CAU</i>
4	99.201	1127	0	1	151177	152303	104968	103851	0	2023	<i>ycf2-frag</i>
5	98.645	812	3	1	58844	59655	66177	66980	0	1432	<i>rbcl-frag</i>
6	91.264	538	16	6	810	1347	8495	9001	0	704	<i>psbA-frag</i>
7	79.528	508	65	23	70077	70562	123337	122847	3.83E-88	326	<i>trnW-CCA/trnP-UGG</i>
8	74.128	889	175	42	104570	105433	308769	307911	2.30E-85	316	<i>rrn16-frag</i>
9	93.59	156	5	2	59617	59771	54013	53862	1.11E-58	228	<i>rbcl-frag</i>
10	93.243	148	9	1	36875	37021	181333	181480	2.40E-55	217	<i>psbC-frag</i>
11	99.145	117	0	1	135055	135171	278600	278485	4.02E-53	209	<i>trnN-GUU</i>
12	97.561	82	1	1	32478	32559	325102	325182	5.35E-32	139	<i>trnD-GUC</i>
13	94.937	79	4	0	55359	55437	27011	26933	1.50E-27	124	<i>trnM-CAU</i>
14	90.805	87	8	0	93360	93446	122383	122297	2.51E-25	117	<i>ycf2-frag</i>
15	90.323	62	4	2	146898	146959	78200	78141	3.29E-14	80.5	<i>ndhB-frag</i>
16	95.652	46	0	1	46052	46097	70630	70673	5.50E-12	73.1	<i>pafl-frag</i>
17	95.349	43	2	0	89640	89682	67014	66972	7.12E-11	69.4	<i>rpl2-frag</i>

Note: “Length (bp)” indicates the segment corresponding to the cp genome, while -frag is abbreviated for fragments transferred from the cp genome to the mt genome.

Synten Analysis—Figure 6 illustrates the varying arrangements of colinear blocks across the *Morus* mitogenomes. The major PCG pairs forming the collinear blocks include, *atp4-nad4L*, *ccmFN-mttB*, and *cox3-sdh4*, *nad3-rps12*, and *nad6-rps4*, respectively. Despite the PCGs being highly conserved across the sister species, our finding highlights the extensive rearrangement of the gene orders across mitochondrial genomes.

Phylogenetic Inference—The 23 conserved mitochondrial PCGs, including *atp1*, *atp4*, *atp6*, *atp8*, *atp9*, *ccmB*, *ccmC*, *ccmFC*, *ccmFN*, *cob*, *cox1*, *cox2*, *cox3*, *matR*, *mttB*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *nad7*, and *nad9* were used for phylogenetic analysis with *Amborella trichopoda* (Amborellales) as the outgroup. We found the phylogenetic tree topology similar to that of the Angiosperm Phylogeny Group (APG) IV (Stevens 2001 onwards). We observed that Magnoliids and Monocots formed a single clade with a BS support of 87, which includes the orders Magnoliales (*Magnolia biondii* and *Liriodendron tulipifera*), Alismatales (*Spirodela polyrhiza*), Asparagales (*Crocus sativa* and *Asparagus officinalis*), Arecales (*Phoenix dactylifera* and *Cocus nucifera*), and Poales (*Triticum aestivum*, *Oryza sativa* Indica, *Zea mays*, and *Sorghum bicolor*). A 100 BS support was observed for the orders of monocots, while within monocots, the commelinids (Arecales and Poales) showed a common ancestry with a 98 BS support. Similarly, the dicots were found to be clustered together with a 100 BS support, where the basal eudicot order Ranunculales (*Pulsatilla dahurica* and *Aconitum kusnezoffii*) was found to be separating from a common ancestor of eudicots. Interestingly, the Vitales (*Vitis vinifera*) belonging to Rosid I/Fabidae was observed to be separated from the Rosid II/Malvidae by the insertion of Santalales (*Malania oleifera* and *Santalum album*). We also observed that Rosid II formed a distinct clade with BS support of 62, comprising Fagales (*Betula pendula* and *Fagus sylvatica*) and Rosales, as indicated by green branches. Within Rosales, the order Moraceae (*Morus rubra*, *M. notabilis*, *M. alba*, *M. multicaulis*, and *M. atropurpurea*) formed a distinct clade with a BS support value of 100, shown in red. However, the North

American *Morus rubra* forming a cluster with non-sister *Morus notabilis* seems to be interesting, albeit with a BS value of 57. The rest of the Rosales families included Rosaceae (*Prunus armeniaca* and *Malus domestica*), Rhamnaceae (*Ziziphus jujuba*), Ulmaceae (*Hemiptelea davidii*), and Cannabaceae (*Cannabis sativa*). Similarly, the Asterids' clade formed a distinct cluster with 100 percent BS support. Asterid II, Escalloniales (*Ilex rotunda*) and Asterales (*Helianthus annuus*) were found in isolation from Lamiales (*Lavandula angustifolia* and *Salvia miltiorrhiza*) and Solanales (*Nicotiana tabacum* and *Capsicum annuum*) of Asterid I. The clustering of Asterid I was supported with a BS value of 100. **Figure 7** shows the phylogenetic tree based on the conserved mitochondrial PCGs across 37 species.

Discussion

The first mitochondrial genome to be sequenced was the human mitogenome in 1981 (~16 kb) (Anderson et al. 1981), whereas the first plant mitogenome (~186 kb) was reported in 1992 from the liverwort *Marchantia polymorpha* (Oda et al. 1992). Since then, several species' mitogenomes have been assembled. While animal mitogenomes are relatively smaller and highly conserved, plant genomes show great variations, including shape and size, varying even within the closely related species (Allen et al. 2007; Gualberto et al. 2014; Gualberto and Newton 2017; Kubo and Newton 2008; Wang et al. 2024). Within genus *Morus*, *M. rubra* mitogenome is much smaller (359,221 bp) (**Figure 1**) compared to that of *M. notabilis* (362,069 bp), *M. multicaulis* (361,546 bp), and *M. atropurpurea* (395,421 bp) (Liangliang et al. 2021). The GC content in all four species was ca. 45%, while the total number of genes and PCGs detected in *M. rubra* and *M. atropurpurea* were 57 and 32, higher than the other two. *M. rubra* and *M. notabilis* both have 21 tRNAs, while the total number of genes encoding rRNAs is higher in *M. rubra* compared to other accessions [see **Table 3** for details]. The mitogenome of *M. rubra* was ca. 2.27 times larger than its chloroplast genome (Adhikari et al. 2025).

Table 3. Comparative overview of four *Morus* mitogenomes.

Characteristics	<i>M. rubra</i> [*]	<i>M. alba</i> var. <i>atropurpurea</i> ^a	<i>M. alba</i> var. <i>multicaulis</i> ^a	<i>M. notabilis</i> ^a
GenBank Accession #	PX233331	MW924383	MW924382	NC041177
Genome size (bp)	359,221	395,412	361,546	362,069
Total number of genes	57	57	54	54
PCGs	32	32	31	30
tRNA	21	22	20	21
rRNA	4	3	3	3
GC content (%)	45.80	45.50	45.42	45.66

^{*}This study and ^aLiangliang et al. (2021)

Repeat Regions in *Morus* Mitogenomes— Repeat regions, including tandem, long, and short sequence repeats, are common in the *Morus* mitogenome (Guo et al. 2017). These sequences are known to play a significant role in mitochondrial genome rearrangement (Cole et al. 2018). Notably, the relatively higher number of SSRs and repeats were found in the *M. rubra* mitogenome (95 and 178, respectively) (**Figure 2**) compared to its chloroplast genome (75 SSRs and 46 repeats), likely reflecting its larger genome size and extensive non-coding regions. Similar to that in the *M. rubra* chloroplast genome, SSRs and repeats in the mitogenome were distributed across introns and intergenic spacer regions (Adhikari et al. 2025). Interestingly, the total SSRs detected in *M. multicaulis* are ca. four times more than in *M. rubra* (Liangliang et al. 2021), suggesting a wide interspecific variation in repeat content. In *M. rubra*'s mt genome, SSRs were the most abundant repeats, aligning with reports in various other plants (Ke et al. 2023; Xie et al. 2024). Previously, SSRs from the pigeon pea (*Cajanus cajan*) mitogenome were used as molecular markers for genotype identification (Khera et al. 2015). We believe that the diverse SSRs and tandem repeats detected in this study may serve as valuable genetic markers for DNA fingerprinting, studying population structure of *M. rubra* as well as phylogenetic relationships among species in the genus *Morus*.

Codon Usage— Relative Synonymous Codon Usage (RSCU) is the relative frequency of synonymous codons used for encoding amino acids in a genome (Sharp and Li 1986). An RSCU value of less than one means rarity in the use of the codon, equal to one means unbiased use of the codon, while greater than one means preference of a specific codon over another (Sharp and Li 1986). Since methionine (AUG) and tryptophan (UGG) are both coded by their respective single designated codons, the RSCU value is equal to one as expected (Sharp and Li 1986). In this study, CGA (encoding Glutamine) showed the lowest RSCU value, and GCU (encoding Alanine) had the highest. These patterns align with Liangliang et al. (2021)'s findings in *M. multicaulis* and *M. atropurpurea* mitogenomes, suggesting conserved codon usage bias across *Morus* species. Unlike 22,775 codons detected in the *M. rubra* chloroplast genome (Adhikari et al. 2025), its mitogenome has codons reduced by two-fifths (9,084 codons in total) (**Figure 3**), which aligns with the reduced number of PCGs (32 in the mitogenome and 83 in the chloroplast). The GC content of the PCGs in the mitogenome (37.63%) was observed to align with the reported value for the *M. rubra*'s chloroplast (~37%), indicating

compositional consistency across organellar genomes. These findings provide insight into codon usage dynamics and genome architecture in *M. rubra*, and may be implemented in future studies on translational efficiency, evolutionary constraints, and organelle-specific gene expression.

RNA Editing and Translational Regulation—Proper folding of cp and mt proteins in higher plants is accomplished by the process of RNA editing, a crucial post-transcriptional modification tool (Bi et al. 2016). The number of RNA editing sites in plants can vary, with the highest number recorded in the clubmoss *Selaginella moellendorffii* with 2,152 sites (Zhang et al. 2020), while they can range between 300 to 500 in angiosperms [reviewed in (Grewe et al. 2014)]. In this study, we identified 372 C-to-U editing sites in the 27 PCGs with a confidentiality threshold above 0.9 (Figure 4). The number of C to U editing sites in the mitogenome of *M. rubra* is comparable to that of *M. multicaulis* (377) (Liangliang et al. 2021), suggesting conserved post-translational regulation within the genus. A high binding free energy characterizes translational accuracy; thus, to regulate the molecular function, the G/C codon, with a higher binding affinity, is preferred through RNA editing (Hao et al. 2021; Hu et al. 2025). In *M. rubra*, RNA editing is required to create start codons in *rpl16* and *rps4* for translation initiation, and to generate stop codons in *atp9*, *ccmFN*, and *sdh4* for transcript termination. This finding is consistent with observations in other plant species such as *Meniocus linifolius*, where the start codon is introduced in *rpl16*, while in *Crucihimalaya lasiocarpa* and *Lepidium sativum*, the stop codon is introduced in the *ccmFN* genes through RNA editing (Liu et al. 2024). Similarly, in *Arabidopsis thaliana*, two exonucleases act simultaneously to process the 3'-end of *atp9* mRNA, as it lacks a stop codon (Perrin et al. 2004). Handa et al. (1998) reported the need of RNA editing for the expression of the *rps4* gene in *Rubus* sp. and *Oryza sativa*. Additionally, in *Solanum tuberosum*, the *sdh4* gene undergoes RNA editing along with co-transcription with the *cox3* gene (Siqueira et al. 2002). Varré et al. (2019) also reported the requirement of RNA editing to create a stop codon for transcriptional termination of *atp9* in *S. tuberosum*. Together, these findings highlight the conserved and dynamic role of RNA editing in regulating mitochondrial gene expression.

Chloroplast-to-Mitochondria DNA Transfer—The *M. rubra* mitogenome demonstrated gene remodeling with the incorporation of ~13 kb fragments from its chloroplast genome. Such inter-organellar gene transfers have been widely reported across plant species, including *Stemona sessilifolia* (Xie et al. 2024), *Morus multicaulis* (Liangliang et al. 2021), *Apostasia shenzhenica* (Ke et al. 2023), and *Camellia sinensis* (Li et al. 2023). Our analysis revealed a significant proportion of chloroplast to mitochondria DNA transfer, accounting for a total of 3.63% of the sequenced genome (Figure 5 and Table 2). Previously, Liangliang et al. (2021) reported ~7.80% DNA migration in *Morus* species, while Lai et al. (2022) reported ca. 6-10% of the entire genome in closely related three accessions of *Broussonetia* species. Complete gene transfers from cp to mt genome are well documented in angiosperms, mainly comprising tRNA genes, including *trnA-UGC*, *trnI-GAU*, *trnM-CAU*, *trnW-CCA*, *trnN-GUU*, *trmP-UGG*, and *trnD-GUC* (Bi et al. 2016; Sugiyama et al. 2005). Similarly, the transfer of rRNA genes like *rnm4.5* and *rnm5* has been previously reported in a seagrass, *Zostera caespitosa* (Yong et al. 2025). Nevertheless, migration of fragmented genes along with the larger chunk of DNA from the cp to the mt genome is highly significant too. Tang et al. (2024) reported the transfer of *rpl2* and *rnl16* fragments in *Paeonia lactiflora*, which aligns with our results. Recent comparative analyses in sweet potato (*Ipomoea batatas*) further support the prevalence of horizontal gene transfer (HGT) between organelles, revealing 33 mitochondrial segments with high homology to chloroplast sequences (Li et al. 2024). These findings reinforce the evolutionary significance of cp-to-mt DNA migration, which has been occurring in angiosperms since at least 300 million years ago (MYA), or the Carboniferous period (Wang et al. 2007).

Synteny and Conserved PCG Clusters—Synteny analysis of the PCGs in *M. rubra* mitogenome revealed extensive rearrangements, including cpDNA fragment incorporated as foreign sequences and inversions (Figure 6). These rearrangements reflect the dynamic nature and are consistent with patterns observed in other angiosperms. Among the conserved ancestral gene clusters, *atp4-nad4L* and *nad3-rps12* are among the ancestral conserved gene clusters, earlier reported in *A. thaliana* (Schleicher and Binder 2021), *Cryptocarya kwangtungensis* (Huang et al. 2025), and *Ajuga reptans* (Liu et al. 2020). The *cox3-sdh4* gene cluster, along with being ancestrally conserved collinear genes, also engages in co-transcription for transcript termination of *sdh4* as it lacks a stop codon (Siqueira et al. 2002). The gene pairs *nad6-rps4* and *ccmFN-mttB* are newly reported in mulberries in this study. As previously reported by Huang et al. (2025), an interlocking pattern was exhibited in the conserved gene clusters in addition to being encoded in the same direction. Moreover, the presence of highly conserved tRNAs compared to the PCGs indicates their crucial importance in the genome (Liangliang et al. 2021).

Phylogenetic Relationships and Taxonomic Implications

The mitochondrial genome structure in plants continues to evolve dynamically, exhibiting substantial variation in size, gene content, and structural rearrangements; however, it has a slower rate of nucleotide substitution (Allen et al. 2007; Gualberto et al. 2014; Kubo and Newton 2008; Palmer and Herbon 1988). In contrast, the cp genome has a higher evolution rate and is supposed to have a lack of recombination, making it suitable for phylogenetic studies (Duminil and Besnard 2021). Due to this reason, the mitogenome is historically underutilized in phylogenetic studies, particularly at the species and genus levels (Govindarajulu et al. 2015). However, recent large-scale phylogenomic analyses have demonstrated that mitochondrial protein-coding genes (mtPCGs) can effectively resolve deep node relationships across angiosperms, offering robust support for major clades and providing complementary insights to plastid and nuclear datasets. These findings are consistent with our results (Figure 7) and underscore the growing relevance of mitogenomic data in evolutionary biology, especially for taxa with complex or ambiguous histories (Lin et al. 2025).

In our study, phylogenetic reconstruction based on *M. rubra* mtPCGs yielded a tree topology (see **Figure 7**) consistent with the APG IV classification (Stevens), reinforcing the reliability of mitochondrial markers for interspecific analyses. The nesting pattern of the *Morus* species in the phylogenetic tree mostly aligns with the evolutionary perspectives previously reported based on chloroplast and nuclear gene regions (Adhikari et al. 2025; Gardner et al. 2021; Nepal and Purintun 2021). The clustering of *M. alba*, *M. atropurpurea*, and *M. multicaulis* was expected, as the latter two are varieties of the former. In contrast, the nesting of *M. notabilis* with *M. rubra* was an interesting observation. The unexpected nesting of *M. notabilis* with *M. rubra*, however, suggests a possible earlier divergence event, a hypothesis supported by chloroplast-based phylogenies and divergence time estimates (Adhikari et al. 2025). The genus *Morus* includes 13 recognized species distributed worldwide (Nepal and Purintun 2021), yet mitogenomic data remain available for only a subset. Expanding mitogenome sequencing to include all *Morus* species would be instrumental in refining phylogenetic relationships and resolving taxonomic ambiguities. Notably, the current bootstrap support for the *M. rubra*–*M. notabilis* clade is relatively weak (57), indicating that additional data could significantly alter tree topology and improve resolution. Furthermore, with the continuous advancement of phylogenetic tools—such as model-aware partitioning, site-heterogeneous substitution models, and improved alignment algorithms—we strongly recommend reannotation and quality control of publicly available organellar genomes. This step is essential to minimize the risk of false-positive phylogenies and to ensure reproducibility and accuracy in evolutionary studies.

In summary, while mitogenomes have traditionally been overlooked in plant systematics, emerging evidence and analytical innovations are rapidly elevating their status as valuable resources for phylogenetic reconstruction, especially when integrated with plastid and nuclear data.

Conclusion

The mitochondrial genome of *Morus rubra* was assembled and annotated, revealing a circular structure of 359,221 bp, which is smaller than *M. atropurpurea* (395 kb) but comparable to *M. multicaulis* and *M. notabilis*. The GC content was ~45.8%, and the genome encoded 57 genes, including 32 PCGs, 21 tRNAs, and four rRNAs—slightly higher than other *Morus* accessions. Notably, the mitogenome was ~2.3 times larger than its chloroplast genome. We identified 95 SSRs and 178 repeat sequences, substantially more than in the chloroplast genome, highlighting the role of repeats in mitogenome expansion and rearrangement. Codon usage analysis of 9,084 codons showed conserved biases across *Morus*, with GCU (Alanine) being most frequent and CGA least. RNA editing was extensive, with 372 C-to-U sites across 27 PCGs, required for generating start and stop codons in several genes, mirroring patterns in other angiosperms.

Chloroplast-to-mitochondria gene transfer accounted for ~3.6% of the mitogenome (~6.6% including cpIRs), including intact rRNAs and tRNAs as well as fragmented genes. Synteny analysis revealed conserved PCG clusters (e.g., *atp4*–*nad4L*, *cox3*–*sdh4*) alongside extensive rearrangements, underscoring the structural dynamism in *Morus* mitogenomes. Phylogenetic reconstruction based on 23 PCGs produced a topology congruent with APG IV, with strong support for major clades. Within *Morus*, *M. rubra* unexpectedly clustered with *M. notabilis* (BS = 57), whereas *M. alba* grouped with its varieties as expected. These results align with chloroplast-based phylogenies and suggest an earlier divergence of *M. rubra*.

Overall, the *M. rubra* mitogenome expands available resources for Moraceae and demonstrates how SSRs, RNA editing, and inter-organellar DNA transfer shape mitochondrial evolution. Although mitogenomes evolve slowly at the nucleotide level, they retain strong phylogenetic signal, supporting their broader implications in plant systematics and evolutionary studies.

Abbreviations

DNA: Deoxyribonucleic acid(s)

RNA: Ribonucleic acid(s)

PCG: Protein coding gene(s)

tRNA: transfer ribonucleic acid(s)

rRNA: ribosomal ribonucleic acid(s)

mt: mitochondrial/mitochondrion

cp: chloroplast

bp: base pair(s)

TCA: tricarboxylic acid

SSR: Simple sequence repeat(s)

Declarations

Authors' Note

The plant species with their mitogenomes' NCBI GenBank accession numbers (in parentheses) analyzed in this research article include: *Amborella trichopoda* (KF754803), *Magnolia biondii* (NC049134), *Spirodela polyrhiza* (NC017840), *Crocus sativus* (OL804177), *Asparagus officinalis* (NC053642), *Phoenix dactylifera* (NC016740), *Cocos nucifera* (NC031696), *Triticum aestivum* (NC036024), *Oryza sativa* var. *indica* (NC071219), *Zea mays* (NC008332), *Sorghum bicolor* (NC008360), *Pulsatilla dahurica* (NC071219), *Aconitum kusnezoffii* (NC053920), *Vitis vinifera* (NC012119), *Malania oleifera* (MT902145), *Santalum album* (OQ868374), *Ilex rotunda* (NC084321), *Helianthus annuus* (KF815390), *Lavandula angustifolia* (OR296704), *Salvia miltiorrhiza* (NC023209), *Nicotiana tabacum* (NC006581), *Capsicum annuum* (NC024624), *Glycine max* (JX463295), *Arabidopsis thaliana* (NC037304), *Betula pendula* (LT855379), *Fagus sylvatica* (MT446430), *Prunus armeniaca* (NC065228), *Malus domestica* (MN964891), *Ziziphus jujuba* (NC029809), *Hemiptelea davidii* (MN061667), *Cannabis sativa* (NC029855), *Morus rubra* (PX233331), *Morus notabilis* (NC041177), *Morus alba* (PX243397), *Morus alba* var. *atropurpurea* (MW924383), and *Morus alba* var. *multicaulis* (MW924382).

Author Contributions

B.A. performed the experiments, analyses, wrote the scripts and codes, and wrote and reviewed the original manuscript. S.P. assisted in writing and reviewing the draft. E.R., G.S., and R.J., assisted in laboratory tasks and J.D.C. assisted with field sampling and contributed to revising the manuscript. M.P.N. conceived and supervised the project, framed the study and analyses, and assisted in writing, reviewing, and finalizing the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest to be disclosed.

Ethics statement

Not applicable.

Data availability

The mitochondrial genomes assembled in this study, with accession numbers **PX233331** for *Morus rubra* and **PX243397** for *Morus alba*, have been deposited into the NCBI GenBank repository.

Code availability

The codes and scripts used in this study are publicly available from the author's GitHub repository at https://github.com/abibek52/Morus_rubra_mitochondria.

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Figures

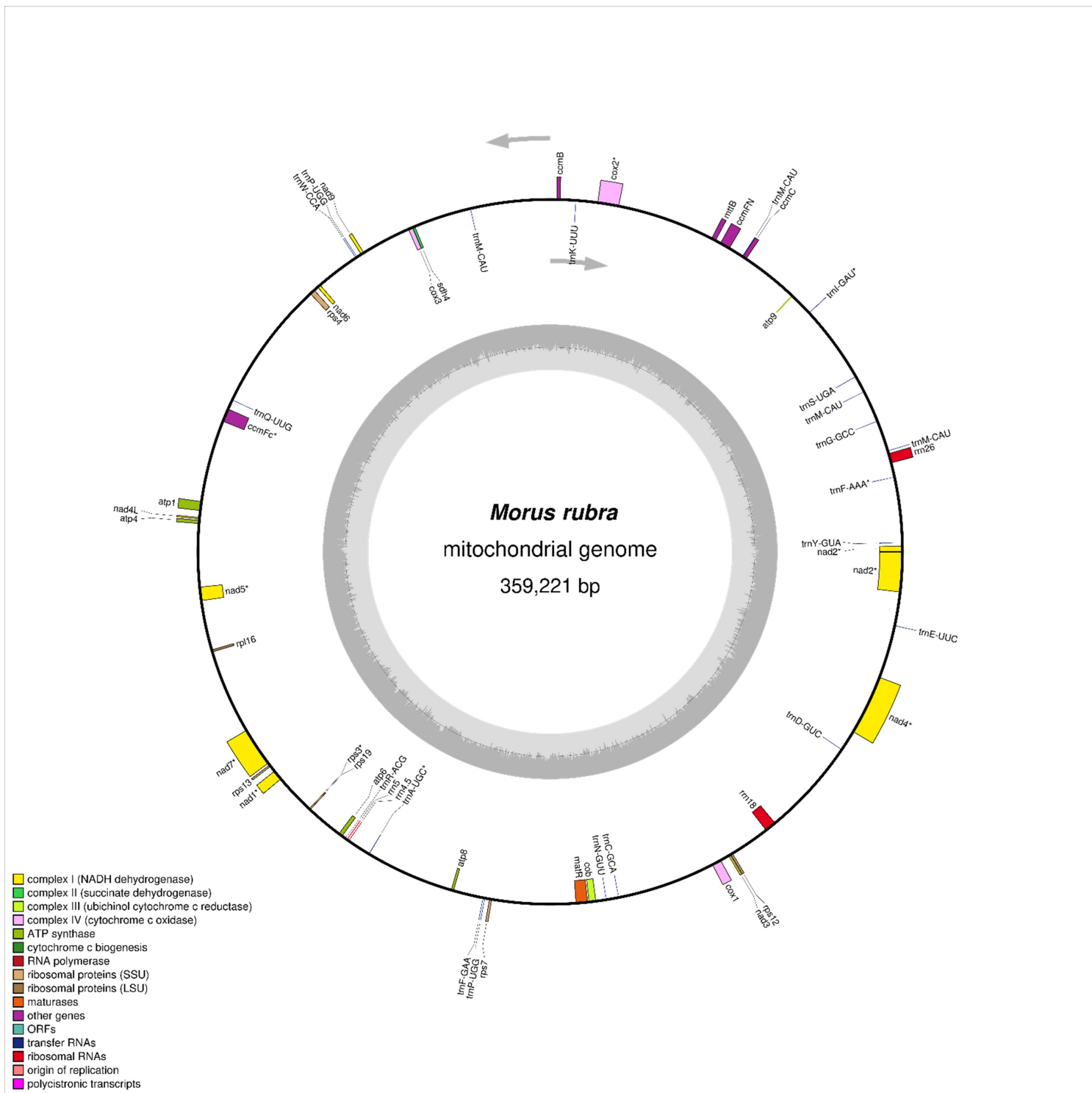


Figure 1

Morus rubra mitochondrial genome map. The dark gray internal concentric circle represents the GC content, while light gray represents AT content. The gray arrows show the downstream direction of the genes. The genes with '*' contain introns, while the genes in different colors denote the functional association of genes as defined by the accompanying legend.

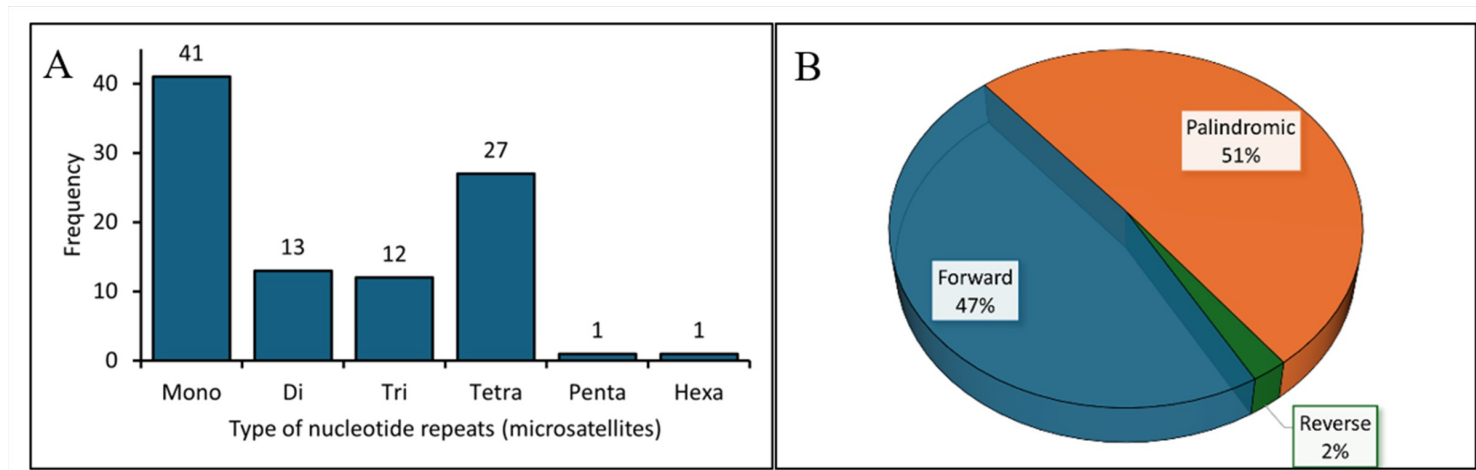


Figure 2

Distribution of repeat sequences in the mitochondrial genome of *Morus rubra*. Panel (A) presents a bar graph showing the frequency of simple sequence repeats (SSRs), while panel (B) displays a pie chart illustrating the proportions of different repeat types identified in the mitogenome.

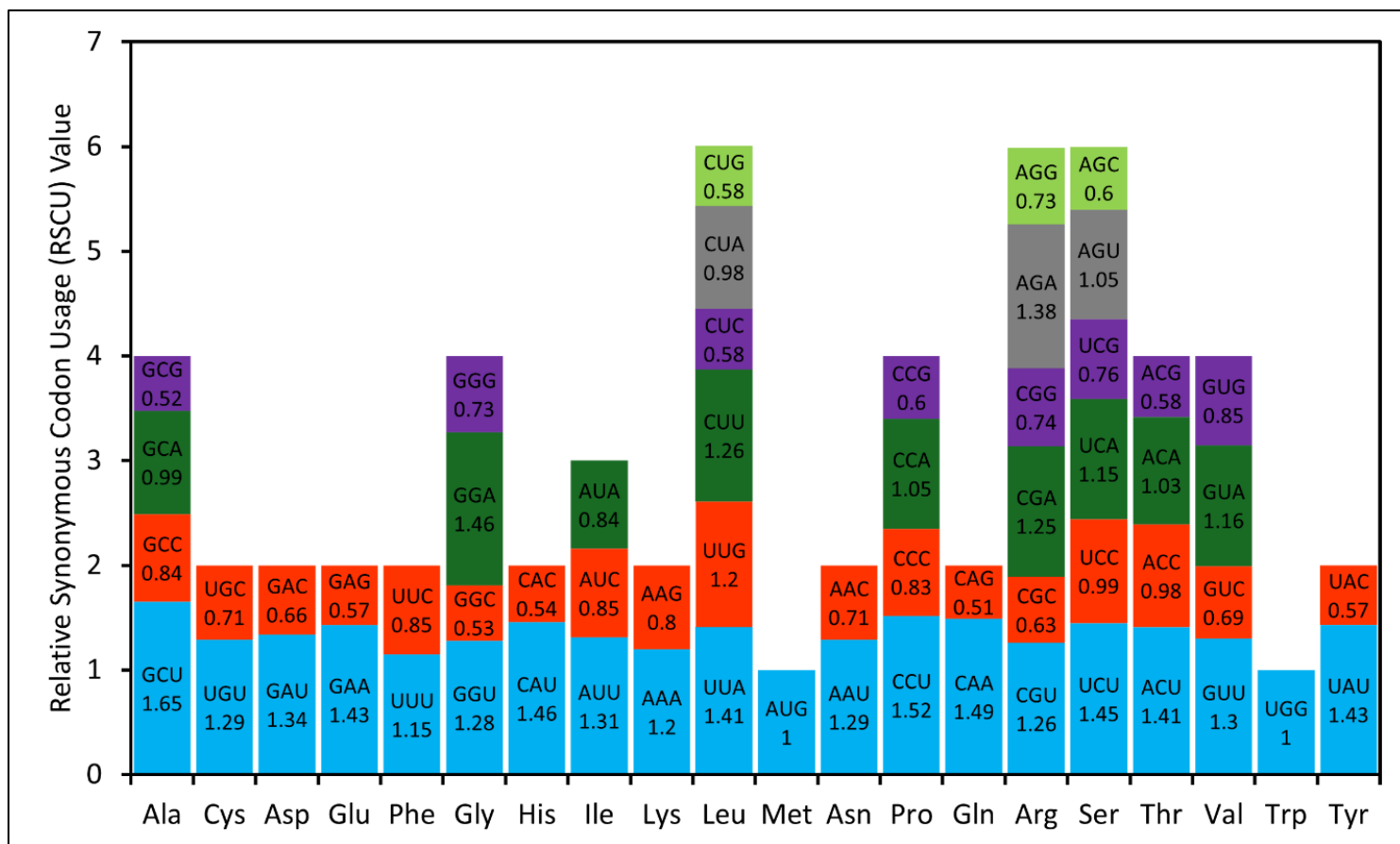


Figure 3

M. rubra mitogenome's relative synonymous codon usage (RSCU). The X-axis represents the amino acid with the associated codon and RSCU values on the Y-axis.

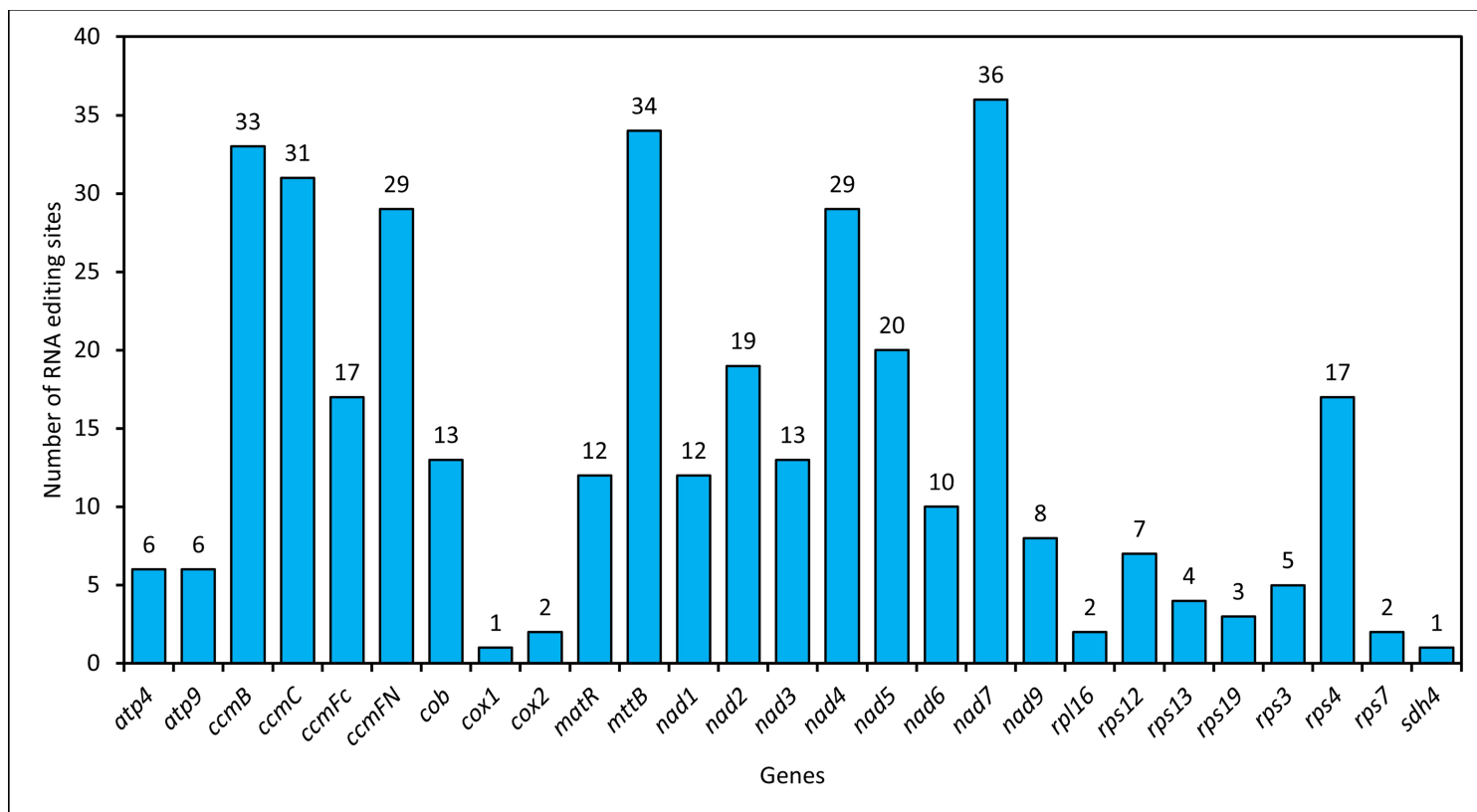


Figure 4

Predicted C-to-U RNA editing sites in the genes of the *M. rubra* mitogenome (cutoff ≥ 0.9). Labels on the X-axis are the gene names, while the number of editable RNA sites is on the Y-axis.

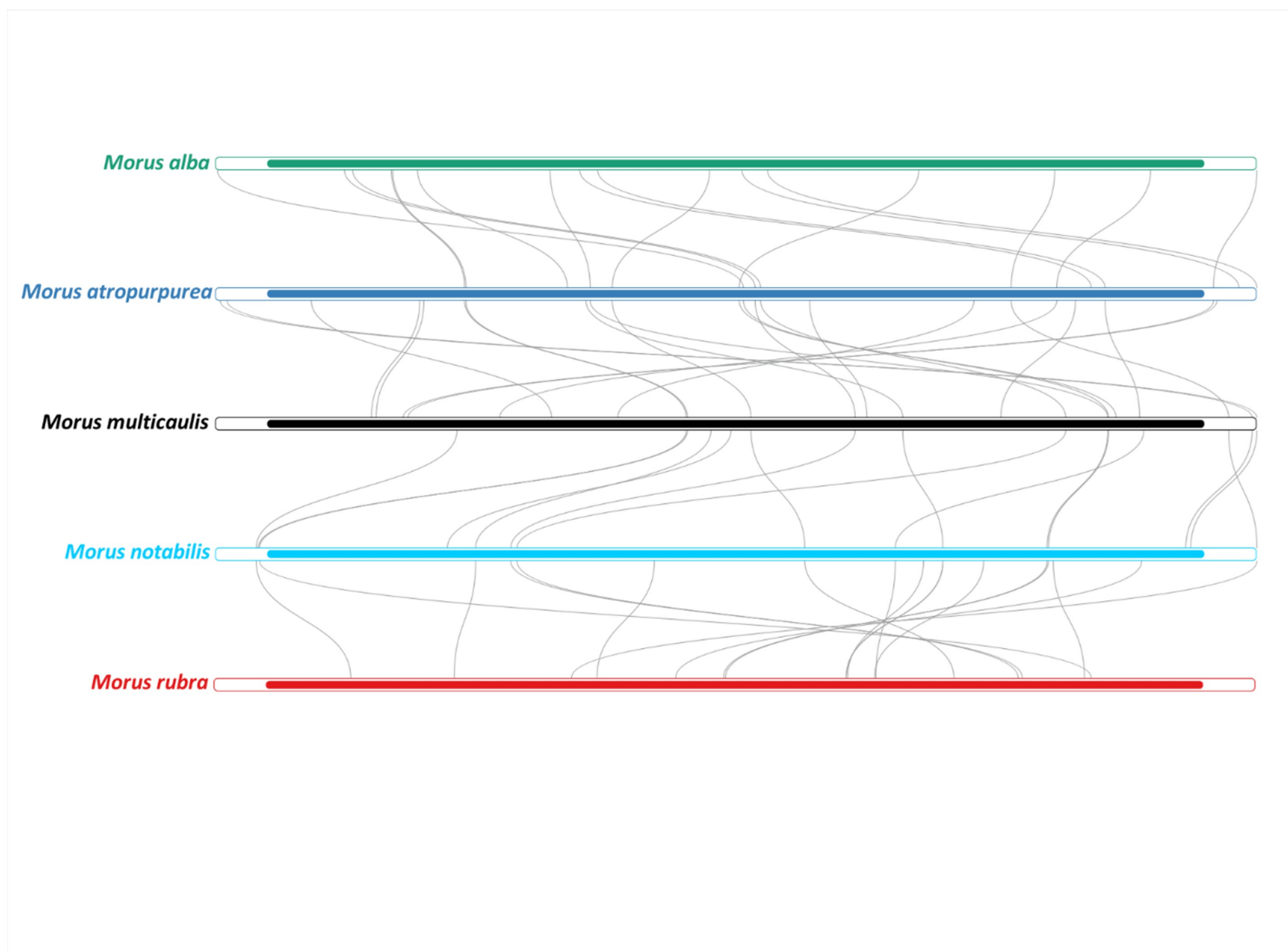


Figure 6

Mitogenome synteny plot. Horizontal bars indicate the mitogenomes of different *Morus* species, while curves show homologous PCG sequences between adjacent species.

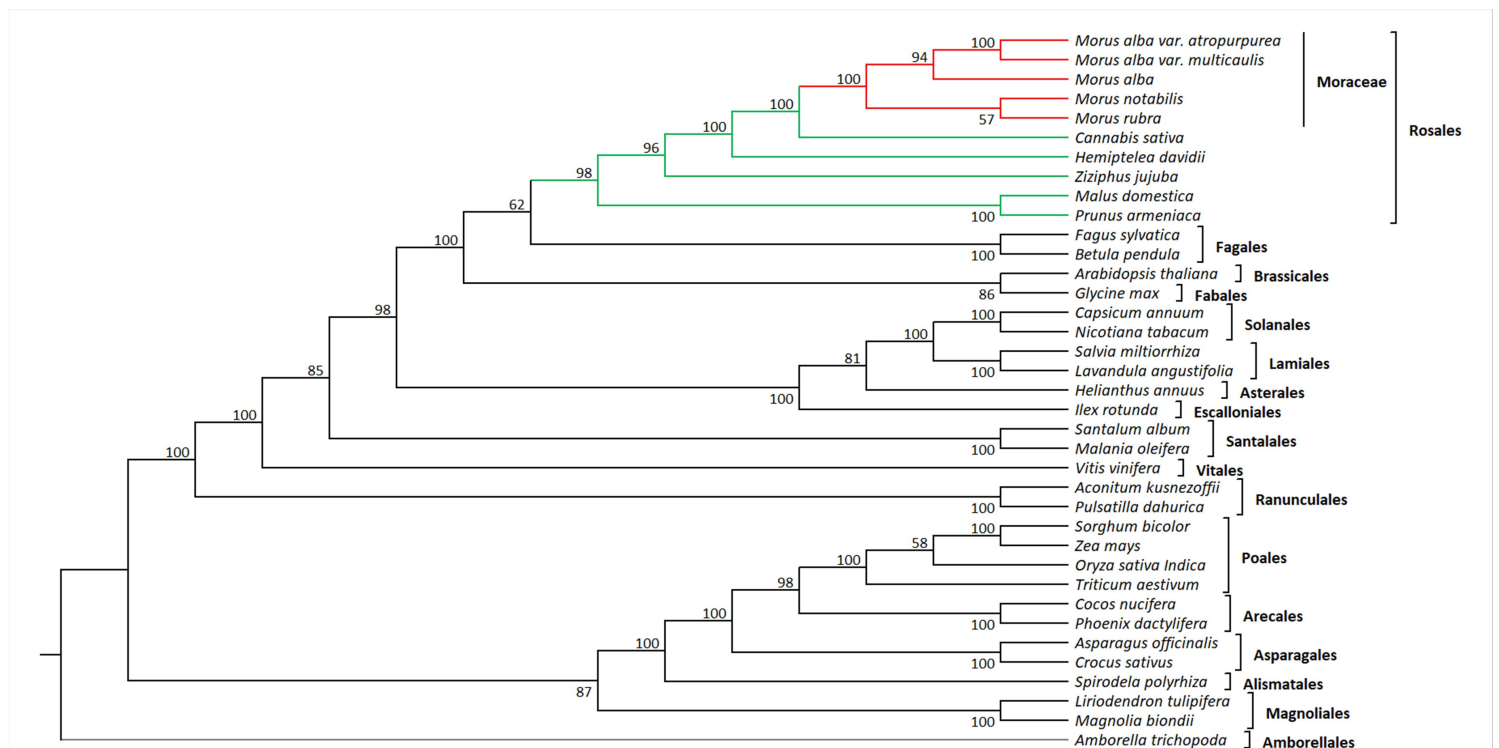


Figure 7

Maximum-Likelihood phylogenetic tree based on 23 mitochondrial protein-coding genes (PCGs) illustrating the relationship among 37 different species, aligning with the Angiosperm Phylogeny Group (APG) IV classification. The phylogenetic analysis employed the GTR + F + R4 substitution model with 1000 bootstrap (BS) replicates. Numerical values along the branches indicate percentage BS support. Accessions marked with an asterisk (*) represent the genomes reported in this study.

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