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Complete chloroplast genomes and phylogenetic analysis of 7 *Murraya* species in China

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Abstract

Background: *Murraya*, a genus of shrubs and trees in the Rutaceae family, consists of approximately 9 species in China with significant medicinal and horticultural value. However, the phylogeny and taxonomy of *Murraya* species remain controversial, particularly with respect to *Murraya exotica* and *M. paniculata*.

Objective: This study aimed to provide insights into the taxonomy, phylogeny, and identification of *Murraya*.

Methods: In this study, the chloroplast (CP) genomes of 7 *Murraya* species were sequenced, assembled, and subjected to comparative and phylogenetic analyses.

Results: The CP genomes of *Murraya* ranged from 158,573 to 160,817 bp in length and encoded 112 unique genes, including 78 protein-coding genes, 30 tRNA genes, and 4 rRNA genes. Similar to other angiosperms, the inverted repeat regions of the CP genomes exhibited lower sequence divergence than the single-copy regions, and coding regions were more conserved than noncoding regions. Comparative analysis identified several highly variable regions (eg, *matK*, *ycf1*, *ndhI–ndhA*, *trnH-GUG-psbA*, *rpl32–trnL*) that could serve as molecular markers for species identification in *Murraya*. Among these, the *ycf1* gene was validated as a useful marker for distinguishing *M. exotica* from *M. paniculata*. Positive selection was detected in 10 genes, including *rbcL*, *psaJ*, *ndhD*, *ndhF*, *rpl2*, *rpl20*, *ycf1*, *accD*, *ccsA*, and *rpl32*. Phylogenetic analysis based on CP genomes supported the recognition of *M. exotica* and *M. paniculata* as independent species. Moreover, the phylogenetic trees indicated that *Murraya* is not monophyletic, with sect. *Bergera* showing a closer relationship to *Clausena*. Molecular dating results suggested that the diversification of *M. paniculata*, *M. alata,* and *M. exotica* occurred approximately 9.11 Mya (95% highest posterior density: 4.90–13.87 Mya).

Conclusion: These findings provide valuable CP genome data for clarifying the phylogenetic relationships between *M. exotica* and *M. paniculata*, and for advancing the study of DNA markers and the evolutionary history of *Murraya*.

Key words: Chloroplast genome; Comparative analysis; *Murraya*; *Murraya exotica*; *Murraya paniculata*; Phylogenetic analysis

Abbreviations: MFC, Murrayae Folium et Cacumen; CP, chloroplast; ISSR, inter-simple sequence repeats; DAMD, directed amplification of minisatellite DNA; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeats; dN, nonsynonymous substitution; dS, synonymous substitution; ML, maximum likelihood; LRT, likelihood ratio test; BEB, Bayesian empirical Bayes; BI, Bayesian inference; CDS, coding sequence; NCS, noncoding sequence; LSC, large single-copy region; SSC, small single-copy region; BS, bootstrap support; PP, posterior probability; Mya, million years ago; MCMC, Markov Chain Monte Carlo; HPD, highest posterior density; PCR, polymerase chain reaction

1. Introduction

Murraya, comprising about 17 species, is a genus in the family Rutaceae distributed across East, South, and Southeast Asia, as well as Australia and the Southwest Pacific Islands. *Murraya* species possess medicinal, horticultural, and economic value. According to the *Chinese Pharmacopoeia* (2020 Edition), Murrayae Folium et Cacumen (MFC) is derived from *Murraya exotica* or *M. paniculata*, and is used in the treatment of stomachache, rheumatism, arthralgia, and toothache.[1]

The circumscription of species within *Murraya* has long been controversial. Based on morphological taxonomy and chemotaxonomy, some scholars divided the genus *Murraya* into 2 sections. Species with white-gray branches were classified as sect. *Murraya*, which includes *M. exotica*, *M. paniculata*, *M. alata*, and others.[2] Sect. *Bergera* comprises species with dark-brown branches, such as *M. euchrestifolia*, *M. kwangsiensis*, *M. kwangsiensis* var. *macrophylla*, and *M. koenigii* (Fig. 1).[3] Some researchers further proposed elevating the 2 sections to the rank of genera, thereby restoring the genus *Bergera* Koenig ex L. (1771).[4–6] Currently, molecular phylogenetic and morphological evidence generally supports revising the 2 sections as separate genera.[7,8]

At the species level, the taxonomic status of *M. exotica* L. and *M. paniculata* (L.) Jack remains controversial and has

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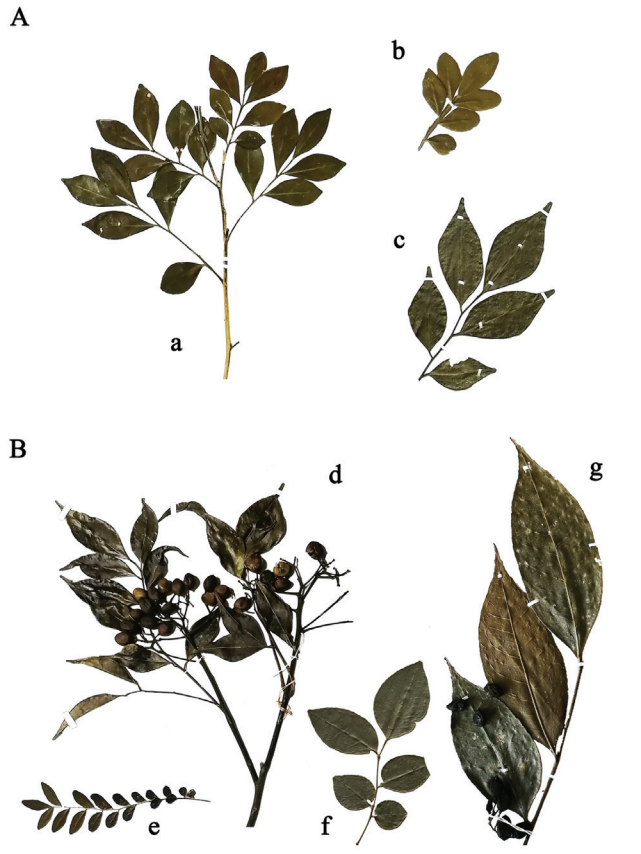

Figure 1. Foliar morphology of *Murraya* species herbarium specimens. (A) sect. *Murraya*: (a) *M. exotica*; (b) *M. paniculata*; (c) *M. alata*. (B) sect. *Bergera*: (d) *M. euchrestifolia*; (e) *M. microphylla*; (f) *M. kwangsiensis*; (g) *M. kwangsiensis* var. *macrophylla*.

been revised several times. Due to the lack of type specimens and the presence of morphological intermediates in cultivation, some scholars have suggested that the 2 species should be synonymized or treated as a single species.[9–11] Others have maintained them as distinct species based on morphological and phytochemical differences.[12] The primary morphological distinction lies in the leaflets: *M. exotica* has elliptic-obovate or obovate leaflet blades, whereas *M. paniculata* exhibits suborbicular to ovate or elliptic leaflets. Additionally, the 2 species differ significantly in chemical composition.[13] The main constituents of *M. exotica* leaves are coumarins,[14,15] while *M. paniculata* leaves are rich in polymethoxylated flavones.[16,17] In the current market, *M. paniculata* serves as the main source of Murrayae Folium et Cacumen (MFC), whereas *M. exotica* is primarily used as a horticultural plant in southern China. Therefore, clarifying the relationship between *M. exotica* and *M. paniculata* is essential to ensure the safety and efficacy of MFC in clinical applications.

Molecular systematic studies have resolved some of the taxonomic issues within the genus *Murraya*. Analyses based on the nuclear ribosomal DNA ITS region and partial chloroplast (CP) DNA sequences indicate that *M. koenigii*, *M. kwangsiensis*, and *M. microphylla* are more closely related to *Clausena* than to other *Murraya* species.[18–20] Inter-simple sequence repeats, directed amplification of minisatellite DNA, and random amplified polymorphic DNA profiles also suggest the presence of distinct genotypes in *M. exotica* and *M. paniculata*.[21] Nevertheless, the relationship between *M. exotica* and *M. paniculata* remains unclear. In this study, we sequenced and assembled the complete CP genomes of *M. exotica*, *M. paniculata*, *M. alata*, *M. euchrestifolia*, *M. kwangsiensis*, *M. kwangsiensis* var. *macrophylla*, and *M. microphylla* from different regions. We analyzed genome features, long repeat sequences, and simple sequence repeats (SSRs), and conducted comparative and phylogenetic analyses based on the CP genomes. This work provides new insights into the taxonomy and phylogeny of *Murraya*.

2. Materials and methods

2.1. Plant material, DNA extraction, and CP genome sequencing

Thirty-two samples representing 7 *Murraya* species were collected from the Herbarium of the Institute of Botany, China Academy of Chinese Medical Sciences (PE), and the voucher specimens were deposited at the same herbarium (Supplemental Table S1, https://links.lww.com/STCM/A68). All samples were identified by Yan Jin. No specific permissions were required for the collection of *Murraya*. In addition, 6 complete CP genomes of Rutaceae were obtained from GenBank: *M. koenigii* (NC_032684.1), *Citrus medica* (NC_050939.1), *C. maxima* (MN782007.1), *Merrillia caloxylon* (NC_032688.1), *C. excavata* (NC_032685.1), and *Zanthoxylum armatum* (NC_050250.1). Total genomic DNA was extracted from 20 to 50 mg of dried leaves using the DNAsecure Plant Kit (Tiangen, Beijing, China). For each accession, 5.0 Gb of raw data were generated using the Illumina NovaSeq 6000 platform (Novogene, Beijing, China) with 150 bp paired-end reads.

2.2. CP genome assembly and annotation and feature analyses

Raw sequencing data were filtered using fastp version 0.19.7. The complete CP genomes of the 32 *Murraya* samples were then assembled from the clean data using GetOrganelle v1.6.2d[22] with the following parameters: maximum extension rounds = 30, max-reads = 2×10^7, and SPAdes *k*-mer values = 45, 65, 85, 115. Genome annotation was performed on 33 CP genomes (including *M. koenigii*, NC_032684.1) using PGA v3 and the online program GeSeq[23] (https://chlorobox.mpimp-golm.mpg.de/geseq.html), with *Amborella trichopoda* (curated by PGA) and *M. paniculata* (NC_052700.1) serving as reference genomes. The search identity threshold was set to 85%. Annotation results were manually reviewed and corrected. Circular CP genome maps were generated using OGDRAW[24] (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html).

2.3. Repeat analysis

REPuter[25] (https://bibiserv.cebitec.uni-bielefeld.de/reputer) was used to identify forward, reverse, complement, and palindromic repeats in the CP genomes. The parameters were set as follows: hamming distance = 3 and minimum repeat size = 30 bp. SSRs were identified using MISA[26] with the following thresholds: 8 repeats for mononucleotide SSRs, 4 repeats for dinucleotide and trinucleotide SSRs, and 3 repeats for tetranucleotide, pentanucleotide, and hexanucleotide SSRs.

2.4. Genomic comparison and divergence analysis

IRscope[27] (https://irscope.shinyapps.io/irapp/) was used to visualize the expansion and contraction of the inverted repeat (IR) regions in the CP genomes. To compare *Murraya* CP genomes, mVISTA[28] (https://genome.lbl.gov/vista/mvista/submit.shtml) was employed to analyze and visualize sequence divergence, using the annotation of *M. exotica* as the reference. MAVUE V2.4.0[29] was used to assess CP genome collinearity. Nucleotide diversity (π) of gene and intergenic regions was calculated using DnaSP v6.[30]

2.5. Positive selection analysis

Selective pressure was analyzed for 78 shared protein-coding genes among the CP genomes of *Murraya*. EasyCodeML v1.41[31] was used to calculate nonsynonymous (dN) and synonymous (dS) substitution rates, as well as their ratio (ω). Each protein-coding gene was aligned separately using ClustalW (codons) in MEGA11[32] after removal of stop codons. Based on these alignments, a maximum likelihood (ML) tree was constructed using IQ-TREE v2.0.5.[33] The preset mode and site models (M0, M3, M1a, M2a, M7, M8, M8a) in EasyCodeML were applied to calculate dN, dS, ω, and likelihood ratio test values. The Bayesian empirical Bayes method was used to estimate posterior probabilities (PP) and detect positive selection in the genes. PPs greater than 0.95 and 0.99 indicate sites under positive selection and strong positive selection, respectively.

2.6. Phylogenetic analysis

Phylogenetic trees were constructed using ML and Bayesian inference (BI) methods. Three datasets were analyzed as follows: (1) complete CP genomes, (2) concatenated coding sequences (CDSs) of 78 shared protein-coding genes (including only 1 IR region), and (3) 109 shared noncoding sequences (NCS) of intergenic regions (including only 1 IR region). Sequence alignments were performed using MAFFT v7.453,[34] and aligned sequences were trimmed with TrimAl v1.4[35] using a gap threshold of 0.9 and a consistency score (cons) of 60. ML analysis was conducted in IQ-TREE with 1000 ultrafast bootstrap replicates, and the best-fitting substitution model was selected using the ModelFinder plugin. BI analysis was performed in MrBayes v3.2.6[36] via PhyloSuite v1.2.2[37] for 10,000,000 generations, with the optimal substitution model also determined using ModelFinder[38] in PhyloSuite. Phylogenetic trees were visualized and edited using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). *Zanthoxylum armatum* (NC_050250.1) was used as the outgroup to root the trees.

2.7. Divergence time estimation

Divergence times of *Murraya* species were estimated using MCMCtree in PAML v4.9[39] based on the complete CP genomes. The ML tree constructed by IQ-TREE was used as the input topology. Three fossil calibrations were applied: the node of *Citrus* species was set at 8 million years ago (Mya)[40]; the node of the branch containing *Clausena excavata* was set at 27.23 Mya[41]; and the node of the outgroup (*Z. armatum*) was set at 35–40 Mya.[42] The Markov Chain Monte Carlo process was run for 20,000 generations with a sampling frequency of every 100 iterations, discarding the first 20,000 samples as burn-in. Divergence times were estimated using the independent rates clock model and the HKY85 substitution model, with 95% highest posterior density (HPD) intervals. The resulting divergence times were then mapped onto the ML tree.

2.8. Molecular marker development

Gene and intergenic regions among *Murraya* species were analyzed using mVISTA and DnaSP. Primers for molecular markers were designed with Primer Premier 5.0,[43] and their accuracy was verified through polymerase chain reaction (PCR) amplification. The 25 μL PCR reaction contained 12.5 μL 2× M5 Super FastTaq PCR MasterMix (Mei5 Biotechnology Co., Ltd., Beijing, China), 0.5 μL forward primer (10 μmol/L), 0.5 μL reverse primer (10 μmol/L), 0.5 μL DNA template, and 11.0 μL dH_2O. The PCR amplification was performed on a Veriti 96 PCR system (Applied Biosystems, Waltham, Massachusetts, USA) under the following conditions: initial denaturation at 95 °C for 3 minutes; 35 cycles of 94 °C for 10 seconds, 58 °C for 15 seconds, and 72 °C for 10 seconds; and a final extension at 72 °C for 5 minutes. PCR products were evaluated by 1.5% agarose gel electrophoresis, followed by purification and sequencing.

3. Results

3.1. Features of the Murraya CP genomes

All 33 *Murraya* CP genomes exhibited a typical quadripartite structure, consisting of 2 IR regions (IRa and IRb), a large single-copy region (LSC), and a small single-copy region (SSC). The total lengths of the CP genomes ranged from 158,573 bp (*M. kwangsiensis* var. *macrophylla*) to 160,817 bp (*M. exotica*) (Fig. 2). Specifically, the CP genome lengths of *M. exotica* ranged from 160,178 to 160,817 bp, while those of *M. paniculata* ranged from 160,261 to 160,369 bp. The lengths of the LSC, SSC, and IR regions varied from 86,813 bp (*M. microphylla*) to 87,812 bp (*M. kwangsiensis*), 17,983 bp (*M. euchrestifolia*) to 18,620 bp (*M. paniculata*), and 26,328 bp (*M. kwangsiensis* var. *macrophylla*) to 27,577 bp (*M. exotica*), respectively. The total GC content ranged from 38.54% to 38.65%, with average GC contents of 37.04%, 33.46%, and 42.94% for the LSC, SSC, and IR regions, respectively.

These CP genomes contained 112 unique genes, including 78 protein-coding genes, 30 tRNA genes, and 4 rRNA genes (Supplemental Table S2, https://links.lww.com/STCM/A68). The *ndhD* gene was annotated as a pseudogene in *M. exotica*, one sample of *M. paniculata*, *M. euchrestifolia*, *M. kwangsiensis*, and *M. kwangsiensis* var. *macrophylla* due to the absence of a valid start codon. Seven protein-coding genes (*ndhB*, *rpl2*, *rpl23*, *rps12*, *rps19*, *rps7*, and *ycf2*), 7 tRNA genes (*trnA-UGC*, *trnI-CAU*, *trnI-GAU*, *trnL-CAA*, *trnN-GUU*, *trnR-ACG*, and *trnV-GAC*), and 4 rRNA genes (*rrn4.5*, *rrn5*, *rrn16*, and *rrn23*) were duplicated in the IR regions. Interestingly, compared with other *Murraya* species, *trnH-GUG* was duplicated in the LSC region of *M. kwangsiensis*, whereas 1 copy of *rps19* and *ycf2* was lost in *M. kwangsiensis* var. *macrophylla*. Furthermore, 3 protein-coding genes contained 2 introns, while 6 protein-coding genes and 6 tRNA genes contained 1 intron across all *Murraya* samples (Supplemental Fig. S1, https://links.lww.com/STCM/A68). An intron of the *ndhF* gene was observed in 4 samples of *M. exotica*.

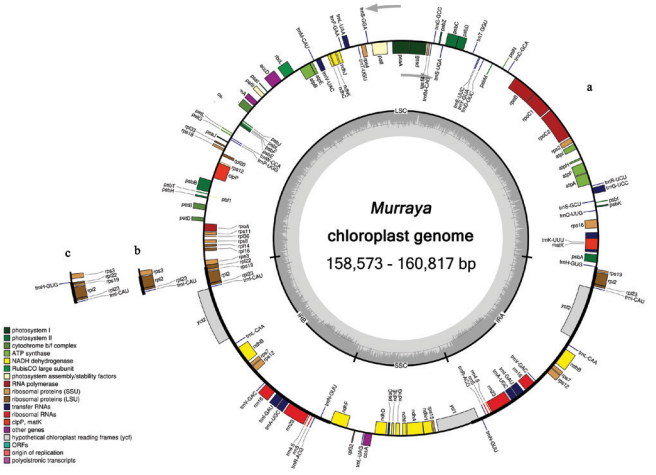

Figure 2. Complete chloroplast genome maps of *Murraya* species. (a) *M. exotica*, *M. paniculata*, *M. alata*, *M. euchrestifolia*, *M. microphylla*, and *M. koenigii*. (b) *M. kwangsiensis* var. *macrophylla*. (c) *M. kwangsiensis*. Arrows represent transcription directions of genes. Distinct genes are shown in different colors.

Nucleotide diversity (π) values were calculated for 112 genes and 109 intergenic regions in *Murraya* CP genomes (Fig. 3). The top 5 genes with the highest π values, in ascending order, were *matK* (0.0100), *ycf1* (0.0098), *ccsA* (0.0097), *ndhF* (0.0096), and *rps15* (0.0084). The top 5 intergenic regions with the highest π values, in ascending order, were *ndhI–ndhA* (0.0351), *trnH-GUG-psbA* (0.0303), *rpl23-rpl2* (0.0288), *rpl2-rpl23* (0.0288), and *trnG-UCC-trnR-UCU* (0.0260). Overall, the average π value of genes was lower than that of intergenic regions.

3.2. Repeat analysis

Four types of long repeats (forward, reverse, complement, and palindromic) were identified in the CP genomes of 33 *Murraya* samples. A total of 1233 long repeats were detected, including 407 forward repeats, 68 reverse repeats, 42 complement repeats, and 716 palindromic repeats (Supplemental Fig. S2, https://links.lww.com/STCM/A68). The number of long repeats per species ranged from 31 (*M. exotica*) to 49 (*M. paniculata*). Most repeats (70.9%) were 30–39 bp in length, 18.7% were 40–49 bp, and 10.4% exceeded 50 bp (Supplemental Table S3, https://links.lww.com/STCM/A68). The longest repeat was a palindromic repeat of 398 bp, which was the only repeat longer than 70 bp.

A total of 7924 SSRs were identified in the CP genomes of 33 *Murraya* samples, including 5738 mononucleotide SSRs, 1544 dinucleotide SSRs, 292 trinucleotide SSRs, 284 tetranucleotide SSRs, 25 pentanucleotide SSRs, and 41 hexanucleotide SSRs. The number of SSRs varied among the 8 species, ranging from 236 (*M. microphylla*) to 254 (*M. kwangsiensis*). Notably, no pentanucleotide SSRs were detected in the CP genomes of *M. exotica* and *M. alata*. Most SSRs were located in the LSC regions, followed by the SSC and IR regions. Among all SSR types, A/T mononucleotide SSRs were the most abundant, accounting for 66.48%, followed by AT/AT dinucleotide SSRs, accounting for 13.24% (Supplemental Fig. S3, https://links.lww.com/STCM/A68).

3.3. Comparative genomes analysis

The boundaries of the LSC/IR and SSC/IR regions were compared among the 8 *Murraya* species (Fig. 4). Four *M. exotica* samples, each approximately 160,816 bp in length, exhibited IR region expansion compared with other samples. In contrast, *M. kwangsiensis* var. *macrophylla*, which had the shortest CP genome, showed IR region contraction. Genes located near the LSC/IR junction included *rps3*, *rpl22*, *rps19*, *rpl2*, and *trnH*. The *rpl22* gene was entirely located in the IRb region in *M. kwangsiensis*, but in the LSC region of *M. kwangsiensis* var. *macrophylla*, whereas in the other 6 species, it crossed the LSC/IRb junction. The *trnH* gene was positioned in the LSC region in all 8 species, with *M. kwangsiensis* containing 2 copies in the LSC, an unusual feature. Due to IR contraction in *M. kwangsiensis* var. *macrophylla*, the *rps19* gene shifted into the LSC region, losing 1 copy, while the 2 *rpl2* genes in the IR regions extended to the LSC/IR boundary, and the *trnH* gene was located farther from the LSC/IRa boundary. The *ndhF* gene contracted inward by 148 bp from the SSC/IRb junction in *M.*

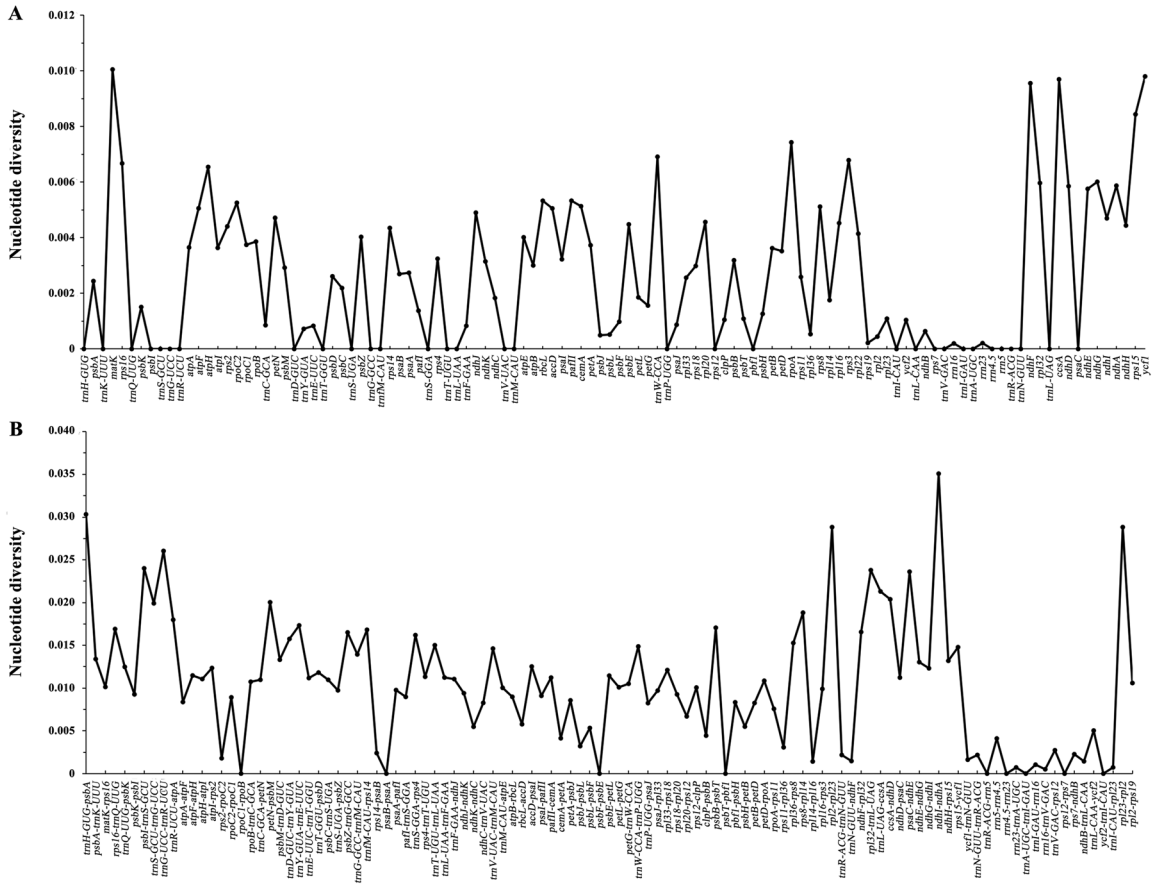

Figure 3. Nucleotide diversity (π) values of 112 genes and 109 intergenic regions in *Murraya* chloroplast genomes. (A) Nucleotide diversity values of 112 genes. (B) Nucleotide diversity values of 109 intergenic regions.

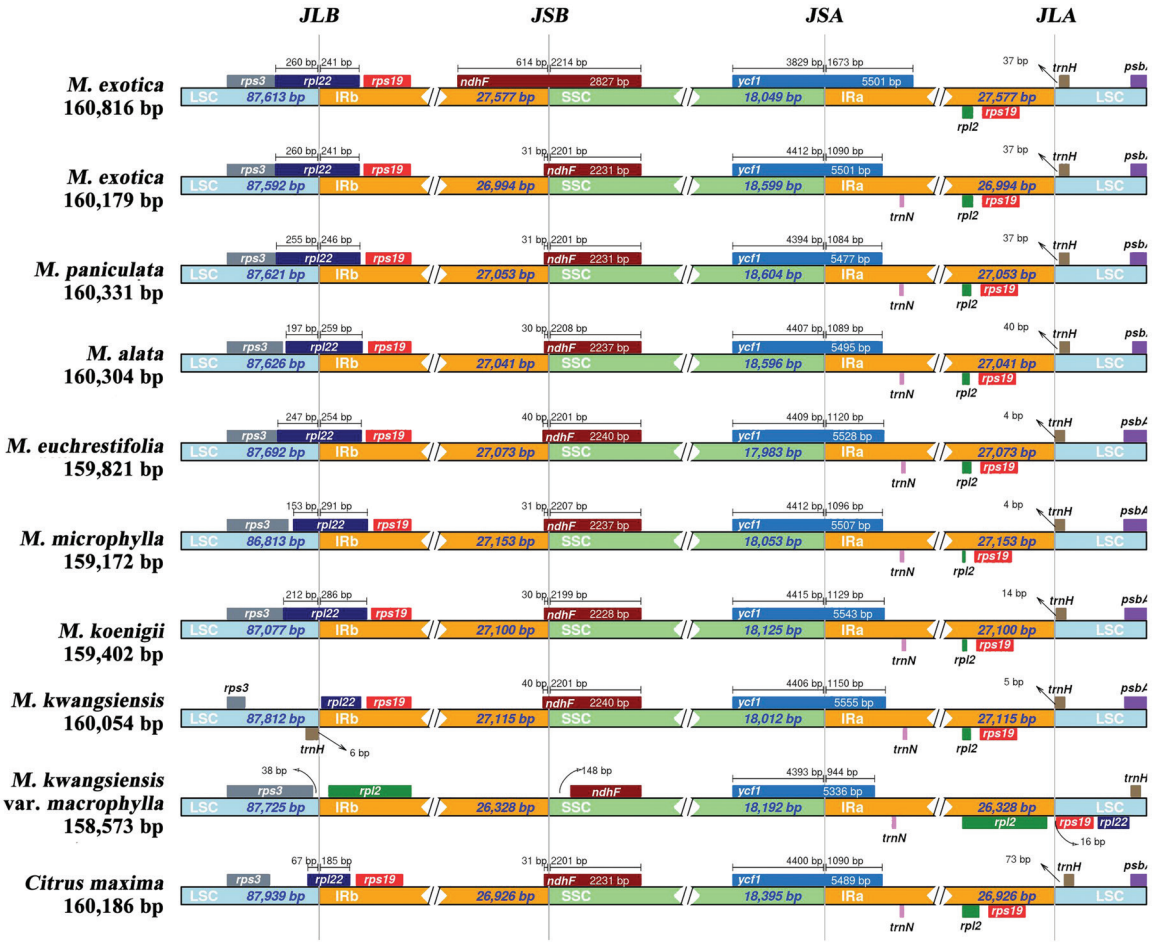

Figure 4. Comparison of the IR/SC boundaries among *Murraya* chloroplast genomes.

kwangsiensis var. *macrophylla*, whereas it expanded in other species. IR expansion in some *M. exotica* individuals resulted in an additional intron in the *ndhF* gene. The *ycf1* gene was located near the SSC/IRa junction, differing only in its distance from the junction among species.

The 8 *Murraya* CP genomes exhibited highly similar sequences. As observed in other angiosperms, the IR regions were more conserved than the LSC and SSC regions, and coding regions were more conserved than noncoding regions. Highly divergent regions were mainly located in intergenic spacers and introns, including *trnH-GUG-psbA*, *petN-psbM*, *rpl32-trnL-UAG*, and *ccsA-ndhD*. Additionally, several genes contained variable regions, such as *matK*, *rps3*, *ndhF*, *ccsA*, and *ycf1* (Fig. 5). Collinearity analysis of the CP genomes, using *M. exotica* as the reference, revealed no obvious rearrangements, indicating that the *Murraya* CP genomes are relatively conserved (Supplemental Fig. S4, https://links.lww.com/STCM/A68).

3.4. Selection pressure analysis

The ratio (ω) of dN to dS substitutions (dN/dS) was calculated for all 78 shared protein-coding genes across 33 complete *Murraya* CP genomes. Using the M8 (β and ω > 1) model, 10 protein-coding genes were identified as being under positive selection, with PP values greater than 0.95 (Table 1). Among these genes, *rbcL* showed the highest number of positively selected amino acid sites (4), followed by *psaJ* (3), *ndhD* (2), *ndhF* (2), *rpl2* (2), *rpl20* (2), *ycf1* (2), *accD* (1), *ccsA* (1), and *rpl32* (1) (Table 1).

3.5. Phylogenetic analysis

Six phylogenetic trees were constructed using ML and BI methods based on complete CP genomes, 78 shared protein-coding genes, and 109 shared NCSs. The topologies of these trees were highly similar, with strong bootstrap support and PP values (Fig. 6; Fig. S5–S7, https://links.lww.com/STCM/A68). The 33 *Murraya* samples were divided into 2 main groups. Three species from sect. *Murraya* (*M. exotica*, *M. paniculata*, and *M. alata*) clustered together, whereas 5 species from sect. *Bergera* (*M. euchrestifolia*, *M. kwangsiensis*, *M. kwangsiensis* var. *macrophylla*, *M. microphylla*, and *M. koenigii*) formed a separate group. Within the sect. *Murraya* group, different populations of *M. exotica* and *M. paniculata* each formed monophyletic clades, with *M. exotica* being the sister lineage to *M. alata*. However, the genus *Murraya* was found to be nonmonophyletic, which is inconsistent with traditional taxonomy. The sect. *Murraya* group showed a closer relationship to *M. caloxylon*, *C. maxima*, and *C. medica*, while the sect. *Bergera* group was more closely related to *C. excavata*.

3.6. Divergence time estimation

The divergence between Aurantieae and Clauseneae was estimated at approximately 35.48 Mya (95% HPD: 30.17–39.82 Mya). The split between *Bergera* and *Clausena* occurred around 31.40 Mya (95% HPD: 25.55–36.67 Mya), while

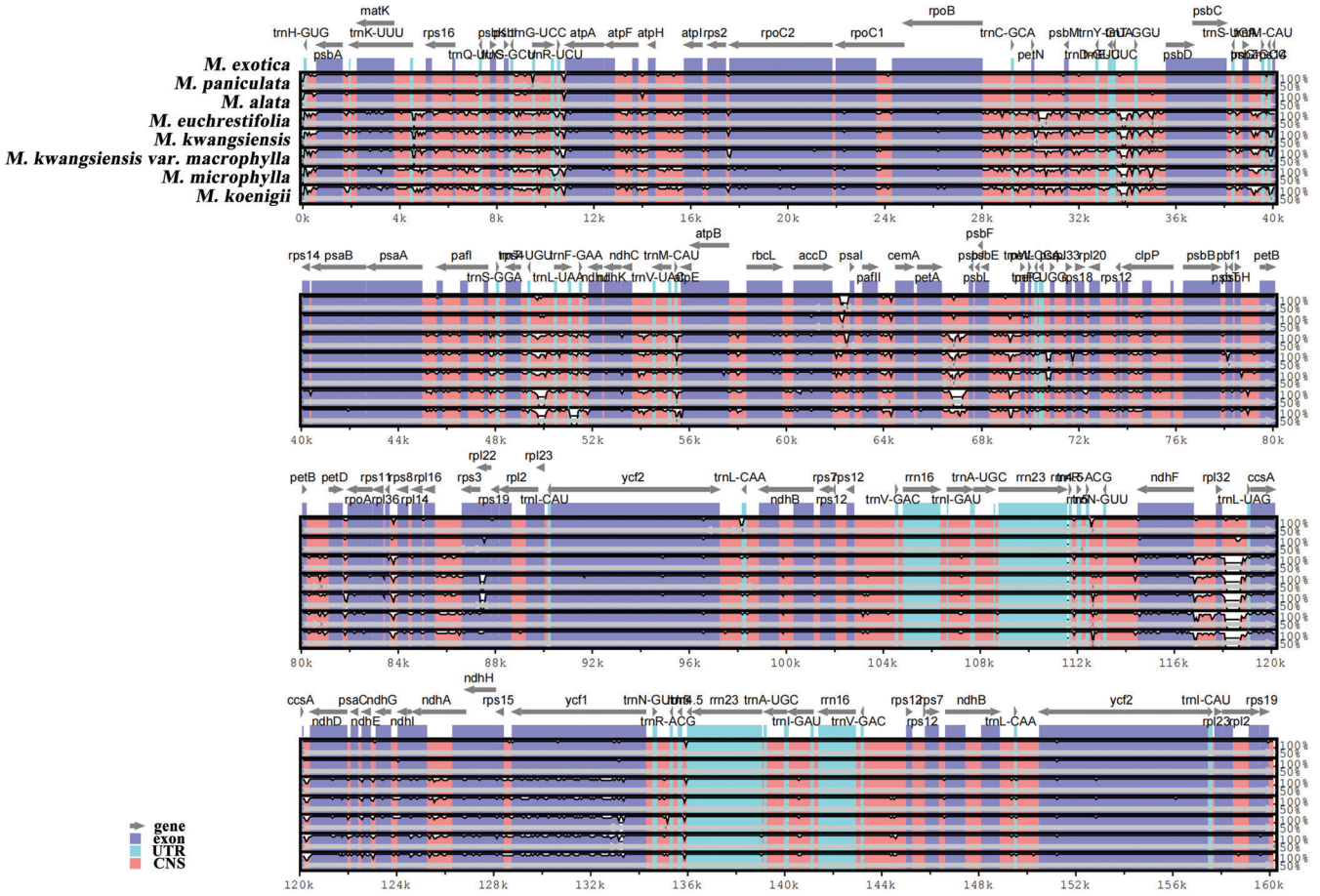

Figure 5. Comparison of *Murraya* chloroplast genomes using mVISTA. Different genetic regions are highlighted in different colors.

species within sect. *Bergera* diverged approximately 21.92 Mya (95% HPD: 15.57–28.15 Mya). Diversification of sect. *Murraya* species and *M. caloxylon* was estimated at 16.13 Mya (95% HPD: 9.55–23.43 Mya). The common ancestor of sect. *Murraya* species diverged around 9.11 Mya (95% HPD: 4.90–13.87 Mya), with the most recent divergence occurring between *M. exotica* and *M. alata* at approximately 5.85 Mya (95% HPD: 2.88–9.47 Mya) (Supplemental Fig. S8, https://links.lww.com/STCM/A68).

3.7. Molecular marker development

To develop a high-resolution molecular marker for distinguishing *M. exotica* and *M. paniculata*, the *ycf1* region was selected, and specific primers were designed targeting conserved sequences (forward primer: 5'-TTTTCTATCTACCCTTACTATTCCA-3'; reverse primer: 5'-GTGGTACTAATCTAGCCCATTTA-3'). To verify the marker's effectiveness at the species level, the primers were used to amplify the *ycf1* region in 6 taxa, including *M. exotica*, *M. paniculata*, *M. alata*, *M. euchrestifolia*, *M.*

Table 1

Positively selected sites detected in *Murraya* chloroplast genomes.

Gene	Ln L	Estimates of parameters	Positive sites
accD	−2243.910390	*p*0 = 0.98499 *p* = 12.91472 *q* = *99.00000* (*p*1 = 0.01501) ω = 9.55667	364 Q 0.977*
ccsA	−1590.450939	*p*0 = 0.99165 *p* = 0.17805 *q* = *0.59846* (*p*1 = 0.00835) ω = 10.13822	178 F 0.995**
ndhD	−2418.152808	*p*0 = 0.99431 *p* = 0.68227 *q* = 1.93972 (*p*1 = 0.00569) ω= 23.00285	409 K 0.986*,499 C 1.000**
ndhF	−3812.591805	*p*0 = 0.99655 *p* = 0.03163 *q* = 0.13975 (*p*1 = 0.00345) ω = 31.31655	491 I 0.999**,737 L 0.996**
psaJ	−199.349805	*p*0 = 0.92680 *p* = 0.00500 *q* = 1.84748 (*p*1 = 0.07320) ω = 999.00000	10 V 0.971*,43 S 0.999**, 44 F 0.972*
rbcL	−2216.349019	*p*0 = 0.93180 *p* = 0.00500 *q* = 2.08469 (*p*1 = 0.06820) ω = 6.27225	219 L 0.967*,461 V 0.963*, 464 E 0.972*,475 L 0.956*
rpl2	−1112.971593	*p*0 = 0.00001 *p* = 3.58318 *q* =0 .00500 (*p*1 = 0.99999) ω = 999.00000	69 K 0.962*,268 L 0.962*
rpl20	−525.714912	*p*0 = 0.87971 *p* = 0.00500 *q* = 2.13535 (*p*1 = 0.12029) ω = 23.96216	73 L 0.975*,76 Y 0.974*
rpl32	−250.281233	*p*0 = 0.97978 *p* = 9.85426 *q* = 99.00000 (*p*1 = 0.02022) ω = 278.94196	48 C 0.999**
ycf1	−9106.672762	*p*0 = 0.97385 *p* = 87.73808 *q* = 99.00000 (*p*1 = 0.02615) ω = 8.65343	461 L 0.957*,1155 L 0.966*

The number of parameters (np) for each gene was 68; *and **indicate posterior probability higher than 0.95 and 0.99, respectively.

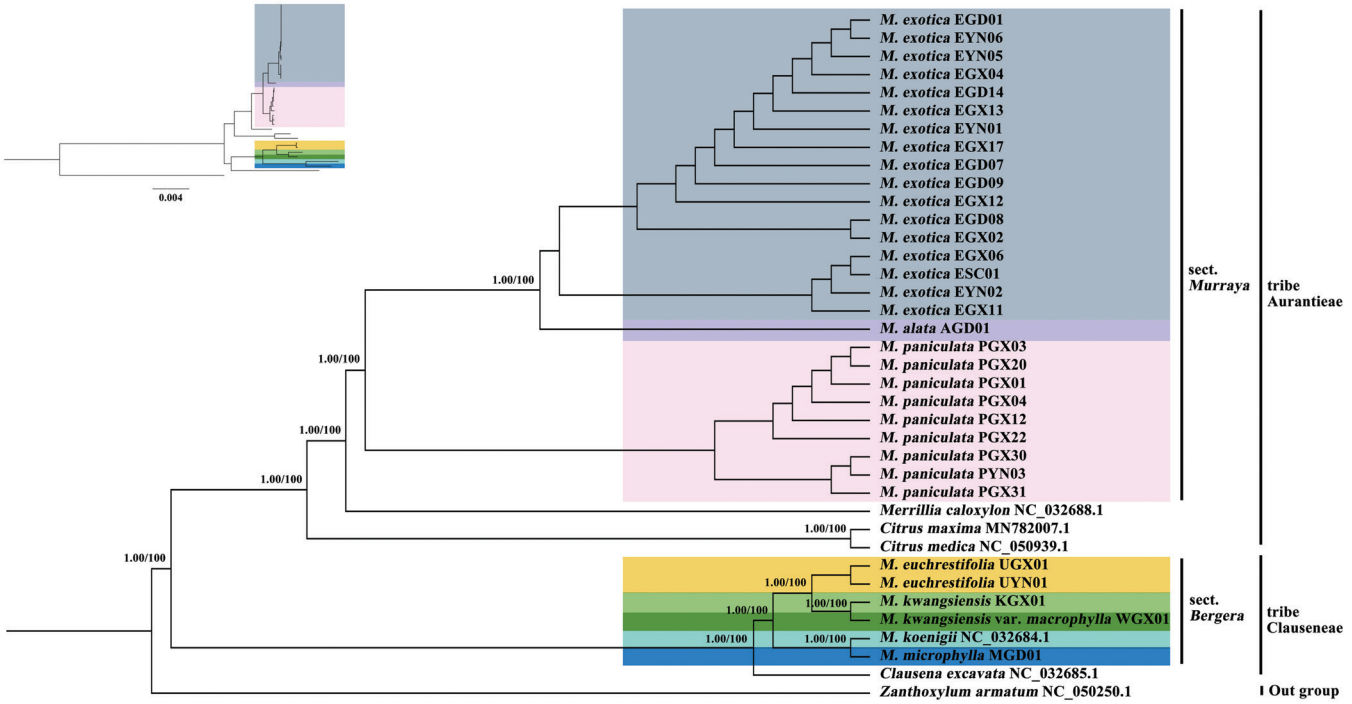

Figure 6. Phylogenetic tree constructed using maximum likelihood (ML) and Bayesian inference (BI) methods based on *Murraya* chloroplast genomes. Bootstrap support (BS) and posterior probability (PP) values are shown above the branches.

kwangsiensis var. *macrophylla*, and *M. microphylla*. The PCR products were approximately 450 bp and shared ≥90% sequence identity with their original sequences (Fig. 7; Supplemental Fig. S9, https://links.lww.com/STCM/A68). One species-specific single-nucleotide polymorphism (SNP) was identified in *M. exotica*, and 2 SNPs were identified in *M. paniculata*. These SNPs provide a reliable molecular basis for the identification of *M. exotica* and *M. paniculata*.

4. Discussion

4.1. Variations among the Murraya species

In this study, we assembled and analyzed the CP genomes of *Murraya* species. As in most angiosperms, all *Murraya* CP genomes exhibited a typical quadripartite circular structure, consisting of 2 SC regions and 2 IR regions.[44] Comparison of the 33 CP genomes revealed that their structures were relatively conserved, with only minor differences in genome size, composition, and gene order. The previously reported total length of *M. koenigii* was 159,402 bp,[45] while the CP genomes assembled in this study ranged from 158,573 to 160,817 bp, consistent with that of *M. koenigii*. Gene composition and arrangement are often related to evolutionary processes. All *Murraya* CP genomes contained 112 unique genes, including 78 protein-coding genes, 30 tRNA genes, and 4 rRNA genes. Similar to other angiosperms, *chlB*, *chlL*, *chlN*, and *trnP-GGG* were absent in the CP genomes of *Murraya*.[46] Additionally, the *infA* gene, a translation initiation factor, was lost in *Murraya*, as reported in some other Rutaceae species such as *C. sinensis*[47] and *Z. bungeanum*,[48] although it remains present in *Camellia japonica*.[49] This suggests that the loss of *infA* may have occurred independently within Rutaceae. Although the loss of *ndh* genes is widely reported in terrestrial plant CP genomes,[50,51] the *Murraya* CP genomes retained 10 complete *ndh* genes, except for a pseudogenized *ndhD* in some samples. Further investigation into the mechanisms and functional consequences of gene loss, pseudogenization, or transfer could provide valuable insights into the evolution of *Murraya* and the Rutaceae family.

Long repeat sequences and SSRs are widely distributed in CP genomes and can contribute to genome variation and rearrangement, significantly influencing species evolution.[52,53] These features have been extensively applied in population genetics and phylogenetic studies.[54,55] In the *Murraya* CP genomes, 4 types of long repeats were detected, including forward, reverse, complement, and palindromic repeats. Among these, palindromic repeats were the most abundant, and the majority of repeats were 30–39 bp in length, consistent with reports from other land plant CP genomes.[56,57] The types and distribution of SSRs were similar across *Murraya species*. A/T mononucleotide repeats were the most common, and other SSR types also exhibited high A/T content, reflecting the generally high A/T composition of CP genomes in most plants.[58] The abundance of SSRs varied among genomic regions, but, as in other Rutaceae species,[49] most were located in the LSC region. These long repeats and SSRs provide valuable information for assessing genetic diversity, evolutionary patterns, and phylogenetic relationships in *Murraya*, particularly for *M. exotica* and *M. paniculata*.

4.2. Expansion and contraction of IR regions in the Murraya species

Contraction and expansion of the IR regions in angiosperm CP genomes are common evolutionary events.[59,60] Even closely related species often exhibit slight differences in IR boundaries, and such contraction or expansion is an important factor affecting CP genome size.[61] In this study, the IR/SC boundaries of 8 *Murraya* species were analyzed. The results showed that, except for *M. kwangsiensis* var. *macrophylla*, the other 7 *Murraya* species had highly similar gene distributions at the IR/SC boundaries, resembling those of *C. maxima*. Significant differences were observed at the LSC/IR boundary of *M. kwangsiensis* var. *macrophylla*, where *rps19* and *rpl22* were entirely located in the LSC, and one copy of *rps19* was lost, a pattern similar to that reported in *Duabanga grandiflora*.[62] Additionally, the *ycf1* gene extended further into

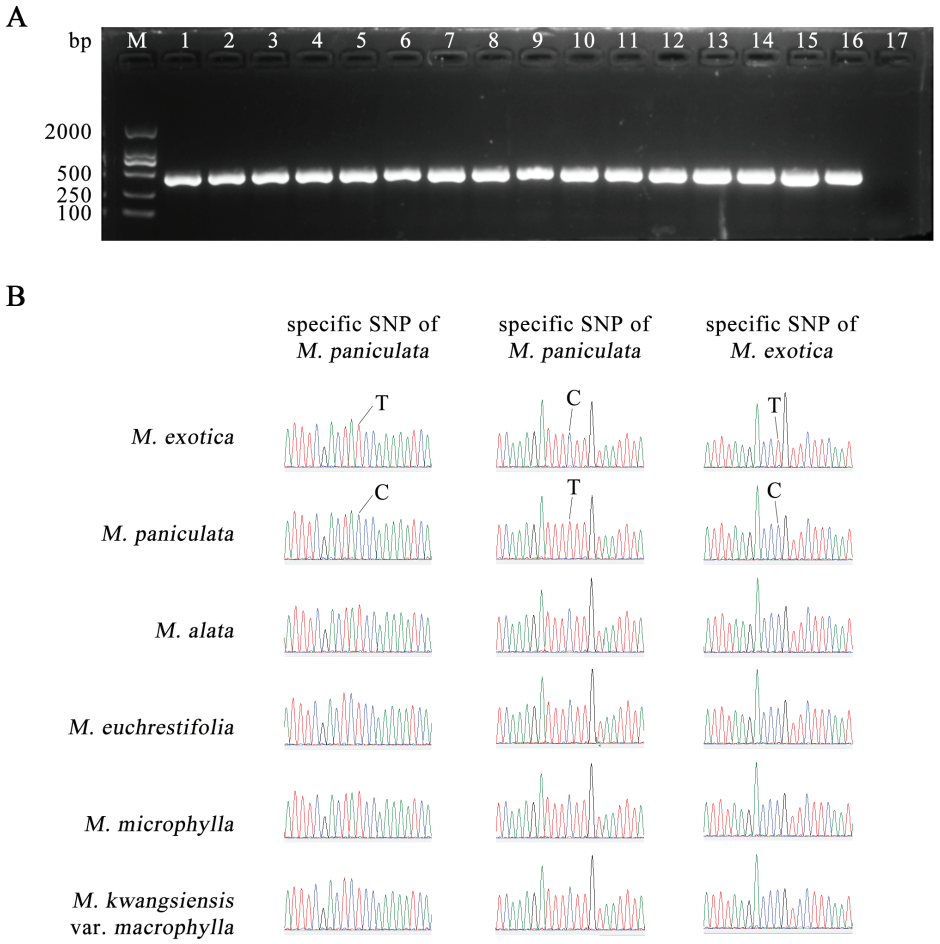

Figure 7. Validation of molecular markers derived from the *ycf1* sequence of *Murraya*. (A) Amplification of molecular markers; M. 2000 bp DNA marker; lanes 1–6, *M. exotica*; 7–12, *M. paniculata*; 13, *M. alata*; 14, *M. euchrestifolia*; 15, *M. microphylla*; 16, *M. kwangsiensis* var. *macrophylla*; 17, dH_2O; (B) SNP sites effective for distinguishing *M. exotica* and *M. paniculata*.

the IR region. In most monocots (except Alismatales and a few Acorales), the *trnH-rps19* cluster is partially or completely located in the IR regions, whereas in dicots it is typically situated in the LSC region.[63] Interestingly, in Elaeagnaceae (eg, *Elaeagnus* and *Shephedia*) and Rhamnaceae (*Rhamnus*), duplication of the *trnH* gene has been reported.[64] In contrast to other *Murraya* species, *M. kwangsiensis* contains 2 copies of the *trnH* gene in the LSC region, positioned 5–6 bp away from the IR region. Further studies are warranted to elucidate the evolutionary significance of IR region contraction and expansion in *Murraya*.

4.3. DNA markers from the comparative analysis

Comparative analysis of the CP genomes showed that the *Murraya* species had highly similar genomes with conserved structures and no obvious structural rearrangements. As in most angiosperms, the IR regions were more conserved than the SC regions,[65] while the SSC regions exhibited the highest sequence divergence among the *Murraya* species. Moreover, coding regions were more conserved than noncoding regions.[66] Sequence divergence analysis indicated that the 8 *Murraya* species could be divided into 2 groups, consistent with the traditional classification. *M. paniculata* and *M. alata* showed higher sequence similarity to *M. exotica*, whereas *M. euchrestifolia*, *M. kwangsiensis*, *M. kwangsiensis* var. *macrophylla*, *M. microphylla*, and *M. koenigii* exhibited higher divergence relative to *M. exotica*. Several highly divergent regions were identified within the *Murraya* CP genomes. In particular, the intergenic region *trnH-psbA* showed a high degree of divergence, consistent with its reported utility as a candidate DNA barcode in grasses and legumes from temperate sub-alpine grasslands.[67] Additionally, the coding region *ycf1* exhibited substantial variation. For land plants, *ycf1* has been recognized as one of the most promising plastid DNA barcodes, generally outperforming *matK*, *rbcL*, and *trnH-psbA* at the species level.[68] In this study, *ycf1* was demonstrated to be effective for the accurate identification of *M. exotica* and *M. paniculata*, both of which are used for anti-inflammatory purposes. Several specific SNPs were also observed in other *Murraya* species, though additional samples are required for further validation. The molecular marker developed here will facilitate the identification of *M. exotica* and *M. paniculata*, supporting the efficient utilization and conservation of wild resources. Future research on other highly variable regions may yield additional DNA markers for *Murraya* identification and phylogenetic studies.

4.4. Adaptive evolution of Murraya species

The ratio ($\omega = dN/dS$) is widely used to assess selective pressure,[69] where $\omega > 1$, $\omega = 1$, and $\omega < 1$ indicate positive, neutral, and purifying selection, respectively. In this study, most protein-coding genes of *Murraya* had ω values below 1, indicating

purifying selection. However, 10 genes were identified as undergoing positive selection. Similar findings have been reported in other angiosperms,[70–72] suggesting that genes under positive selection play crucial roles in CP biogenesis and function. The *accD* gene encodes the β-carboxyl transferase subunit of acetyl-CoA carboxylase, which is involved in maintaining plastid compartments.[73] The *ccsA* gene encodes a c-type cytochrome synthesis protein, functioning in the delivery of heme from the stroma to the lumen and catalyzing heme ligation reactions.[74] *NdhD* and *ndhF* encode subunits of NADH-plastoquinone oxidoreductase, which mediate cyclic electron transport in photosystem I by forming a supercomplex with the photosystem I.[75] The *psaJ* gene encodes a photosystem I subunit necessary for the stability and optimal excitation of photosystem I.[76] The *rbcL* gene encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, which is essential for CO_2 fixation in plants.[77] Notably, *rbcL* contained the highest number of positively selected amino acid sites (4) among the *Murraya* species, indicating a potential role in their adaptive evolution. The *rpl2*, *rpl20*, and *rpl32* genes encode ribosomal proteins, which are essential for maintaining ribosome integrity.[78] Finally, the *ycf1* gene encodes Tic214, a protein crucial for CP protein import in green tissues.[79] Given that *Murraya* species are predominantly distributed in warm, high-light environments, positive selection on these genes may contribute to their adaptation to such conditions.

4.5. Phylogenetic inference and divergence time of the *Murraya* species

CP genomes exhibit highly conserved structure and gene composition, with variable nucleotide substitution rates across different regions, making them highly suitable for phylogenetic analysis at multiple taxonomic levels and providing significant advantages in plant phylogenetic studies.[80] In contrast to traditional classifications of *Murraya*, the monophyletic groups of sect. *Murraya* and sect. *Bergera* did not cluster into a single clade. Consistent with previous molecular studies, sect. *Murraya* was monophyletic and sister to *M. caloxylon*, whereas sect. *Bergera* was monophyletic and sister to *C. excavata*.[18–20,81] Phylogenetic analysis based on the pangenome also supported the close relationship between sect. *Bergera* and *Clausena*.[82] This taxonomic arrangement is further supported by chemotaxonomic evidence.[83,84] Sect. *Murraya* and sect. *Bergera* produce distinct alkaloids, namely, yuehchukene in one group and girinimbine in the other, and differ in volatile oil composition. Notably, yuehchukene has been isolated from the stem and root barks of *M. caloxylon*, reinforcing the close relationship between *M. caloxylon* and sect. *Murraya*.[85] Collectively, evidence from morphotaxonomy, chemotaxonomy, and molecular phylogeny supports a generic-level division within *Murraya* rather than a sectional classification.[86] Accordingly, *Merrillia* and sect. *Murraya* should be placed in the tribe Aurantieae, while sect. *Bergera* should be assigned to the tribe Clauseneae. Species including *M. euchrestifolia*, *M. kwangsiensis*, *M. kwangsiensis* var. *macrophylla*, *M. microphylla*, and *M. koenigii* should be transferred to *Bergera*. Furthermore, the phylogenetic trees confirmed that *M. exotica* and *M. paniculata* are distinct taxa,[21] with *M. exotica* showing a closer relationship to *M. alata* than to *M. paniculata*. These results provide robust phylogenetic evidence supporting the recognition of *M. exotica* and *M. paniculata* as independent species and contribute to resolving longstanding taxonomic confusion within the genus. However, the CP genome and nuclear genome data for *Murraya* and other Rutaceae species remain limited. Additional genomic data will be necessary to further elucidate the evolution and phylogenetic relationships within *Murraya* and the broader Rutaceae family.

Based on the complete CP genome, we estimated the divergence times of *Murraya* species. The most recent common ancestor of sect. *Murraya* and *Bergera* was estimated to have existed approximately 35.48 Mya (95% HPD: 30.17–39.82 Mya), which is earlier than the 22.76 Mya estimated by Shi et al.[87] In contrast, Nguyen et al., using ITS sequences of Rutaceae, estimated the divergence of sect. *Murraya* and *Bergera* at 66.9 Mya.[19] These discrepancies likely reflect differences in molecular data selection, taxon sampling, fossil calibrations, and analytical methods. Yang et al., using 4995 SC gene families, estimated the diversification of *M. paniculata* and other Aurantieae species to be approximately 25 Mya,[88] which is close to our estimate of 28.01 Mya (95% HPD: 21.00–34.70 Mya). Our estimate for the divergence of *M. exotica* and *M. paniculata* (9.11 Mya, 95% HPD: 4.90–13.87 Mya) was also consistent with the previous estimate of 5.86 Mya.[87] These divergence time estimations based on complete CP genomes provide valuable insights into the evolutionary history of *Murraya*. Nevertheless, further validation using whole-genome data is warranted.

5. Conclusion

In this study, we analyzed the complete CP genomes of 33 samples representing 8 *Murraya* species, 5 of which were reported for the first time. The CP genomes are highly conserved, exhibiting the typical quadripartite structure with similar genome size, gene content, and gene order. We identified long repeat sequences and SSRs, and observed that contraction and expansion of the IR regions were associated with genome size, gene duplication, and intron occurrence. Comparative analyses revealed highly variable regions, including *ndhI–ndhA*, *trnH-GUG-psbA*, *rpl32-trnL*, *matK*, and *ycf1*. Ten protein-coding genes (*rbcL*, *psaJ*, *ndhD*, *ndhF*, *rpl2*, *rpl20*, *ycf1*, *accD*, *ccsA*, and *rpl32*) showed evidence of positive selection. Phylogenetic analyses and divergence time estimations based on CP genomes supported the classification of *M. exotica* and *M. paniculata* as 2 distinct species. Additionally, a molecular marker based on *ycf1* was developed for the accurate identification of these 2 species.

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Statement of ethics

None declared.

Conflict of interest statement

The authors declare that they have no conflicts of interest with regard to the content of this report.

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Data availability

The data that support the findings of this study are available upon request and are also openly accessible in the Chloroplast

Genome Information Resource at https://ngdc.cncb.ac.cn/cgir/ (accessed 10 February 2023). Accession numbers for all sequences are provided in the supplementary materials.

Author contributions

Ziyuan Chen: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data Curation, Writing—Original Draft, Visualization. Yan Jin: Resources. Yuyang Zhao: Resources, Project administration. Chao Jiang: Conceptualization, Methodology, Data Curation, Writing—Review & Editing, Visualization, Project administration. Yuan Yuan: Conceptualization, Writing—Review & Editing, Supervision, Project administration, Funding acquisition.

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