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RESEARCH ARTICLE OPEN ACCESS

DNA Barcoding Reveals Cryptic Diversity and Informs Conservation Priorities in Chinese *Firmiana* Species (Malvaceae) Using Genome Skimming Data

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Received: 9 June 2024 | **Accepted:** 21 July 2025

Editor-in-Chief: Binbin Li | **Handling Editor:** Ahimsa Campos-Arceiz

Funding: This study was supported by Natural Science Foundation of Yunnan (202201AT070232, 202401AT070224 and 202201AS070049), Natural Science Foundation of Hainan (ZDYF2023RDYL01), Hainan Institute of National Park Program (KY-24ZK02), the 14th Five-Year Plan of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (XTBG-1450303/E3ZKFF8B01), the Key Program of Yunnan, China (202101BC070003), Ministry of Science and Technology of China Basic Resource Investigation Program (2021FY100200), Yunnan Revitalization Talent Support Program "Young Talent" and "Innovation Team" Projects, and Ecological and Environmental Conservation Program from the Department of Ecology and Environment of Yunnan Province.

Keywords: conservation | DNA barcode | identification | phylogeny | plastome

ABSTRACT

Accurate species delineation is crucial for biodiversity conservation. The genus *Firmiana* Marsili (Malvaceae) comprises deciduous trees and shrubs, many of which are rare and endangered in China, underscoring the urgent need for effective conservation measures. However, morphological similarities among closely related *Firmiana* species complicate taxonomic identification, and traditional morphology-based approaches are often insufficient for taxa with complicated evolutionary histories. To address this, we conducted genome skimming on 62 *Firmiana* samples representing all 10 recognized Chinese species and two unidentified taxa, assembling plastome and nuclear ribosomal DNA sequences. We evaluated the effectiveness of super-barcodes (plastid genomes and nrDNA), plastid hyper-variable barcodes, and four universal barcodes. Our results show that nrITS exhibited the highest discriminatory power, successfully identifying all 10 recognized *Firmiana* species, and is thus recommended as the primary barcode for the genus. In addition, two cryptic lineages were discovered within the *Firmiana major* complex. These results provide critical insights for the conservation, management, and sustainable use of endangered *Firmiana* species. This study underscores the urgent need to revise species boundaries within the *F. major* complex, and highlights the potential of DNA barcoding as an efficient tool for species identification and conservation of the genus *Firmiana*.

[Corrections added on 26 September 2025, after first online publication: In Affiliation 9, "Chinese Academy of Scienc" was corrected to "Chinese Academy of Sciences." In Section 2.1, "Illumina Novoseq. 6000 platform" was corrected to "Illumina Novaseq 6000 platform." In the References, the follow corrections were made: (1) "Frimiana kerrii" was corrected to "Firmiana kerrii"; (2) "Firminia major" was corrected to "Firmiana major"; (3) in Ya et al. (2018), "(Vol 10, pg 713, 2018)" was removed and the page range was corrected from "915–915" to "713–715.".]

Summary

Protecting endangered tree species requires being able to tell them apart accurately—something that is not always possible when species look very similar. In China, the genus *Firmiana*, which includes several rare and threatened trees, presents this exact challenge. To address it, we analyzed DNA from 62 samples representing all 10 recognized Chinese *Firmiana* species and two unidentified groups. We tested a range of genetic markers, including complete chloroplast DNA sequences and nuclear DNA regions. We found that one nuclear marker, called nrITS, could perfectly distinguish all 10 species. We also discovered two previously unknown, genetically distinct groups within what was thought to be a single species, *F. major*. These discoveries will enable more accurate species identification and guide more effective conservation planning. Our study highlights DNA barcoding as a reliable and efficient method for identifying and conserving rare and endangered plants such as *Firmiana*.

- **Practitioner Points**
  - NrITS is recommended as a reliable and efficient DNA barcode for accurate identification of *Firmiana* species in taxonomic and conservation work.
  - The discovery of cryptic taxa within the *Firmiana major* complex highlights the need to re-evaluate species boundaries and update classification systems.
  - Genetic diversity and threat-status assessments for the cryptic lineages within the *F. major* complex require further investigation, and the national conservation list of *Firmiana* species in China should be revised accordingly.

1 | Introduction

Species are the fundamental units of study in life sciences and provide the basis for addressing questions in biogeography, ecology, evolutionary biology, systematics, and conservation biology (Sutherland et al. 2013; Reydon 2019). Precise species delineation is therefore essential for biodiversity conservation (Agapow et al. 2004; Hong 2016; Reydon 2019; Allendorf et al. 2022). While traditional morphology-based taxonomy remains valuable in fieldwork and herbarium studies, it is often insufficient for identifying species with complex evolutionary histories and mechanisms. Moreover, it relies heavily on the expertise of taxonomists and the availability of diagnostic characters, which are often limited to specific life stages or sexes (Hebert et al. 2003; Kress et al. 2015; Hollingsworth et al. 2016). Trees, with their long life histories, frequently take years or decades to bear flowers or fruits—traits typically required for morphological identification. This delay hinders accurate identification, use, and conservation. Over the past two decades, however, the advent of molecular identification approaches, particularly DNA barcoding, has filled this gap by facilitating the identification of sterile woody plants (Hollingsworth et al. 2016; Kress 2017; Le et al. 2020; Hu et al. 2022). In particular, low-coverage whole genome sequencing, or genome skimming, using next-generation sequencing (NGS) technology, can obtain organellar genomes and nuclear ribosomal DNA (nrDNA) sequences. These data improve species delimitation and provide complementary insights alongside standard barcodes (Straub et al. 2012; Coissac et al. 2016; Hollingsworth et al. 2016; Chen, Yin, et al. 2023).

Firmiana Marsili (Malvaceae) comprises 18 recognized species of deciduous trees and shrubs, distributed from continental Asia throughout Southeast Asia to New Guinea and Fiji (POWO. 2024). The taxonomy of *Firmiana* has long been controversial. Based on floral morphology, flowering phenology (flowering before or after leaf emergence), and seed number per carpel, the genus was initially divided into two genera: *Firmiana* and *Erythropsis* Lindley ex Schott & Endlicher (Ridley 1934; Xu and Xu 2000). However, Kostermans (1954) argued that variation in floral traits and seed number is common in Sterculiaceae, and that these features alone were insufficient to warrant recognition of *Erythropsis* as a separate genus. Consequently, *Erythropsis* has since been subsumed under *Firmiana* (Tang et al. 2007). Recent phylogenetic studies of *Firmiana* (Abdullah et al. 2019; Lu et al. 2019; Lu, Huang, et al. 2021; Lu and Luo 2022; Li et al. 2024) divide the genus into two clades: one containing species previously classified under *Erythropsis* (e.g., *Firmiana colorata*, *Firmiana pulcherrima*, *Firmiana kwangsiensis*) and another comprising traditional *Firmiana* species (e.g., *Firmiana hainanensis*, *Firmiana simplex*, *Firmiana major*, *Firmiana danxiaensis*). The relationship between the pantropical genus *Hildegardia* and *Firmiana* also remains unresolved. Phylogenetic analyses of plastid DNA regions indicate that two *Hildegardia* species are nested within *Firmiana* (Wilkie et al. 2006; Li et al. 2022). In the absence of comprehensive species sampling for phylogenomic analyses, *Hildegardia* continues to be treated as a distinct genus, while *Erythropsis* remains in synonymy with *Firmiana* (Tang et al. 2007; Li 2018, 2020; POWO 2024). In this study, we follow the treatment of *Firmiana*, including *Erythropsis*, as adopted in the *Flora of China* (FOC) and *Plants of the World Online* (POWO).

To date, 10 species of *Firmiana* are recognized in China (Figure 1, Table S1) (Huang et al. 2011; Tang et al. 2007; Wang et al. 2019; Zhang, Cai, et al. 2020). Among them, *Firmiana simplex* is widely cultivated, but no reliable evidence of extant wild populations has been reported. In addition, *F. colorata* and *Firmiana kerrii* are distributed in Southeast Asia. The remaining seven species have restricted ranges in southern and southwestern China, with most being considered rare and endangered (Huang et al. 2011; Tang et al. 2007; Wang et al. 2019; Zhang, Cai, et al. 2020). Within the genus, *F. kwangsiensis* is listed as a first-class protected species in the *List of National Key Protected Wild Plants* (NKPWP) in China, while the other species (excluding *F. simplex*) are classified as second-class protected species (Lu, Qin, et al. 2021). In the 2004 *China Species Red List*, five species were assessed as Threatened (CR: *F. danxiaensis* and *F. kwangsiensis*; EN: *F. major* and *F. pulcherrima*; VU: *F. hainanensis*) (Wang and Xie 2004). In the more recent *Red List of Chinese Biodiversity: Higher Plants*, five species were also categorized as Threatened (CR: *Firmiana calcarea*; EN: *F. kwangsiensis*, *F. major*, and *F. pulcherrima*; VU: *F. hainanensis*) (Ministry of Ecology and Environment of the People's Republic of China, & Chinese Academy of Sciences 2013).

In 1998, *Firmiana major* was assessed as "Extinct in the Wild" by the *IUCN Red List of Threatened Species* (Sun 1998). However, a wild population was rediscovered in 2001 at the National Nature

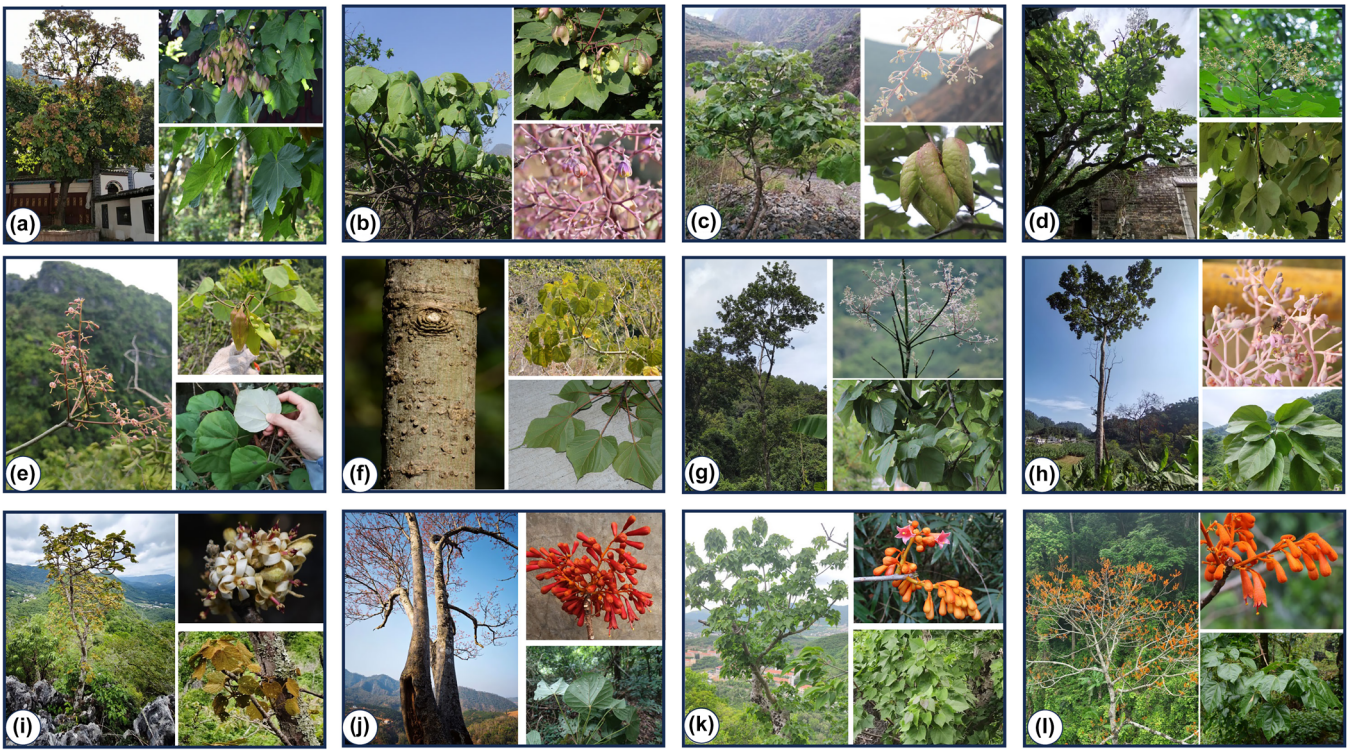

FIGURE 1 | Color photographs of all 12 *Firmiana* taxa used in this study. (a) *F.* sp.1; (b) *F.* sp.2; (c) *F. major*; (d) *F. simplex*; (e) *F. calcarea*; (f) *F. danxiaensis*; (g) *F. hainanensis*; (h) *F. daweishanensis*; (i) *F. kerrii*; (j) *F. colorata*; (k) *F. pulcherrima*; (l) *F. kwangsiensis*.

Reserve of *Cycas panzhihuaensis* in Sichuan Province, Southwest China (Wang 2001). More recently, it has been added to the *List of Yunnan Protected Plant Species with Extremely Small Populations* (PSESP) in China (Sun 2021). Extensive field surveys have since documented approximately 25 localities of *F. major* in Southwest China. In these areas, wild populations occur as shrubs or small trees, whereas cultivated populations can reach over 10 m in height (Li, Chen, et al. 2020; Ma et al. 2022; Cha et al. 2023). Genetic analyses of *F. major* using microsatellite loci revealed that three cultivated tree populations differ genetically from eight wild shrub populations, suggesting they may represent distinct genetic lineages (Ma et al. 2022). Moreover, individuals from populations in the upper Jinsha River (e.g., Dadong village, Lijiang) produce larger, nearly indehiscent fruits (Figure 1c) and have densely hairy abaxial leaf surfaces, distinguishing them from populations in the lower reaches of the Jinsha River (e.g., Panzhihua). These morphological and genetic differences suggest that the recently rediscovered wild populations and cultivated plants may represent distinct species. This underscores the urgent need to revise the species boundaries of *F. major* to better inform and support conservation efforts. Furthermore, most *Firmiana* species are rare and regionally endemic, typically confined to ecologically sensitive habitats. Human activities in these regions exacerbate the risk of species extinction (Wang et al. 2019; Li, Chen, et al. 2020; Miu et al. 2020; Zhang, Cai, et al. 2020). To ensure the effective protection of endangered *Firmiana* species, the development of efficient DNA barcodes for rapid and accurate identification across life stages and plant material types is crucial.

In this study, we conduct genome skimming on 62 *Firmiana* samples, representing all recognized Chinese *Firmiana* species, to assemble plastome and nuclear ribosomal DNA (nrDNA) sequences. The main objectives were to: (1) validate species delineation in Chinese *Firmiana* through phylogenetic reconstruction; (2) evaluate the effectiveness of super-barcodes (plastid genome and nrDNA), plastid hyper-variable barcodes, and the universal plant barcodes (*rbcL*, *matK*, *trnH-psbA*, nrITS) (Kress et al. 2005; Hollingsworth et al. 2009; Li, Gao, et al. 2011; Li, Liu, et al. 2011) for species identification; and (3) delimit species boundaries within the *F. major* complex.

2 | Materials and Methods

2.1 | Sampling, DNA Extraction, and Sequencing

Two to ten individuals were collected for each recognized Chinese *Firmiana* species. In total, we sampled 62 *Firmiana* individuals, representing 10 accepted species and two unknown taxa from the *F. major* group, along with *Cola acuminata*, *Pterocymbium javanicum*, and *Scaphium scaphiferum* (subfamily Sterculioideae) as outgroups (Table S1; Figures 1 and 2). Fresh leaves of sampled individuals were collected and dried immediately in silica gel. Total genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987). Approximately 5 μg of purified genomic DNA was used to construct shotgun libraries, and paired-end sequencing was performed on the Illumina NovaSeq 6000 platform (San Diego, CA, United States) using the PE150 mode. Raw reads were filtered to remove adaptors and low-quality reads with the NGS QC Toolkit (Patel and Jain 2012) under default parameters. Approximately 4.0 GB of raw data was generated per sample.

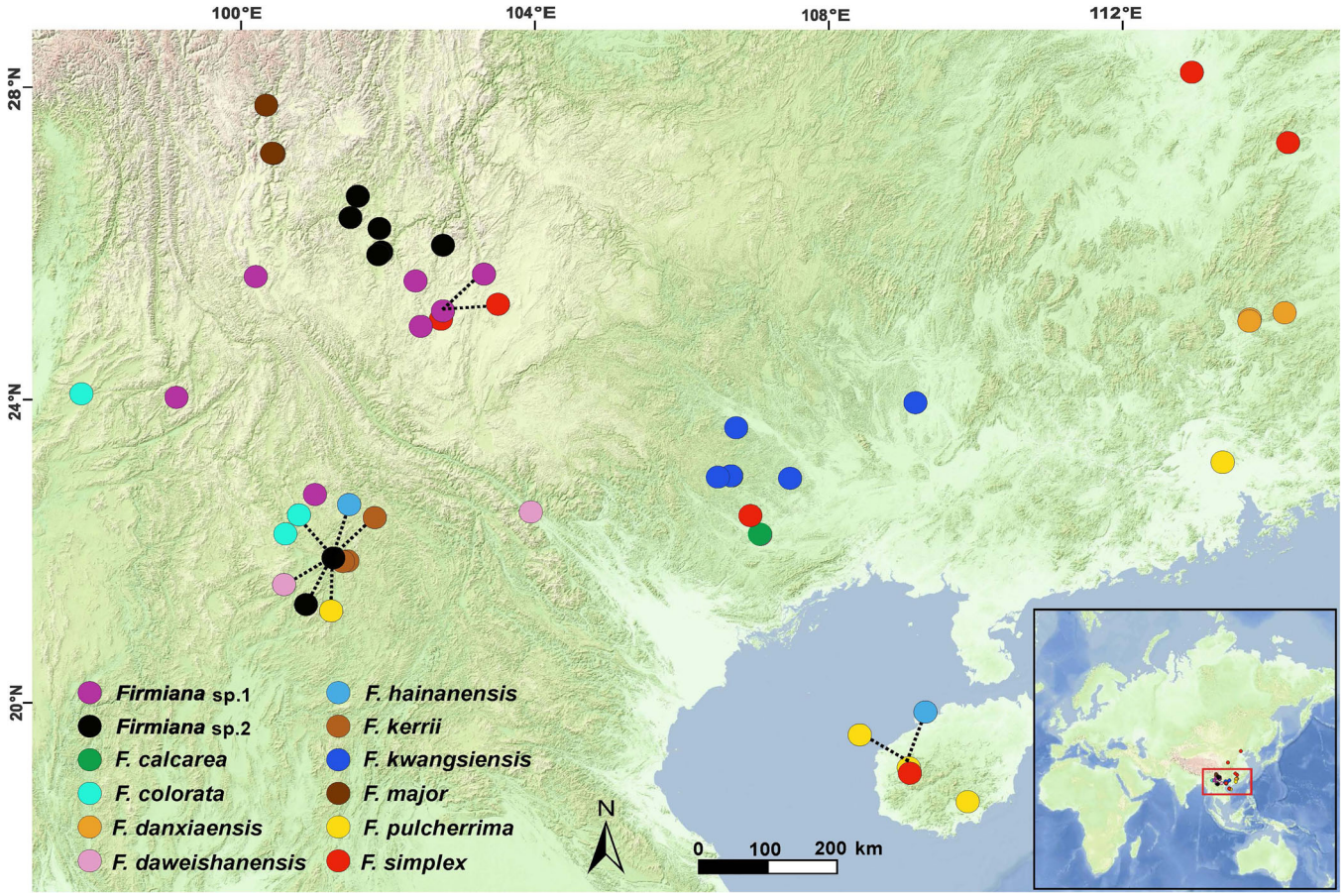

FIGURE 2 | Geographical distribution of all *Firmiana* collection sites used in this study. The two red circles outside the red rectangular box in the inset represent *F. simplex* samples collected from Huaibei town, Beijing (upper circle) and Xieyang, Shanxi Province (lower circle).

2.2 | Plastome and nrDNA Assembly, Annotation, and Comparative Analyses

Whole plastome sequences of all newly sequenced samples were de novo assembled using GetOrganelle v1.7.5 (Jin et al. 2020) with the parameters: "-w 0.65 -R 30 -k 21, 33, 45, 65, 85, 105 -F embplant_pt." A previously published plastome sequence from the *F. major* group (Accession No. NC_037242/MG229069; code: tr2) (Ya et al. 2018) was included in the analyses and served as the reference for gene annotation. All assembled plastomes were annotated and manually corrected in Geneious v8.0.2 (Kearse et al. 2012), with a similarity threshold of 75% set relative to the reference. Nuclear ribosomal DNA (nrDNA) sequences were also assembled using GetOrganelle with the parameters: "-w 0.65 -R 30 -k 21, 33, 45, 65, 85, 105 -F embplant_nr."

All plastomes (with one IR region) and nrDNA sequences were aligned using MAFFT v7.471 (Katoh and Standley 2013). Four universal barcodes, *matK* (M), *rbcL* (R), *trnH-psbA* (T), and nrITS (I), were extracted from the plastome and nrDNA matrices, respectively. To detect boundary variations among *Firmiana* plastomes, the position of each gene at the junctions of the large single-copy (LSC), small single-copy (SSC), and inverted repeat (IR) regions was examined and visualized in Geneious.

2.3 | Hyper-Variable Region Identification

To assess sequence divergence among *Firmiana* plastomes, DNA Sequence Polymorphism (DnaSP) v.5.10.01 (Librado and Rozas 2009) was used to calculate the nucleotide diversity value (π) based on an alignment of all plastomes (with one IR region). The sliding window length was set at 600 bp with a 200 bp step size. The eight regions with the highest π values were identified as hyper-variable regions (Wang et al. 2022). These regions were extracted from the plastome matrix in Geneious and subsequently used for phylogenetic analysis to evaluate species delimitation and discrimination.

2.4 | Phylogenetic Inference for *Firmiana*

To reconstruct phylogenetic relationships among *Firmiana* species, we used three datasets comprising all 62 *Firmiana* individuals and three outgroup species: (1) the whole plastome data matrix with one IR region (WPM), (2) the complete nuclear ribosomal DNA (nrDNA), which includes the small-subunit (SSU) ribosomal RNA (rRNA) gene (18S), the internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, the internal transcribed spacer 2 (ITS2), and the large-subunit (LSU) rRNA gene (28S), and (3) the nuclear ribosomal internal transcribed spacer region (nrITS), which includes ITS1, 5.8S, and ITS2.

Both maximum likelihood (ML) and Bayesian inference (BI) approaches were used for phylogenetic inference. ML phylogenetic trees were constructed using RAxML-HPC 8.2.12 (Stamatakis 2014) under the GTR + Γ substitution model, with 1000 bootstrap replicates to assess support values (MLBS). BI was implemented using MrBayes v3.2.7 (Ronquist et al. 2012), using the best-fit nucleotide substitution model for each data set selected by ModelFinder in PhyloSuite v1.2.2 (Zhang, Gao, et al. 2020). Two parallel Markov Chain Monte Carlo (MCMC) runs were performed for 2,000,000 generations, sampling every 1000 generations. The first 25% of trees were discarded as burn-in, and the remaining trees were used to construct a majority-rule consensus tree with posterior probability values (BIPP). Convergence was confirmed by ensuring that the effective sample size (ESS) of all parameters exceeded 200. All tree files were visualized using the tvBOT online tool (Xie et al. 2023). We considered MLBS values ≥70 as strong support in ML analyses and BIPP values ≥0.95 as strong support in BI analyses (Hillis and Bull 1993; Alfaro 2003; Erixon et al. 2003; Kolaczkowski and Thornton 2007).

2.5 | Species Delimitation via DNA Barcoding Analyses

We evaluated species identification efficiency across three categories of barcodes: super barcodes, hyper-variable regions, and universal barcodes. Six super-barcode datasets (WPM, nrDNA, WPM + nrDNA, IR, SSC, and LSC), eight hyper-variable plastid regions, and 15 universal barcode datasets (four single barcodes and 11 combinations; Table 1) were used to construct ML trees in RAxML, employing the same parameters as described above.

TABLE 1 | Sequence matrix characteristics and species discrimination power of different barcode categories for 12 *Firmiana* taxa (62 samples; outgroups excluded).

Barcodes	DNA regions	Length	No. of variable sites	No. of parsimony informative sites	Proportion of identified taxa
	Plastome + nrDNA	143,671	1925	1679	10/12
Super barcode	nrDNA	5909	137	126	9/12
	Plastome	137,762	1788	1553	9/12
	LSC	91,723	1336	1153	9/12
	SSC	20,336	376	331	7/12
	IR	25,692	75	68	6/12
Universal barcode*	**I**	**787**	**88**	**82**	**11/12**
	I + T	1122	95	87	10/12
	I + M + T	1988	115	103	10/12
	I + M + R + T	2587	117	105	10/12
	I + M	1653	108	98	9/12
	I + R + T	1721	97	89	9/12
	I + M + R	1386	90	84	8/12
	I + R	2252	110	100	7/12
	M + R + T	1800	29	23	2/12
	M + R	1465	22	18	2/12
	T	335	7	5	2/12
	M + T	1201	27	21	1/12
	R + T	934	9	7	1/12
	M	866	20	16	0
	R	599	2	2	0
Hyper-variable regions	*trnG*$^{\mathrm{UCC}}$-*atpA*	1188	33	27	6/12
	ycf1	1001	34	30	5/12
	ndhD-CCSA	643	19	17	3/12
	psaJ-rpl20	1159	28	23	2/12
	psbZ-trnfM$^{\mathrm{CAU}}$	937	19	19	2/12
	rpl32-ndhF	707	24	19	2/12
	trnL$^{\mathrm{UAA}}$-*ndhJ*	627	17	16	1/12
	ycf3-trnS$^{\mathrm{GGA}}$	628	18	17	1/12

Note: *Universal barcode represents four DNA regions, nrITS (I), *matK* (M), *rbcL* (R), and *trnH-psbA* (T).

Species were considered successfully identified when all individuals of a species formed a monophyletic group with MLBS ≥ 70, a conservative threshold for reliable support (Hillis and Bull 1993; Fazekas et al. 2008). Furthermore, to test the accuracy and reliability of published plastomes, we retrieved 48 *Firmiana* plastome sequences from the NCBI database (Table S2). After removing one IR region, we aligned these plastome sequences with our WPM data set and constructed an ML tree. This analysis was used to confirm whether published *Firmiana* plastome sequences clustered within their corresponding species clades.

2.6 | Genetic Distance Estimation and nrITS Haplotype Comparisons for Potential Cryptic Species

Phylogenetic analyses revealed two potential cryptic species within the *Firmiana* major complex: *Firmiana* sp.1 (shrub type) and *Firmiana* sp.2 (tree type). To examine genetic divergence, we calculated intra- and interspecific pairwise genetic distances using the Kimura 2-parameter (K-2P) model, based on WPM and nrITS datasets, in MEGA X (Kumar et al. 2018). To assess whether genetic distances differed significantly between intra- and interspecific comparisons, we applied non-parametric Mann–Whitney tests in the "stats" package of R v4.3.1, with results visualized in the "ggplot2" package (R Core Team 2023).

To further evaluate nuclear variability within the *Firmiana major* complex, we mapped raw genome-skimming reads from all 18 samples of *F. major*, *Firmiana* sp.1, and *Firmiana* sp.2 to the ITS reference sequence of *F. major* (ma1) in Geneious. This mapping-based approach allowed us to detect early-generation hybrids by identifying multilocus heterozygosity.

3 | Results

3.1 | Plastome Characteristics of *Firmiana*

We analyzed 62 complete *Firmiana* plastome sequences (Table S1), including 61 newly generated in this study and one previously published plastome (MG229069/NC_037242; Ya et al. 2018). The plastomes of *Firmiana* spp. were highly conserved in terms of genome structure and gene content. Plastome sizes ranged from 160,509 (*Firmiana colorata* co3) to 161,377 bp (*Firmiana* sp.2 sh8) (Figure S1; Table S1). All plastome sequences displayed the typical quadripartite structure, consisting of a pair of inverted repeat (IR) regions (25,521–25,583 bp) separated by a large single-copy (LSC) region (89,360–90,226 bp) and a small single-copy (SSC) region (19,960–20,087 bp) (Table S1). The overall GC content varied narrowly, from 36.9% to 37.1% (Table S1). Each plastome encoded 114 unique genes, including 80 protein-coding genes, 30 tRNA genes, and four rRNA genes (Figure S1). Seventeen genes were duplicated in the IR regions, comprising six protein-coding, four rRNA, and seven tRNA genes (Figure S1).

The boundaries of the IR/SC regions were highly conserved across *Firmiana* plastomes, with no evidence of significant IR contraction or expansion (Figure S2). The IRa/LSC junction was consistently intersected within the *rps19* gene, with 6 bp of the gene extending into IRa. The *ycf1* gene spanned the Ira/SSC junction, with 36 bp located within IRa. The *ndhF* gene was fully contained within SSC, 135–213 bp away from the SSC/IRb boundary. The distance between $trnN^{GUU}$ and the SSC/IRb junction ranged from 352 to 366 bp across species. Similarly, *rpl2* was located entirely within IRb, positioned 115–190 bp from the IRb/LSC boundary, while $trnH^{GUG}$ of the LSC was located just 2 bp away from this junction (Figure S2).

Nucleotide diversity analysis indicated that *Firmiana* plastomes are highly conserved, with no region exceeding a nucleotide diversity value (π) of 0.02. The eight most variable regions, ranging from 627 to 1188 bp in length and each exceeding $\pi = 0.01$, were selected as hyper-variable regions for subsequent barcoding analyses (Figure 3a ; Table 1).

3.2 | Phylogenetic Relationships of *Firmiana* Species

The WPM alignment (excluding the three outgroup species) was 137,762 bp in length, containing 1788 variable sites and 1553 parsimony-informative sites. The nrDNA alignment was 5909 bp in length, with 137 variable sites and 126 parsimony-informative sites. The nrITS alignment was 787 bp in length, comprising 88 variable sites and 82 parsimony-informative sites (Table 1).

Phylogenetic analyses using WPM and nrITS/nrDNA datasets strongly supported the division of *Firmiana* into two clades (Figures 4 and S3b). Clade I corresponds to the *Erythropsis* group and includes four accepted species: *F. colorata, F. kerrii*, *F. kwangsiensis*, and *F. pulcherrima*. This grouping was strongly supported in both WPM and nrITS datasets (WPM: MLBS/BIPP = 93/1.00; nrITS: MLBS/BIPP = 100/1.00). Clade II corresponds to the *Firmiana* group, comprising six accepted species: *F. calcarea, F. danxiaensis*, *F. daweishanensis*, *F. hainanensis*, *F. major*, and *F. simplex*. It also encompasses two undescribed taxa: *Firmiana* sp.1 and *Firmiana* sp.2. This clade was also strongly supported by both WPM and nrITS datasets (WPM: MLBS/BIPP = 93/1.00; nrITS: MLBS/BIPP = 100/1.00). The nrDNA and nrITS phylogenies yielded identical overall topologies, though the nrITS tree displayed higher bootstrap values for certain nodes that were poorly resolved in the nrDNA phylogeny. In the nrITS-based ML tree, all 10 accepted *Firmiana* species, along with *Firmiana* sp.1, formed well-supported monophyletic groups. However, the WPM tree did not support the monophyly of *F. calcarea*, as one sample was grouped with *F. simplex*. Moreover, *Firmiana* sp.2 did not form a monophyletic group in either the nrITS or WPM phylogenies.

The interspecific relationships within the two clades were incongruent between WPM and nrITS phylogenies (Figure 4). In Clade I, the WPM data set strongly supported *F. colorata* and *F. kerrii* (MLBS/BIPP = 87/1.00) and *F. kwangsiensis* and *F. pulcherrima* (MLBS/BIPP = 89/1.00) as sister groups. By contrast, nrITS resolved *F. kerrii* as the earliest diverging lineage (MLBS/BIPP = 100/1.00), followed by *F. pulcherrima* (MLBS/BIPP = 98/1.00), and then *F. colorata* and *F. kwangsiensis* as sister taxa (MLBS/BIPP = 83/0.97) (Figure 4). In Clade II, WPM strongly supported *F. major* as the earliest diverging lineage (MLBS/BIPP = 93/1.00), whereas nrITS placed *F. calcarea* in that position with full support (MLBS/BIPP = 100/1.00). The interspecific

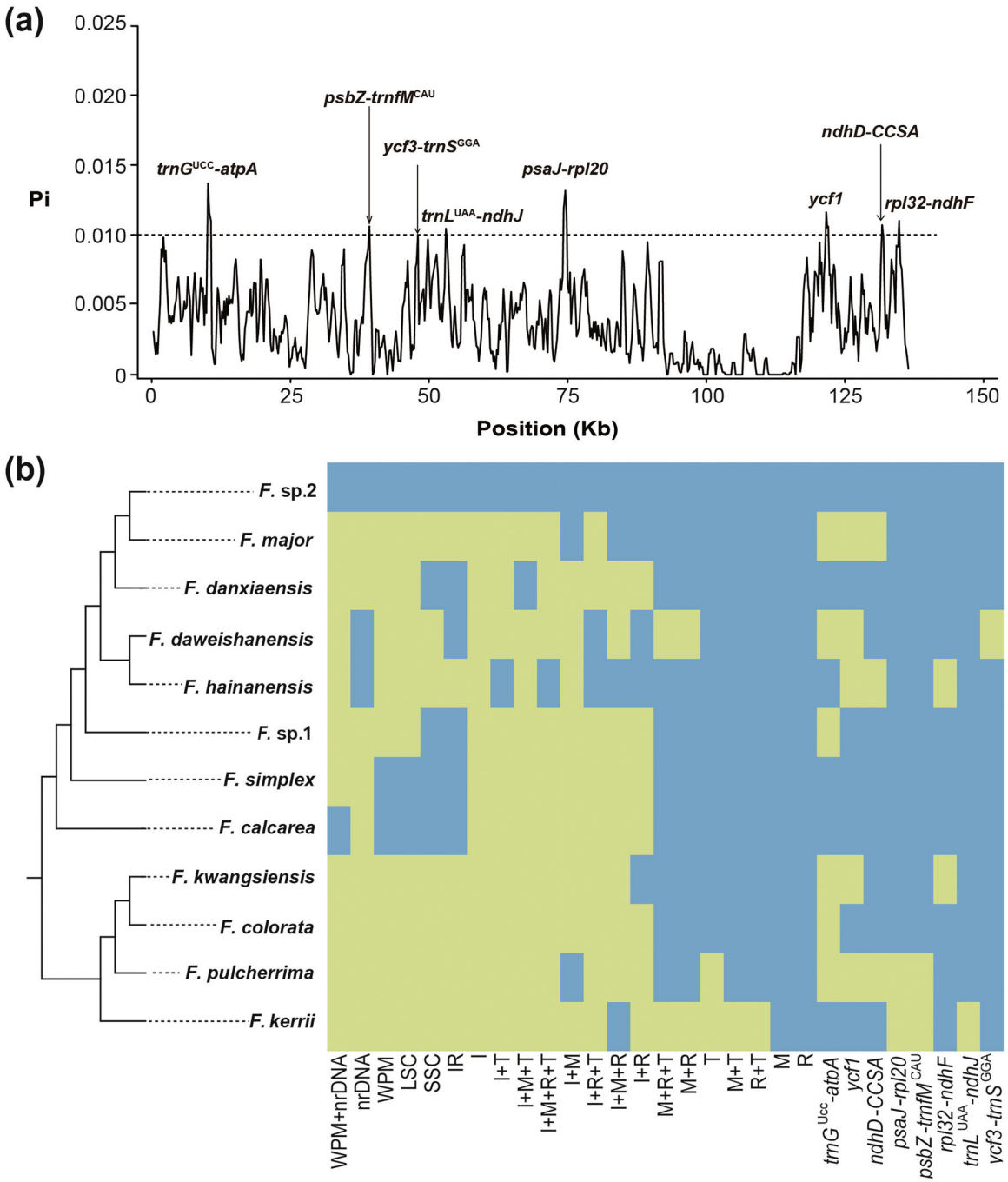

FIGURE 3 | (a) The eight hyper-variable regions identified based on nucleotide diversity of the plastomes; (b) their species identification effectiveness compared with six super-barcodes (WPM + nrDNA, nrDNA, WPM, LSC, SSC, IR), four universal barcodes (nrITS [I], *matK* [M], *rbcL* [R], *trnH-psbA* [T]), and their combinations using the maximum likelihood (ML) method.

relationships among the remaining species in Clade II remained unresolved in both datasets (Figure 4).

3.3 | Performance of the Super-, Hyper-Variable, and Universal Barcodes

The species identification efficiency of six super-barcodes, eight hyper-variable regions, and 15 universal barcodes for all 10 *Firmiana* species is summarized in Table 1 and Figures 3b, S3–S5. Among all the markers, nrITS (I) showed the highest discriminatory power, successfully identifying 11 out of 12 taxa (Figures 3b, S3–S4; Table 1). In comparison, plastid super-barcode (WPM), with or without nrDNA, resolved 9–10 taxa (Figures 3b, S3; Table 1). The eight hyper-variable plastid regions displayed relatively low to moderate discriminatory capacity, ranging from only one species (e.g., *trnL*^{UAA}*-ndhJ*) to six species (i.e., *trnG*^{UCC}*-atpA*) (Figure S5; Table 1).

Phylogenetic analyses incorporating 48 *Firmiana* plastomes retrieved from Genbank revealed two cases of misidentification (MN533966 and ON813240) (Figure S6). Specifically, sample MN533966, labeled as *F. simplex*, clustered with *F. colorata* within Clade I, while sample ON813240, labeled as *Firmiana*

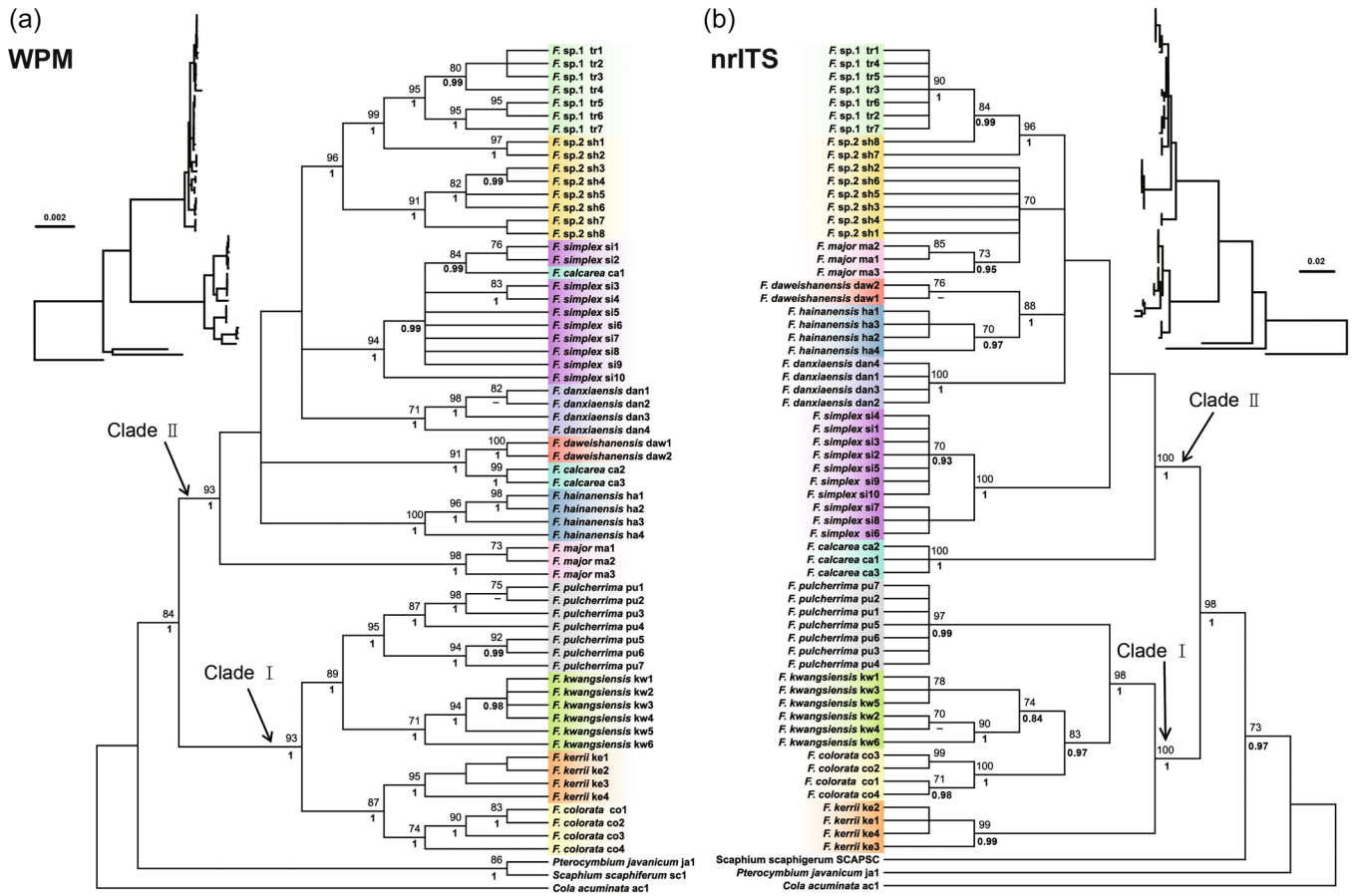

FIGURE 4 | Maximum likelihood phylogeny of *Firmiana* based on the whole plastome data matrix with one IR region (WPM) (a) and nrITS (b). ML bootstrap support (MLBS) and Bayesian posterior probability (BIPP) values are shown above and below branches, respectively. Scale bar indicates substitutions per site.

hainanensis, grouped with the outgroup species, suggesting that it does not belong in *Firmiana*. After incorporating these additional plastome data, the monophyly of nine species identified in our data set was further supported, with higher MLBS values confirming species-level resolution (Figures 4a and S6).

3.4 | Intra- and Inter-Specific Genetic Distance Estimation

Genetic distances between *Firmiana* sp.1, *Firmiana* sp.2, and the other *Firmiana* species are shown in Figure 5. For *Firmiana* sp.1, intraspecific genetic distances were significantly lower than interspecific distances to the 10 accepted species and *Firmiana* sp.2 across both WPM and nrITS datasets ($p < 0.05$). Similarly, *Firmiana* sp.2 showed significantly lower intraspecific distances compared to interspecific distances with the 10 taxa and *Firmiana* sp.1 ($p < 0.05$). Exceptions occurred in two cases: no significant difference was observed in the interspecific distance between *Firmiana* sp.2 and either *Firmiana* sp.1 (WPM data set) or *F. major* (nrITS data set) (Figure 5).

3.5 | nrITS Haplotypes in *F. major* Complex

The nrITS haplotypes identified in the *F. major* complex revealed nine variable sites distinguishing *F. major* from *Firmiana* sp.1. In contrast, *Firmiana* sp.2 exhibited greater nrITS haplotype diversity (Table S3). Three samples (sh3, sh7, and sh8) displayed more than two heterozygous sites. Among these, the ITS heterozygous sites in samples sh3 and sh8 were exact combinations of the corresponding haplotypes from *F. major* and *Firmiana* sp.1, suggesting potential hybrid ancestry. One sample (sh7) carried a unique haplotype with distinct substitutions at positions 511 and 712. Additionally, five other samples of *Firmiana* sp.2 contained unique combinations of variable sites characteristic of both *Firmiana major* and *Firmiana* sp.1 (Table S3).

4 | Discussion

4.1 | Phylogenetic Validation of Species Delineation in *Firmiana*

This study represents the first comprehensive phylogenetic reconstruction of Chinese *Firmiana*, covering all 10 recognized species and two potential cryptic taxa. Both plastome and nrDNA/nrITS phylogenies robustly support the division of Chinese *Firmiana* into two clades (Figures 4 and S3b), consistent with prior research that sampled only three to eight species with limited individuals (Abdullah et al. 2019; Lu et al. 2019, Lu, Huang, et al. 2021; Lu and Luo 2022; Li et al. 2024).

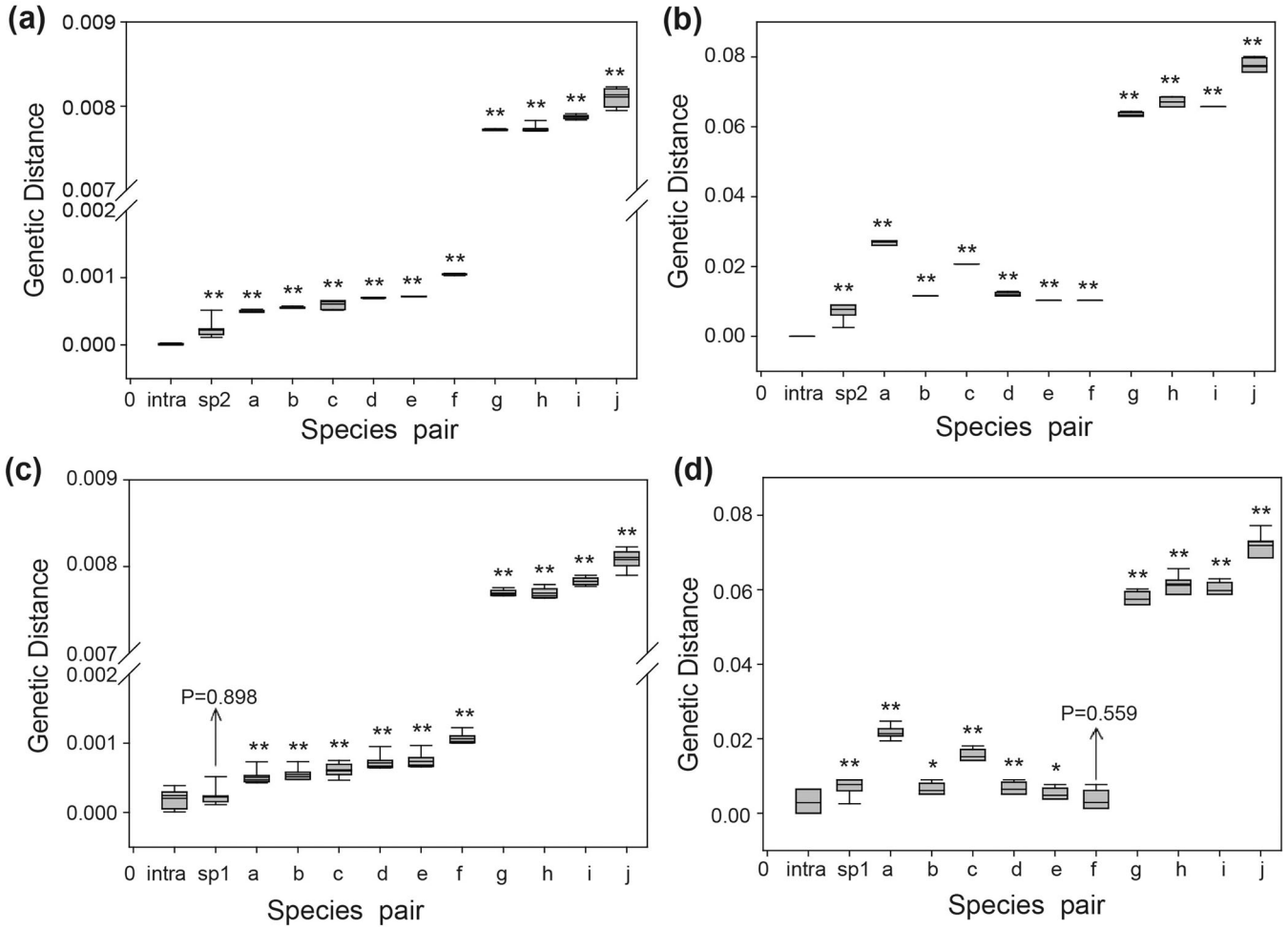

FIGURE 5 | Mann–Whitney tests using intra- and interspecific genetic distances for *F.* sp.1 (a, b) and *F.* sp.2 (c, d) using plastome (a, c) and nrITS (b, d) datasets. Statistical significance is indicated (**$p < 0.01$, *$p < 0.05$). Intra: intraspecific genetic distances; sp1: *Firmiana* sp.1; sp2: *Firmiana* sp.2; a: to *F. simplex*; b: to *F. danxiaensis*; c: to *F. calcarea*; d: to *F. hainanensis*; e: to *F. daweishanensis*; f: to *F. major*; g: to *F. kerrii*; h: to *F. kwangsiensis*; i: *F. pulcherrima*; j: to *F. colorata*.

Historically, *Erythropsis* was separated from *Firmiana* based on traits such as flowering while leafless and a campanulate calyx with short lobes (Ridley 1934; Kostermans 1954, 1957; Xu and Xu 2000). In our results, however, *F. daweishanensis* and *F. kerrii*, which share the leafless flowering trait with *Erythropsis*, and their calyces are similarly divided almost to the base with reflexed lobes, fall in different clades, indicating that these characters are phylogenetically uninformative. Moreover, *Hildegardia barteri* and *H. populifolia* were respectively nested within the *Erythropsis* and *Firmiana* groups (Li et al. 2022; Wilkie et al. 2006). This suggests that the boundaries among *Erythropsis*, *Firmiana*, and *Hildegardia* remain unresolved, requiring broader sampling and nuclear genomic data for clarification.

Overall, both WPM and nrITS phylogenies strongly support the current taxonomic framework for all 10 recognized Chinese *Firmiana* species, including two recently described species and one newly recorded taxon. *Firmiana calcarea* C.F. Liang & S.L. Mo ex Y.S. Huang, described in 2011, is restricted to the Longgang National Nature Reserve in Guangxi (Huang et al. 2011). *F. daweishanensis* Gui L. Zhang and J. Y. Xiang, described in 2020, is confined to the Daweishan area in southeastern Yunnan (Zhang et al. 2020). *Firmiana kerrii* (Craib) Kosterm. was newly recorded in Mengla county, southern Yunnan (Wang et al. 2019). These three species were originally described mainly on morphological grounds, and our comprehensive phylogeny provides strong molecular support for their taxonomic placement. Importantly, the results also suggest that additional cryptic or undescribed *Firmiana* species may yet to be discovered.

4.2 | DNA Barcoding of *Firmiana* and Discovery of Potential Cryptic Species

Rapid and accurate identification of endangered species is crucial for their conservation, management, and sustainable use. Morphological identification, though widely used, is often limited by phenotypic plasticity and developmental variation (Sommer 2020). This is especially apparent in *Firmiana*, where key diagnostic traits such as flowering characteristics, calyx morphology, and leaf shape can vary considerably (Tang et al. 2007). In field surveys, pronounced variation in leaf morphology and similarities in fruit and flower structures among closely related species made identification difficult for nonspecialists (Figure 1).

A prime example is the *Firmiana major* complex, which was previously treated as a single species due to its morphological resemblance across corolla, leaf, and fruit traits. DNA barcoding, however, reclassified this complex into two independent species (*F. major* and *Firmiana* sp.1) and one genetically complicated group (*Firmiana* sp.2). Upon closer examination, these three taxa can also be distinguished by their geographical distribution and growth habits: *F. major*, distributed in the upper reaches of the Jinsha River (e.g., Muli, Sichuan; Dadong village, Lijiang, Yunnan), typically appears as a short arbor; *Firmiana* sp.1, cultivated in central Yunnan, grows into large trees exceeding 10 m in height; whereas *Firmiana* sp.2, found in the lower reaches of the Jinsha River (e.g., Panzhihua, Sichuan; Chuxiong, Yunnan), presents as short arbors or herbs. This evidence highlights DNA barcoding as an efficient complement to morphology for species discrimination (Ferri et al. 2009; Hernández-Triana et al. 2019). Our study further shows that nrITS alone can successfully distinguish all 10 accepted *Firmiana* species (Figures 3b, 4b, S4a; Table 1). Therefore, we recommend nrITS as the primary barcode for the genus, suitable for both taxonomic and conservation applications.

Globally, plant conservation lags behind that of vertebrates (Corlett 2023), and as many as 39% of vascular plants may be threatened with extinction (Nic Lughadha et al. 2020). This gap contributes to "dark extinctions," where species go extinct before being formally described (Boehm and Cronk 2021). Recognizing cryptic species— distinct species erroneously classified as one—can significantly improve biodiversity assessments and conservation priorities (Bickford et al. 2007). DNA barcoding has proven effective at detecting and distinguishing cryptic taxa across many groups (Ragupathy et al. 2009; Carolan et al. 2012; Funk, Caminer, et al. 2012; Saitoh et al. 2014).

In this study, through DNA barcoding, we identified two potential cryptic species within the *F. major* group. The first species, *Firmiana* sp.1, is a tall tree exceeding 10 m in height and is predominantly cultivated in old temples and botanical gardens in Yunnan. The second species, *Firmiana* sp.2, is a shrub or small tree found in southern Sichuan and northern Yunnan. All samples of *Firmiana* sp.1 formed a monophyletic clade in both WPM and ITS phylogenies (Figure 4). The intraspecific K-2P genetic distance of *Firmiana* sp.1 was significantly lower than the interspecific distances to the 10 accepted Chinese species of *Firmiana* (Figure 5). This finding aligns with the population genetic analysis of *F. major* (Ma et al. 2022), which showed cultivated "*F. major*" samples (our *Firmiana* sp.1) as genetically distinct from typical *F. major* and *Firmiana* sp.2. Collectively, this evidence supports recognition of *Firmiana* sp.1 as a genetically distinct species. In contrast, eight samples of *Firmiana* sp.2 did not form a monophyletic clade, instead clustering either with *Firmiana* sp.1 or *F. major* (Figure 4). nrITS haplotype analyses revealed admixed ancestry in *Firmiana* sp.2: two individuals (sh3, sh8) were heterozygous at multiple nrITS loci, bearing haplotypes derived from both *Firmiana* sp.1 and *F. major*, consistent with early-generation hybridization. The remaining specimens exhibited either species-specific haplotypes or non-parental variants at diagnostic sites (Table S3). This indicates that *Firmiana* sp.2 represents a genetically complex group requiring further population genomic analyses to resolve its taxonomic status.

Finally, our evaluation of publicly available *Firmiana* plastomes revealed two misidentified sequences in GenBank (MN533966 and ON813240; Figure S6). MN533966, labeled as *F. simplex*, and collected in Ruili, Yunnan, China (Wang et al. 2021), clustered with *F. colorata* in Clade I rather than with other *F. simplex* individuals in Clade II, indicating a case of mislabeling. ON813240, labeled as *F. hainanensis* and sourced from the South China Botanical Garden, Chinese Academy of Sciences (Guangzhou, China). This sample grouped outside all other *Firmiana* species in both Tan et al. (2023) and the present analysis, pointing to a second clear case of taxonomic misassignment. These cases underscore the need for rigorous verification of genomic data in public repositories and suggest that accompanying specimen photographs would aid specialists in ensuring accurate species assignments.

4.3 | Implications for Conservation and Management

Species represent the fundamental evolutionary and ecological units in biology, and their conservation must safeguard genetic diversity to enable adaptation to ongoing environmental change (Hausdorf 2021). Identifying appropriate conservation units (CUs)—such as evolutionarily significant units (ESUs), adaptive units (AUs), designable units (DUs), and management units (MUs)—is therefore essential (Funk, McKay, et al. 2012; Hausdorf 2021). ESUs, the most widely used intraspecific CUs, are defined as populations that demonstrate significant reproductive isolation. This isolation has resulted in adaptive differences, making such populations important evolutionary components within a species (Funk, McKay, et al. 2012). Phylogenetic inference provides an important tool for identifying ESUs in threatened species, as they often correspond to distinct clades within phylogenetic trees (Li, Milne, et al. 2020). Our WPM phylogenies, for instance, provide insights into the intraspecific classification of possible ESUs within *Firmiana*. For *F. danxiaensis*, one sample collected from Nanxiong County (dan4) formed a distinct clade apart from other samples collected in Renhua County (dan1, dan2, and dan3) (Figure 4a; Table S1). This pattern is consistent with recent population genetic studies (Wu et al. 2018; Chen, Zhao, et al. 2023) that identified two different genetic groups (DX: populations collected from Danxia Mountain and NX: populations collected from Nanxiong basin), suggesting that these populations could be classified as separate ESUs in forthcoming conservation planning.

Firmiana species are typically pollinated by birds, insects, or wind, with varying levels of self-compatibility. Inbreeding depression has been reported in species such as *F. kwangsiensis* and *F. colorata* (Solomon Raju et al. 2004; Huang et al. 2018; Ma et al. 2022; Chen, Zhao, et al. 2023). Consequently, interspecific hybridization and gene flow are possible in areas of sympatry. Hybridization can have contrasting evolutionary outcomes: it may threaten rare and endangered species through genetic or demographic swamping (Levin et al. 1996; Todesco et al. 2016), or it may enhance genetic diversity and morphological plasticity, rescuing species with extremely small populations and promoting adaptation to changing environments (Rius and Darling 2014; Hamilton and Miller 2015; Grant and Grant 2019). The latter scenario has significant potential in species evolution,

especially when habitats are extremely limited or degraded (Dittrich-Reed and Fitzpatrick 2013). In the case of *Firmiana*, two individuals (sh3 and sh8) were identified as hybrids between *F. major* and *Firmiana* sp.1 (Figure 4; Table S3). Therefore, ex-situ conservation strategies for the genus *Firmiana* must account for potential genetic risks posed by hybridization.

Population genetic analyses have also revealed strong genetic differentiation among *F. major* populations along the Jinsha River. Typical *F. major* populations from the upper Jinsha River (i.e., EY, LJ, and PJ) form distinct genetic clusters compared to those found in the middle reaches (PD, PZH, WB, and YM), where *Firmiana* sp.2 occurs (Ma et al. 2022). Restricted to hot, arid valleys along the Jinsha River, these populations face limited gene flow due to rugged topography restricting seed dispersal. This restriction has resulted in high genetic differentiation between these populations within the species (Ma et al. 2022). Previous surveys estimated the population sizes to be approximately 5000 individuals for *F. major* and 1000 for *Firmiana* sp.2 (Ma et al. 2022), while a newly discovered *F. major* population in Shangri-La County, Yunnan Province, exceeds 10,000 individuals (Cha et al. 2023). However, further genetic analyses are required to ascertain whether this Shangri-La population is distinct.

Given the strong genetic differentiation and limited gene flow between *F. major* and *Firmiana* sp.2, and considering that *Firmiana* sp.1 is only found in cultivation, hybridization between *F. major* and *Firmiana* sp.1 is expected to be rare and unlikely to result in the extinction of *F. major* due to genetic swamping. Instead, *Firmiana* sp.2 represents unknown and underestimated genetic resources in *Firmiana*, which need urgent taxonomic assessment. At present, *F. major* and *Firmiana* sp.2 should be treated as distinct CUs, with ex situ programs designed to avoid interbreeding and prevent genetic contamination. Concurrently, in situ conservation of *F. major* should also incorporate cross-population transplants to increase genetic variation, as suggested by Ma et al. (2022). Moreover, since wild populations of *Firmiana* sp.1 may be extinct or undiscovered, the cultivated *Firmiana* sp.1 trees constitute a valuable genetic resource for landscape gardening, particularly due to their large tree form and cultural presence in temples and botanical gardens. Therefore, concerted efforts should be made to protect these ancient trees in situ, ensuring preservation of their unique genetic traits.

The application of genomic data offers exciting opportunities for conservation and the management of species with complex evolutionary histories (vonHoldt et al. 2018; Taylor and Larson 2019). Future studies of *Firmiana* should integrate genomic approaches to assess genetic diversity, detect detrimental mutations, quantify gene flow, and explore genotype–environment interactions. Such efforts will enable customized conservation strategies to safeguard the long-term persistence of this genus.

5 | Conclusions

In this study, we systematically evaluated DNA barcoding for Chinese *Firmiana* plants using genome skimming data. We assessed the discriminatory power of plastomes and nrDNA super barcodes, plastid hyper-variable regions, and universal barcodes. Our species discrimination analyses showed that nrITS, either alone or in combination with *trnH-psbA*, *rbcL+trnH-psbA*, or *matK+rbcL+trnH-psbA*, was able to discriminate all 10 recognized species and should therefore be recommended as the primary barcode for the genus *Firmiana*. Moreover, we identified two cryptic taxa within the *F. major* complex, providing important conservation implications for several *Firmiana* species. Beyond identifying the most effective DNA barcodes for accurate species identification, this study also provides valuable insights for future conservation, management, species discovery, and sustainable utilization of *Firmiana*. Further research with broader sampling is needed to clarify phylogenetic relationships among *Erythropsis*, *Hildegardia*, and *Firmiana*. In addition, population-level studies employing whole-genome resequencing are necessary to resolve the taxonomy of *Firmiana* sp.1 and *Firmiana* sp.2. Overall, this case study highlights the potential of genome skimming—simultaneously generating plastome, nrDNA, and traditional universal barcodes—as a promising tool for developing efficient species DNA barcodes, facilitating credible taxonomic revisions, biodiversity assessments, and conservation planning for *Firmiana* and other endangered plant taxa.

Author Contributions

Li-Jun Yan: conceptualization, funding acquisition, data curation, formal analysis, methodology, visualization, writing – original draft, writing – review and editing. **Ruo-Zhu Li:** data curation, investigation, methodology, resources, writing – review and editing. **Yun-Xue Xiao:** investigation, resources, writing – review and editing. **Richard T. Corlett:** conceptualization, writing – review and editing. **Yan Liu:** investigation, resources, writing – review and editing. **Zhi-Xiang Yu:** investigation, resources, writing – review and editing. **Rong-Sheng Luo:** visualization, writing – review and editing. **Jing Yang:** conceptualization, supervision, funding acquisition, writing – review and editing. **Wen-Bin Yu:** conceptualization, funding acquisition, investigation, project administration, resources, supervision, writing – review and editing.

Acknowledgments

We are grateful to Qing Chen, Hong-Bo Ding, Jia-Xin Fu, Tian-Ping Huang, Yu-Song Huang, Lu-Xiang Lin, En-De Liu, Ji-Feng Long, Jian-Yong Shen, Guang-Da Tang, Shu-Yuan Wan, Ji-Dong Ya, Ping Yang, Yong-Qiong Yang, and Qiang Zhang for fieldwork assistance and providing materials; to Yu-Kai Chen, Hong-Bo Ding, and Zhi-Fa Chen for providing some *Firmiana* pictures; to Jing Yang and Zhi-Rong Zhang for their help and suggestions in the lab work; to Wei-Bang Sun for his valuable comments and suggestions; and to the physical support from the Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, the Public Technology Service Center and Center for Gardening and Horticulture, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Daweishan National Nature Reserve, Hainan National Park, Nonggang National Nature Reserve, and Sichuan Panzhihua Cycas National Nature Reserve. This study was supported by Natural Science Foundation of Yunnan (202201AT070232, 202401AT070224 and 202201AS070049), Natural Science Foundation of Hainan (ZDYF2023RDYL01), Hainan Institute of National Park Program (KY-24ZK02), the 14th Five-Year Plan of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (XTBG-1450303/E3ZKFF8B01), the Key Program of Yunnan, China (202101BC070003), Ministry of Science and Technology of China Basic Resource Investigation Program (2021FY100200), Yunnan Revitalization Talent Support Program "Young Talent" and "Innovation Team" Projects, and Ecological and Environmental Conservation

Program from the Department of Ecology and Environment of Yunnan Province.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All newly assembled *Firmiana* plastomes have been submitted to GenBank with their accession numbers given in Table S1.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Figure S1: Map of *Firmiana* chloroplast genomes. Genes outside the circle are transcribed clockwise; those inside are transcribed counterclockwise. The dark gray area in the inner circle indicates GC content. **Figure S2:** Comparison of LSC, SSC, and IR junction borders across *Firmiana* species distributed in China. **Figure S3:** Phylogenetic relationships of *Firmiana* species based on six super-barcodes (WPM+nrDNA, nrDNA, WPM, LSC, SSC, IR) using the Maximum Likelihood (ML) method. Branches with < 50 bootstrap support collapsed; MLBS ≥ 70 shown. Successfully identified species highlighted in bold if all individuals formed a monophyletic group with MLBS ≥ 70. **Figure S4:** Phylogenetic relationships of *Firmiana* species based on four universal barcodes (nrITS, *matK*, *rbcL*, and *trnH-psbA*) and their combinations using the Maximum Likelihood (ML) method. Branches with < 50 bootstrap support collapsed; MLBS ≥ 70 shown. Successfully identified species highlighted in bold if all individuals formed a monophyletic group with MLBS ≥ 70. **Figure S5:** Phylogenetic relationships of *Firmiana* species based on eight hypervariable regions (*trnG*^{UCC}*-atpA*, *ycf1*, *ndhD-CCSA*, *psaJ-rpl20, psbZ-trnfM*^{CAU}, *rpl32-ndhF*, *trnL*^{UAA}*-ndhJ*, *ycf3-trnS*^{GGA}) extracted from the plastid genome using the Maximum Likelihood (ML) method. Branches

with < 50 bootstrap support collapsed; MLBS ≥ 70 shown. Successfully identified species highlighted in bold if all individuals formed a monophyletic group with MLBS ≥ 70. **Figure S6:** The Maximum Likelihood (ML) tree of 110 *Firmiana* plastomes with one IR region, including 48 retrieved from NCBI. Arrows indicate misidentified sequences (MN53396 and ON813240). **Table S1:** Sampling information and plastid genome details for 65 samples used in this study. **Table S2:** Information for 48 *Firmiana* plastomes downloaded from the NCBI website. **Table S3:** nrITS haplotypes of *Firmiana major* complex.