Plastid Phylogenomics Resolves Phylogenetic Relationships of Convolvulaceae Postprint

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Abstract

Convolvulaceae is a pantropical group with worldwide distribution, characterized by rich morphological diversity and significant economic importance. However, the phylogenetic relationships among the major clades or tribes within this family have long remained unresolved. To elucidate the phylogenetic relationships within Convolvulaceae, this study conducted representative sampling of 40 species from eight tribes within the family, and performed phylogenetic analyses based on whole plastid genome data using maximum likelihood and Bayesian inference. The results showed: (1) The plastid genomes of Convolvulaceae all exhibit a quadripartite structure, with genome sizes ranging from 113,273 to 164,112 bp and the number of protein-coding genes ranging from 66 to 79. (2) Phylogenetic analyses based on five DNA matrices (i.e., WCG, CDS, LSC, IR, SSC) revealed that the topologies of the WCG and CDS matrices were essentially consistent, with only slight differences in support values for a few branches; the topological difference between the LSC region and the WCG matrix concerned the phylogenetic positions of the Cuscuteae, Dichondreae, and Cresseae tribes; AU and SH tests indicated significant topological conflicts between the WCG matrix and the SSC and IR region matrices. (3) All phylogenetic analysis results demonstrated that Cuscuta and the Dichondreae tribe are both included within Convolvuloideae and should be treated at the tribal rank. (4) Based on the WCG and CDS matrices, the phylogenetic relationships among the eight tribes of Convolvulaceae were well resolved, i.e., Cardiochlamyeae and Erycibeae clustered together and diverged first from Convolvuloideae, followed by Cuscuteae, with the remaining five tribes splitting into two clades. (5) Phylogenomic analyses confirmed that Merremieae is a polyphyletic group, particularly the genus Merremia, and therefore the taxonomic status of this tribe and the delimitation of Merremia require revision.

Full Text

Preamble

Plastid Phylogenomic Insights into the Phylogeny of Convolvulaceae

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Abstract

Convolvulaceae is a pantropical family with rich morphological diversity and significant economic value. However, phylogenetic relationships among its major lineages or tribes remain unresolved. To address this, we sampled 40 species representing eight tribes within Convolvulaceae and performed phylogenetic analyses using maximum likelihood and Bayesian inference based on complete plastid genome data. Our results show that: (1) All Convolvulaceae plastomes exhibit a quadripartite structure, with genome sizes ranging from 113,273 to 164,112 bp and containing 66–79 protein-coding genes. (2) Phylogenetic analyses based on five DNA matrices (WCG, CDS, LSC, IR, and SSC) revealed that topologies from WCG and CDS matrices were largely congruent, with only minor differences in support values for a few branches. Topological differences between LSC and WCG matrices concerned the phylogenetic positions of Cuscuteae, Dichondreae, and Cresseae. AU and SH tests indicated significant topological conflicts between WCG matrix and those from SSC and IR regions. (3) All phylogenetic analyses confirmed that both Cuscuta and Dichondreae are nested within Convolvuloideae and should be treated at tribal rank. (4) Phylogenetic relationships among the eight tribes were well resolved using WCG and CDS matrices: Cardiochlamyeae and Erycibeae form a clade that diverged first from Convolvuloideae, followed by Cuscuteae, with the remaining five tribes splitting into two major clades. (5) Phylogenomic analyses confirmed that Merremieae is polyphyletic, particularly the genus Merremia, necessitating taxonomic revision of both the tribe and the genus.

Key Words: Convolvulaceae, phylogenetic relationships, plastome, Cuscuta, Merremieae

Introduction

Convolvulaceae, belonging to Solanales, comprises approximately 56 genera and 1,900 species distributed worldwide from tropical to temperate regions, with centers of diversity in the Americas and Asia. Some genera are endemic to tropical regions. The family includes important resource plants for food (e.g., sweet potato *Ipomoea batatas*), medicine, and horticulture, while some species play crucial roles in maintaining community biodiversity.

As a significant plant group, Convolvulaceae has attracted early taxonomic attention. Choisy published the first classification system in 1834, and at least 16 subsequent systems have been established based on morphological taxonomy. Divergent weighting of characters by different scholars has led to substantial discrepancies in both the circumscription and ranking of the family. Currently, two concepts are widely used: narrow and broad circumscriptions. The narrowly defined Convolvulaceae are typically annual or perennial vines with milky latex in stems and leaves, bicollateral vascular bundles, alternate spirally arranged leaves, actinomorphic bisexual flowers with sympetalous corollas usually bearing five distinct midpetaline bands, undivided ovaries, and one or two terminal styles. Due to unique habitats and life habits, some morphological characters have undergone parallel evolution, complicating familial delimitation and inframilial classification. The main controversies center on whether to include *Humbertia*, *Cuscuta*, and Dichondreae. Although taxonomists recognized their close relationship with Convolvulaceae, these groups were often treated as separate families because they lack some characteristics of the narrowly defined family. For instance, the parasitic genus *Cuscuta* possesses haustoria, lacks roots, has scale-like leaves, and non-green twining stems—features that differ markedly from typical Convolvulaceae. Roberty (1952) treated it as a separate family based on racemose inflorescences and slightly fleshy capsules, a classification supported by Austin (1973) based on chromosome numbers. Similarly, Dichondreae was segregated as a distinct family due to its deeply 2- or 4-lobed ovary and gynobasic style. However, Austin' s (1998) cladistic analysis of 128 characters (including floral, vegetative, anatomical, embryological, and cytological features) revealed extensive parallel evolution, suggesting that classification systems based solely on morphology would be inconsistent.

With rapid advances in DNA sequencing technology, molecular phylogenetic analyses can trace evolutionary relationships and provide robust evidence for taxonomic delimitation. The most recent molecular systematic studies in Convolvulaceae have primarily used organellar and nuclear gene fragments, supporting the monophyly of the broadly defined family and dividing it into two subfamilies and twelve tribes: Humbertioideae (containing one tribe, Humbertieae, with one genus and one species) and Convolvuloideae (containing eleven tribes: Cardiochlamyeae, Erycibeae, Dichondreae, Cresseae, Maripeae, Jacquemontieae, Cuscuteae, Aniseieae, Convolvuleae, Merremieae, and Ipomoeeae). Nevertheless, relationships among major lineages within Convolvuloideae remain unresolved. For example, Stefanović et al. (2002) initially used four plas-

tid genes to position Cardiochlamyeae as sister to the rest of Convolvuloideae, but subsequent analyses incorporating nuclear and mitochondrial genes yielded a "comb-like" structure among major lineages. Refulio-Rodriguez & Olmstead (2014) supported a clade comprising Cardiochlamyeae and Erycibeae as the basal group, but the phylogenetic position of Cuscuteae and the taxonomic status of Merremieae remain unclear.

Molecular systematic studies using gene fragments (e.g., *rbcL*, *matK*, *trnL-F*, ITS) have resolved relationships at various taxonomic levels, greatly advancing our understanding of plant phylogeny. However, limited phylogenetic information and varying evolutionary rates among genes often result in topological incongruence. To construct more robust phylogenetic trees, integration of additional genes or genomic data is necessary. While sequencing technologies have enabled increasing numbers of plant genomes to be sequenced, the large size, assembly difficulties, and complexity of nuclear genomes limit their utility in phylogenetic studies. In contrast, plastid genomes offer advantages including high copy number, moderate mutation rate, conserved structure, predominantly uniparental inheritance, and absence of recombination, making them widely used in plant phylogenomics. For example, phylogenetic analysis of 78 genes from 1,827 plastid genomes resolved relationships among major lineages of green plants, and subsequent analysis of 80 genes from 2,881 plastid genomes comprehensively updated the angiosperm phylogenetic framework.

This study sampled representative species from major lineages of broadly defined Convolvulaceae to explore its circumscription and accurately reconstruct the phylogenetic framework of Convolvuloideae, with particular focus on clarifying the phylogenetic position of *Cuscuta* and the taxonomic status of Merremieae. Our findings will enhance understanding of Convolvulaceae evolution and provide a foundation for studying the evolution of parasitism in *Cuscuta* and the origin and evolution of floral coloration in the family.

Materials and Methods

1.1 Materials

Following the revised classification framework of Stefanović et al. (2003), we sampled 40 species from 21 genera across eight tribes of Convolvulaceae, representing major lineages within Convolvuloideae but lacking material from Humbertioideae. Second-generation sequencing data for 23 species from 16 genera were obtained from the Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences. Molecular materials and voucher specimens for newly sequenced samples are deposited in the Germplasm Bank and the Herbarium of Kunming Institute of Botany (KUN) . Plastid genome data for 17 species from eight genera were downloaded from GenBank , with *Nicotiana tabacum* (NC001879) from Solanaceae used as the outgroup.

1.2 Plastid Genome Sequencing and Assembly

Total DNA was extracted from silica-dried fresh material or herbarium specimens using the Plant Genomic DNA Kit [DP320, Tiangen Biotech (Beijing) Co., Ltd.]. After quantification, shallow genome sequencing (genome skimming) was performed. Sequencing libraries of 350 bp were constructed with 150 bp or 250 bp paired-end reads using Illumina MiSeq or HiSeq 2500 platforms, yielding approximately 2 Gb of data per species. Raw reads were assembled de novo using GetOrganelle (Jin et al., 2020) to automatically generate circular plastid genomes. For species that could not be automatically circularized, manual assembly was performed by importing scaffolds into Geneious (Kearse et al., 2012), using the closest related circularized sequence as a reference for contig ordering and concatenation with LASTZ (Harris, 2007), followed by manual correction. Batch annotation was performed using Geneious with *Ipomoea nil* (AP017304) as reference, with similarity parameters set to 70%. Annotations were manually corrected, with protein-coding gene boundaries determined based on reference sequences and open reading frames (ORFs).

1.3 Sequence Alignment and Phylogenetic Analysis

Five datasets were constructed: (1) whole plastid genome sequence (WCG, with one IR region removed); (2) large single-copy (LSC) region; (3) small single-copy (SSC) region; (4) inverted repeat (IR) region; and (5) protein-coding sequences (CDS). Using tobacco as reference, collinearity was assessed with MAUVE (Darling et al., 2004), with non-collinear sequences manually adjusted and re-evaluated. Boundaries of plastid genome regions (LSC, SSC, IRa, and IRb) were determined in Geneious, and sequences for each region and protein-coding genes were extracted. WCG, LSC, SSC, and IR alignments were performed using MAFFT Online (Katoh & Standley, 2013) with default parameters. The 79 protein-coding genes were aligned using the bacterial codon model, with each gene aligned individually using the MAFFT plugin in Geneious before concatenation. Alignments were manually inspected in Geneious to exclude highly heterogeneous regions. The five final matrices (LSC, SSC, IR, CDS, and WCG) were uploaded to figshare (10.6084/m9.figshare.14329949). Matrix characteristics including length, variable sites, and parsimony-informative sites were calculated using MEGA X (Sudhir et al., 2018).

Maximum likelihood (ML) trees were constructed using RAxML (Alexandros, 2014) with the GTR+GAMMA+I model for all matrices to estimate the best-scoring ML tree and bootstrap support values (BS) from 1,000 replicates. Support was categorized as: BS $\leq$ 50% (unsupported), BS = 51–79% (weak/low support), and BS $\geq$ 80% (strong/high support). Bayesian inference (BI) trees were constructed for WCG and CDS matrices using MrBayes (Huelsenbeck & Ronquist, 2001) via CIPRES Science Gateway (Miller et al., 2010) or supercomputers. The best-fit nucleotide substitution model was selected using ModelFinder (Kalyaanamoorthy et al., 2017) in PhyloSuite (Zhang et al., 2020) based on BIC criteria. MCMC analyses ran for 2,000,000 generations, sam-

pling every 100 generations. The first 25% of trees were discarded as burn-in, and a 50% majority-rule consensus tree was constructed to calculate posterior probabilities (PP). Support was categorized as: PP = 0.51–0.94 (weak/low support) and PP $\geq$ 0.95 (strong/high support). Trees were visualized using Figtree v1.4.0.

1.4 Phylogenetic Conflict Detection

Approximately unbiased (AU) and Shimodaira-Hasegawa (SH) tests were used to assess whether optimal trees from other datasets (LSC, IR, and SSC ML trees) differed significantly from the WCG topology. SH test is conservative for rejecting null hypotheses, while AU test has less bias. Both tests were performed using IQ-TREE (Trifinopoulos et al., 2016) with the RELL algorithm for 1,000 replicates. P-values < 0.05 indicated significant conflict with the WCG topology.

Results

2.1 Plastid Genome Characteristics

All newly sequenced Convolvulaceae species exhibit typical quadripartite structure comprising one LSC region, one SSC region, and two IR regions. Plastome sizes range from 113,273 to 164,112 bp, with LSC regions of 71,518–88,896 bp, SSC regions of 3,087–16,678 bp, and IR regions of 22,409–29,081 bp. GC content varies from 37.2% to 40.1% . Annotation revealed that, except for *Cuscuta*, autotrophic lineages have conserved protein-coding gene content of 78–79 genes. The parasitic *Cuscuta* has lost some genes, retaining only 66 protein-coding genes.

2.2 Matrix Data Characteristics

Among the five plastid genome matrices, the WCG matrix was 166,691 bp long with 53,093 variable sites (31.9% of sequence length) and 28,675 parsimony-informative sites (17.2%), showing the highest sequence diversity. The CDS matrix was 76,883 bp long with 21,748 variable sites and 12,292 parsimony-informative sites, representing 41.0% and 42.9% of those in the WCG matrix, respectively. The IR matrix (37,107 bp) contained 9,908 variable and 5,574 parsimony-informative sites; the SSC matrix (8,232 bp) had 3,596 variable and 1,966 parsimony-informative sites; and the LSC matrix (112,926 bp) had 37,053 variable and 19,699 parsimony-informative sites.

2.3 Phylogenetic Analysis

Within broadly defined Convolvulaceae (including *Cuscuta* and Dichondreae), all seven tribes except Merremieae (genus *Merremia*) were monophyletic across all analyses [FIGURE:1, FIGURE:2]. Cresseae and Dichondreae were strongly supported as sister groups, as were Convolvuleae and *Merremia quinquefolia* within Ipomoeeae, and Convolvuleae with some Merremieae species.

ML and BI analyses of WCG and CDS matrices yielded largely congruent topologies [Figure 1: see original paper], with only a minor conflict in the position of *Tridynamia sinensis* within Cardiochlamyeae in the BI tree based on CDS matrix. Support values for some nodes were slightly lower in CDS than WCG analyses. Cardiochlamyeae and Erycibeae were strongly supported as sister groups across all matrices (BS $\geq$ 86, PP = 1.00), diverging earliest from other Convolvulaceae. This relationship received support from WCG, CDS, and LSC matrices (BS = 100/89/100; PP = 0.99/1.00), while the IR matrix suggested a possible sister relationship with Clade I (BS = 62) [Figure 1: see original paper].

In WCG and CDS matrices, Cuscuteae formed an isolated monophyletic branch sister to all other Convolvulaceae (BS = 68/74; PP = 1.00/1.00). The remaining five tribes formed two strongly supported clades: Clade I (Convolvuleae, Merremieae, and Ipomoeeae) and Clade II (Cresseae and Dichondreae). Within Clade I, Convolvuleae and Ipomoeeae were each monophyletic and nested within Merremieae, with LSC matrix analysis grouping them with Cuscuteae (BS = 60). However, IR (BS = 93) and SSC (BS = 61) matrices supported Cuscuteae as sister to Cresseae + Dichondreae. Notably, four *Merremia* species were distributed across three different clades: *M. hainanensis* grouped with Convolvuleae (BS $\geq$ 96, PP = 1.00), *M. quinquefolia* with Ipomoeeae (BS $\geq$ 98, PP = 1.00), and the remaining two species with other Merremieae genera (BS = 100, PP = 1.00). *Ipomoea* was paraphyletic, forming two strongly supported clades (BS = 100, PP = 1.00).

2.4 Phylogenetic Conflict Detection

Significant topological differences between WCG/CDS matrices and the other three matrices prompted AU and SH tests . Results showed no significant conflict between WCG and LSC topologies, suggesting the sister relationship between Cuscuteae and Clade I is plausible (PAU = 0.0837; PSH = 0.451). However, topologies from IR and SSC matrices were rejected (P $<$ 0.001).

Discussion

3.1 Delimitation of Broadly Defined Convolvulaceae

Traditional classifications of Convolvulaceae have been contentious, particularly regarding *Cuscuta* and Dichondreae, due to differing character weightings among researchers. While most scholars advocated treating parasitic *Cuscuta* as a monotypic tribe or subfamily within Convolvulaceae, some segregated it as a separate family. Similarly, Dichondreae was sometimes recognized as a distinct family due to its deeply lobed ovary and gynobasic style. Our plastid phylogenomic analyses demonstrate that the currently circumscribed Convolvulaceae represents a "natural" group, with both *Cuscuta* and Dichondreae properly included within the family rather than as separate families, consistent with Stefanović et al. (2002). Our results also support the inframilial classification of Stefanović et al. (2003), merging Cuscutaceae into Convolvulaceae as tribe

Cuscuteae and placing Dichondreae within Convolvuloideae.

3.2 Phylogenetic Relationships within Convolvuloideae

Previous molecular systematic studies established a framework of two subfamilies and twelve tribes, but relationships among major lineages within Convolvuloideae, particularly the positions of Cardiochlamyeae, Erycibeae, and Cuscuteae, remained unclear. Stefanović et al. (2002) initially positioned Cardiochlamyeae and Erycibeae as successive basal lineages, but later analyses with additional genes yielded a "comb-like" polytomy. Refulio-Rodriguez & Olmstead (2014) weakly supported Cardiochlamyeae + Erycibeae as a basal clade, but limited sampling and low support left relationships unresolved. Our analyses of four datasets (excluding IR) strongly support Cardiochlamyeae and Erycibeae as sister taxa that likely diverged earliest within Convolvuloideae. Weak support and significant conflicts in IR and SSC matrices may reflect heterogeneous nucleotide substitution rates in these regions.

Regarding Cuscuteae, WCG and CDS matrices strongly support Clade I and Clade II as sister groups that together are sister to Cuscuteae, representing an improvement over previous gene-fragment studies. Stefanović & Olmstead (2004) reported a "comb-like" structure, while McNeal et al. (2007) found Clade I as sister to Cuscuteae based only on *rps2*. Notably, *rbcL* results from McNeal et al. (2007) and our LSC matrix both support a possible Cuscuteae + Clade II relationship that cannot be rejected. These conflicts highlight how different molecular datasets influence the phylogenetic position of *Cuscuta* and suggest that nuclear genomic data are essential for further resolution.

3.3 Polyphyly of Merremieae

The polyphyly of Merremieae, particularly the genus *Merremia*, has been reported previously. Traditional concepts of Merremieae have been revised by removing non-monophyletic taxa, mostly to Aniseieae, yet Ipomoeeae remains nested within a broadly defined Merremieae. Some *Merremia* species remain scattered outside the core tribe, leaving even the revised Merremieae non-monophyletic. Stefanović & Olmstead (2004) and Simões et al. (2015) both found Ipomoeeae nested within Merremieae, with *Merremia* splitting into ten lineages. Simões & Staples (2017) proposed abandoning the tribal classification and using clade concepts, reassigning some *Merremia* species to other genera or establishing new ones, though these changes require further support.

Our plastid phylogenomic analyses show Convolvuleae and Ipomoeeae as monophyletic groups within Clade II, but Merremieae split into three branches: *M. hainanensis* groups with Convolvuleae, *M. quinquefolia* with Ipomoeeae, and the remaining two species with other Merremieae genera. These results differ somewhat from Simões et al. (2015), possibly due to sampling bias, data quantity differences, or regional endemism. Given the low support for backbone relationships in Simões et al. (2015), more comprehensive sampling and new

genomic data are needed for taxonomic revision of *Merremia*.

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Note: Figure translations are in progress. See original paper for figures.