Chromosome Karyotype Analysis of 21 Taxa in the Genus Clematis (Postprint)

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Abstract

Clematis L. is one of the large genera in Ranunculaceae, with important horticultural and medicinal value. To investigate the evolutionary patterns of chromosome sets in Clematis plants and reveal phylogenetic relationships among sections and species within the genus, this study employed the conventional squash method to process and squash root tips of 21 taxa in Clematis, observed morphological characteristics of chromosomes and conducted karyotype analysis, while simultaneously using Ward' s linkage method for cluster analysis. The chromosome morphology of narrow-lobed Taihang clematis, hairy-fruited Yangtze clematis, crisped-sepaled clematis, Sino-Indian clematis, and obtuse-sepaled clematis is reported for the first time. The results showed that all 21 taxa of Clematis were diploid, with chromosome numbers of 16 (2n=2x=16), and all taxa except Sino-Indian clematis possessed satellites; the chromosomes of long-petaled clematis, obtuse-sepaled clematis, parsley-leaved clematis, brown-haired clematis, C. flammula, and hairy-fruited Yangtze clematis were of "2B" type, while those of other taxa were of "2A" type; the karyotype asymmetry index of Clematis ranged between 60.29% and 63.79%; the chromosome sets of Clematis plants were relatively primitive, and extensive karyotypic variation existed among species. Collectively, the results indicated that chromosome numbers in Clematis plants evolved from diploid to polyploid, followed by the production of aneuploids through the diploidization process of polyploids. Chromosome evolution in Clematis occurred primarily at the diploid level, achieved through the generation of chromosomal structural variations, via four evolutionary pathways: production of heterozygous chromosomes, enhancement of karyotype asymmetry, alteration of chromosome types, and changes in satellite chromosomes. Meanwhile, classification at the sectional and species levels based on karyotypic characteristics was largely consistent with traditional taxonomy, indicating that karyotype analysis can provide clues for section-level classification within Clematis. The results of this study provide new reference data for research fields such as systematic classification, genetic evolution, and resource

utilization of Clematis plants.

Full Text

Chromosome Karyotype Analysis of 21 Clematis Taxa

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Abstract

Clematis L. is one of the major genera in Ranunculaceae, possessing significant horticultural and medicinal value. To investigate the evolutionary patterns of chromosome sets in *Clematis* and elucidate phylogenetic relationships among sections and species, this study employed conventional squash techniques to prepare root tip preparations of 21 *Clematis* taxa. Chromosome morphology was observed and karyotypes were analyzed, with cluster analysis performed using Ward' s linkage method. Chromosome morphology of *C. kirilowii* var. *chanetii*, *C. puberula* var. *tenuisepala*, *C. tubulosa*, *C. tibetana*, and *C. peterae* is reported for the first time. All 21 taxa were diploid with a chromosome number of 16 (2n = 2x = 16). Except for *C. tibetana*, all taxa possessed satellites. The chromosomes of *C. fusca*, *C. peterae*, *C. macropetala*, *C. aethusifolia*, *C. flammula*, and *C. puberula* var. *tenuisepala* were classified as "2B" type, while all other taxa were "2A" type. The karyotype asymmetry coefficient ranged from 60.29% to 63.79%. The chromosome complements of *Clematis* appear relatively primitive, with extensive interspecific karyotypic variation. These results suggest that chromosome number evolution in *Clematis* proceeded from diploidy to polyploidy, followed by aneuploid formation through polyploid diploidization. Chromosome evolution in the genus occurs primarily at the diploid level through structural variation, progressing via four pathways: generation of heterozygous chromosomes, increased karyotype asymmetry, changes in chromosome type, and variation in satellite chromosomes. Karyotypic characteristics were largely consistent with traditional classification at both sectional and species levels, indicating that karyotype analysis can provide valuable clues for classification at the sectional rank. These findings provide new reference materials for research on systematics, genetic evolution, and resource utilization in *Clematis*.

Keywords: *Clematis*, chromosome, cluster analysis, karyotype, cytology

Introduction

Clematis plants possess significant economic value and have long been used as medicinal herbs in China, with multiple species exhibiting analgesic, anti-inflammatory, and anti-tumor properties (Ma, 2010). Recent phytochemical studies have revealed the presence of triterpenoid saponins, flavonoids, and lignans (Zhang et al., 2018). With their elegant floral and fruit morphology and strong adaptability, *Clematis* species serve as excellent vertical landscaping plants and are among the commonly used ornamental flowers worldwide, holding exceptional horticultural value (Gao et al., 2017). Taxonomically, *Clematis* L. belongs to Ranunculaceae and represents a cosmopolitan genus with over 300 wild species distributed globally, including more than 150 species in China (Wang & Bartholomew, 2001; Wang and Li, 2005). The genus is taxonomically challenging due to its great diversity, and while numerous systematic studies have been conducted, classifications differ substantially in the delimitation of infrageneric taxa across different systems (Tamura, 1995; Johnson, 1997; Grey-Wilson, 2000; Wang and Li, 2005). Wang and Li (2005) divided the genus into four subgenera and 15 sections based on morphological characteristics, representing the most comprehensive classification system to date with clearly articulated relationships among infrageneric groups. However, morphological systems still face limitations in explaining the origin and evolution of *Clematis*, particularly regarding unclear relationships among sections and substantial discrepancies with molecular phylogenetic studies (Miikeda et al., 2006; Xie et al., 2011; Lehtonen et al., 2016). Therefore, resolving systematic evolution and infrageneric relationships requires integration of additional characters.

Plant karyotypes reflect overall features of chromosome evolution, and studies of chromosome and karyotypic characteristics help determine interspecific relationships and reveal genetic evolutionary mechanisms (Liu et al., 2010). Karyotypic features have proven important for classification at species, generic, and even familial levels in many plants (Wang, 2007). The significance of chromosome and karyotype analysis is particularly evident in the taxonomic history of Ranunculaceae (Langlet, 1927; Gregory, 1941; Tamura, 1995; Kong et al., 1997; Yang, 2000, 2001, 2002). In the 1930s-40s, Western botanists discovered two chromosome types in Ranunculaceae (Langlet, 1927; Gregory, 1941): large chromosomes (R-type) and small chromosomes (T-type), a characteristic subsequently used as an important reference for subfamilial classification (Tamura, 1995).

Clematis belongs to tribe Anemoneae and thus possesses typical large chromosomes (R-type), suggesting that chromosome and karyotypic characteristics may have taxonomic and phylogenetic value for the genus. However, previous cytological studies on *Clematis* based on chromosome morphology have not clearly elucidated their relationship with morphological classification and have been limited in scope. Additionally, with *Clematis* widely distributed globally, previous studies have incomplete taxon coverage, with insufficient chromosome and karyotypic data for certain groups. Therefore, this study investigated chromosome

numbers and karyotypes of 21 *Clematis* taxa, with chromosome data reported for the first time in five taxa. We observed chromosomes of 21 *Clematis* taxa, constructed cluster trees to analyze phylogenetic relationships, and examined reported chromosome numbers and ploidy levels in *Clematis* to address: (1) evolutionary patterns of *Clematis* chromosomes, and (2) the taxonomic value of karyotypic characteristics in the genus.

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1. Materials and Methods

1.1 Experimental Materials

The experimental materials comprised 19 species and 2 varieties of *Clematis*, representing 7 sections according to the latest classification system and covering all sectional taxa from North China. Taxon names and sources are listed in Table 1 . All voucher specimens are deposited in the Herbarium of Hebei Agricultural University (HBAU). Living plants were cultivated uniformly at the experimental farm of Hebei Agricultural University.

1.2 Experimental Methods

During August-September 2020, 10 container seedlings with vigorous growth and well-developed root systems were selected for each taxon. Lateral root tips (1–2 cm) were collected between 8:00–9:00 AM, with at least 30 young root tips sampled per taxon. Root tips were pretreated in an ice-water mixture at 4°C for 24 h, then washed 3–4 times with distilled water and fixed in Carnoy' s fixative (absolute ethanol:glacial acetic acid = 3:1) for 24 h. After fixation, samples were rinsed with distilled water and either processed immediately or stored in 70% ethanol. Stored materials were washed 3–4 times with distilled water before hydrolysis in 1 $mol \cdot L^{-1}$ HCl at 60°C for 10 min. Root tips were then rinsed and subjected to hypotonic treatment in distilled water at room temperature for 1 h to allow cell swelling. Meristematic regions (0.2 mm) were placed on slides, blotted dry, stained with improved carbol fuchsin for 10 min, covered with a coverslip, tapped gently, and squashed. Observations were made using a Leica DM 4000 microscope.

1.3 Data Acquisition and Analysis

Thirty well-spread metaphase cells were selected per taxon to observe and count chromosome numbers. Five cells with the clearest morphology and best dispersion were chosen for detailed analysis. Chromosome arms were measured using Photoshop 2020 for image pairing and ImageJ for arm length measurement (Xu et al., 2019), with mean values from the five cells used as parameters. Karyotype types were determined according to Stebbins (1971) and Li and Chen (1985). The karyotype asymmetry coefficient (AS.K%) was calculated following Arano (1963), and centromere position was classified according to Levan

et al. (1964). Karyotype evolutionary trends were plotted with asymmetry coefficient as the vertical axis and mean arm ratio as the horizontal axis. For cluster analysis, cytological characteristics including karyotype asymmetry coefficient, longest/shortest chromosome ratio, mean arm ratio, centromere index, presence/absence of satellites (quantified as 1/0), and karyotype type (2A = 0, 2B = 1) (Sheng, 2011) were used as classification criteria. Ward' s linkage method was applied using SPSS Statistics software.

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2. Results

2.1 General Karyotypic Characteristics of *Clematis*

Based on metaphase micrographs, chromosome morphology diagrams, and karyotype patterns (Figs. 1, 2, 3), the chromosome number was stable across all 21 *Clematis* taxa, with a consistent count of 2n = 2x = 16 (x = 8), confirming their diploid status. Detailed parameters including relative length range (from chromosome 8 to chromosome 1), longest/shortest chromosome ratio, arm ratio range, karyotype type, karyotype formula, asymmetry coefficient, centromere index, and mean arm ratio are presented in Table 2 . Chromosome types comprised m, sm, st, and t chromosomes, with m-type predominating. All taxa except *C. tibetana* possessed satellites. The mean longest/shortest chromosome ratio was 1.89, the proportion of chromosomes with arm ratios >2:1 was 0.375, and mean arm ratios ranged from 2.54 to 3.85. Two karyotype types were observed: "2A" and "2B" . Taxa with 2A karyotypes included *C. kirilowii*, *C. kirilowii* var. *chanetii*, *C. acerifolia*, *C. tangutica*, *C. intricata*, *C. hexapetala*, *C. grandidentata*, *C. fruticosa*, *C. sibirica* var. *ochotensis*, *C. glauca*, *C. heracleifolia*, *C. brevicaudata*, *C. tubulosa*, *C. tibetana*, and *C. vitalba*. Taxa with 2B karyotypes included *C. macropetala*, *C. peterae*, *C. aethusifolia*, *C. fusca*, *C. flammula*, and *C. puberula* var. *tenuisepala*. Karyotype formulas could be summarized as 2n = 2x = 16 = 10m + 0–2sm + 0–6st + 0–4t. Karyotype asymmetry coefficients ranged from 60.29% to 63.79% across the 21 taxa.

2.2 Analysis of Karyotype Evolutionary Trends

The karyotype coordinate plot (Fig. 4 [Figure 4: see original paper]) with mean arm ratio as the horizontal axis and asymmetry coefficient as the vertical axis revealed bidirectional evolutionary trends. *C. grandidentata* showed rapid evolution along the mean arm ratio axis, while *C. tibetana*, *C. intricata*, and *C. tangutica* (section *Meclatis*) evolved rapidly along the asymmetry coefficient axis. In angiosperms, karyotype evolution generally proceeds from symmetry to asymmetry (Stebbins, 1971). Taxa positioned toward the upper right in Fig. 4 represent more evolutionarily advanced groups. Consequently, *C. aethusifolia* located near the lower left represents a relatively primitive karyotype, whereas *C. peterae*, *C. alpina* var. *ochotensis*, and *C. glauca* positioned near the upper right represent more advanced karyotypes.

2.3 Cluster Analysis

Based on karyotypic parameters (asymmetry coefficient, longest/shortest chromosome ratio, mean arm ratio, centromere index, satellite presence/absence, and karyotype type), cluster analysis was performed (Fig. 5 [Figure 5: see original paper]). Following previous taxonomic treatments (Wang and Li, 2005), at Euclidean distance 10.0, the 21 taxa divided into three clusters. Cluster I primarily comprised members of section *Clematis*, along with *C. acerifolia* (section *Cheiropsis*) and *C. fruticosa* (section *Fruticella*). Cluster II included 12 taxa from sections *Meclatis*, *Atragene*, *Tubulosae*, and *Clematis*. Cluster III contained two species from section *Clematis* and one from section *Viorna*. Sections *Fruticella* and *Cheiropsis*, each represented by a single taxon, both clustered with section *Clematis* taxa. *C. aethusifolia* and *C. fusca* from section *Viorna* did not cluster together even at Euclidean distance 25.0, indicating distant phylogenetic relationships. Notably, *C. kirilowii* and its variety *C. kirilowii* var. *chanetii* showed high similarity in karyotypic parameters and clustered closely, supporting their close relationship.

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3. Discussion

3.1 Chromosome Evolutionary Patterns in *Clematis*

Database queries of reported *Clematis* chromosome numbers reveal a base number of x = 8, with diploid (2n = 16), tetraploid (2n = 32), hexaploid (2n = 48), and octoploid (2n = 64) cytotypes, plus aneuploid numbers (2n = 36, 42, 44, 49, 50). Diploidy (2n = 2x = 16) predominates, while polyploidy and aneuploidy are rare, reported only in seven taxa: *C. paniculata*, *C. terniflora*, *C. huchouensis*, *C. smilacifolia*, *C. bourdillonii*, *C. theobromina*, and *C. chinensis* var. *fujisanensis*. Chromosome number evolution involves polyploidization and aneuploidization (Wang, 2016). Polyploids are considered more environmentally adaptable than their diploid progenitors (Stebbins, 1971; Brochmann et al., 2004), while aneuploid formation is often associated with abnormal meiosis in polyploid gamete production (Comai, 2000). Thus, *Clematis* chromosome numbers likely evolved from diploidy to polyploidy, then to aneuploidy through polyploid diploidization. However, all seven taxa are cytotypic complexes containing both diploid and other ploidy levels, with no exclusively polyploid or aneuploid taxa identified, suggesting limited roles for polyploidy and aneuploidy in *Clematis* evolution.

Chromosome evolution in *Clematis* occurs primarily at the diploid level through structural variation: (1) Generation of heterozygous chromosomes observed in six taxa (*C. kirilowii*, *C. kirilowii* var. *chanetii*, *C. macropetala*, *C. fruticosa*, *C. sibirica* var. *ochotensis*, *C. acerifolia*) at positions 5, 5, 4, 6, 1, and 4, respectively. (2) Increased karyotype asymmetry, with type evolution from "2A" to "2B" in *C. fusca*, *C. macropetala*, *C. peterae*, and *C. puberula* var. *tenuisepala*. (3) Chromosome type changes, particularly transformation from st-type to t-

type chromosomes in pairs 7–8, observed in *C. heracleifolia*, *C. grandidentata*, *C. brevicaudata*, *C. puberula* var. *tenuisepala*, *C. glauca*, and *C. tubulosa* (Gong et al., 1985; Zhang, 1990; Yang, 1994). (4) Polymorphism of satellite chromosomes, with variation in number and type. Following Brat (1965), *Clematis* satellites include types I, II, III, and IV, predominantly type III. Some taxa exhibit multiple evolutionary pathways simultaneously. These structural evolutionary patterns indicate that *C. aethusifolia* is relatively primitive, while *C. macropetala*, *C. puberula* var. *tenuisepala*, *C. fusca*, and *C. heracleifolia* are more advanced, supported by seedling leaf morphology (Cheng et al., 2016) and phylogenetic studies (Miikeda et al., 2006; Lehtonen et al., 2016). Chromosome evolutionary characteristics thus provide important reference value for infrageneric phylogenetic studies.

3.2 Taxonomic Significance of Chromosome Morphology in *Clematis*

The close relationship between *Clematis* and *Naravelia* in Ranunculaceae has long been recognized (Miikeda et al., 2006; Xie, 2011). Recent molecular phylogenetic studies support merging *Naravelia* and *Archiclematis* into *Clematis*, consistent with their shared chromosome number. The monotypic genus *Anemoclema* (tribe Anemoneae) possesses 16 chromosomes with a 3A karyotype, indicating close relationship with *Clematis* but more advanced than the 2A/2B types in *Clematis*, a conclusion supported by molecular phylogenetics showing *Anemoclema* as sister to *Clematis* (Zhang et al., 2015).

Cluster analysis of the 21 *Clematis* taxa revealed that karyotype studies do not support subgeneric classification but provide some reference for sectional delimitation. Previous studies (Sheng, 2011; Peng et al., 2012) showed *Clematis* section taxa clustering together, as did *Cheiropsis* section taxa. However, karyotype studies of section *Viorna* conflict with traditional taxonomy (Wang and Li, 2005). Sheng (2011) found *Viorna* taxa clustering separately, with *C. aethusifolia* and *C. fusca* from this section showing distant relationships in our study. Similarly, ten section *Clematis* taxa separated into three clusters. These results do not support traditional classification but align with seedling leaf morphology and molecular phylogenetic studies, suggesting that sections *Viorna* and *Clematis* may not be monophyletic. Molecular studies (Mu, 2011; Sheng, 2015; Yan et al., 2016; Liu et al., 2018; Choi et al., 2021) support close relationships among *C. tibetana* and *C. tangutica*; *C. brevicaudata*, *C. heracleifolia*, and *C. intricata*; and *C. hexapetala* and *C. kirilowii*, demonstrating consistency among karyotype, seedling morphology, and molecular systematics. Our study and Sheng (2015) also show *C. kirilowii* and its variety *C. kirilowii* var. *chanetii* clustering together, indicating that karyotype analysis provides reference value for species-level classification and can aid species identification. For different populations (Wang et al., 2017), karyotype analysis can provide clues about diversity.

The *C. acerifolia* sampled in this study had a 2A karyotype, representing a relatively primitive group consistent with traditional classification, but differing

from the 2B type reported for Beijing populations. *C. heracleifolia* from Nanjing showed lower asymmetry than those from Beijing and Hebei, representing a more primitive karyotype. *C. fusca* from Shandong also differed from Hebei populations (Gong et al., 1985; Wang et al., 2017). These differences likely reflect varying evolutionary stages among populations: environmental changes may drive higher evolutionary advancement in competitive contexts. The *C. acerifolia* population sampled here represents a newly discovered site in Baoding, Hebei, with suitable, stable conditions and minimal human disturbance, resulting in a more primitive karyotype.

In summary, chromosome number and karyotype analysis provide clear clues for taxonomic delimitation in Ranunculaceae. Within *Clematis*, most species conform to sectional classification, though karyotype evidence alone cannot definitively determine sectional placement. Karyotype analysis offers reference value for species delimitation and identification, while revealing substantial chromosomal variation among populations. Cytological evidence can aid systematic and taxonomic studies of *Clematis*, and future research should expand sampling and strengthen chromosomal investigations.

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