Cytogeography of Caltha palustris (Ranunculaceae) from China (Postprint)

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Abstract

Twenty-three C. palustris accessions and ten C. scaposa accessions have been cytologically investigated using the traditional chromosome tableting technique and flow cytometry (FCM), in order to investigate the evolution of C. palustris and C. scaposa in Caltha of Ranunculaceae. Caltha palustris was found to be a polyploid complex, which contained tetraploids (2n = 4x = 32), hexaploids (2n = 6x = 48), and octoploids (2n = 8x = 64), and C. scaposa were tetraploids (2n = 4x = 32) and octoploids (2n = 8x = 64). Tetraploids were common in C. palustris and C. scaposa; however, hardly any diploids were discovered. This finding may be explained by cytotype adaptive differences to the underlying heterogeneity of environmental factors. Most accessions of C. palustris and C. scaposa were from extreme habitats, such as the alpine mountains in the Qinghai–Tibetan Plateau. Ancestral diploids may have existed in this region during glacial periods and colonized most regions at the end of the glaciation cycles. However, individuals with other ploidy levels may gradually replace diploids, because of their increased fitness in changing environment. Moreover, there were two possible evolutionary colonization routes: one from Gan' su to Yunnan, and the other from Tibet to Yunnan. Molecular phylogeny have shown that C. scaposa is closely related to C. palustris, the chromosome size of C. scaposa was smaller than that of C. palustris, C. scaposa may be a relatively derived evolutionary taxon. More samples need to be analyzed in the future to better elucidate C. scaposa cytogeography because of only 10 accessions.

Full Text

Cytogeography of *Caltha palustris* (Ranunculaceae) from China

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Abstract: To investigate the evolution of *Caltha palustris* and *C. scaposa* within the genus *Caltha* (Ranunculaceae) in China, we conducted cytological investigations of 23 *C. palustris* accessions and 10 *C. scaposa* accessions using traditional chromosome squash techniques and flow cytometry (FCM). *Caltha palustris* was found to be a polyploid complex containing tetraploids (2n = 4x = 32), hexaploids (2n = 6x = 48), and octoploids (2n = 8x = 64), while *C. scaposa* exhibited tetraploids (2n = 4x = 32) and octoploids (2n = 8x = 64). Tetraploids were common in both species, yet no diploids were discovered. This pattern may reflect cytotype-specific adaptive differences to heterogeneous environmental conditions. Most accessions originated from extreme habitats, particularly alpine regions of the Qinghai–Tibetan Plateau. Ancestral diploids likely existed in this region during glacial periods and colonized surrounding areas following deglaciation, but were subsequently replaced by other ploidy levels with higher fitness in changing environments. Two potential evolutionary colonization routes are proposed: one from Gansu to Yunnan, and another from Tibet to Yunnan. Molecular phylogenetic studies have shown that *C. scaposa* is closely related to *C. palustris*. The smaller chromosome size observed in *C. scaposa* suggests it may be a relatively derived taxon. However, additional sampling is needed to fully elucidate the cytogeography of *C. scaposa* given our limited dataset of only 10 accessions.

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Keywords: cytogeography, *Caltha palustris*, *C. scaposa*, polyploidy

Introduction

Polyploidy, the duplication of entire sets of chromosomes, represents a key process in the evolution and diversification of vascular plants (Hegarty et al., 2013; Otto & Whitton, 2000). Previous studies have demonstrated that polyploids

often exhibit superior adaptive capacity to stressful or novel environments compared to their diploid progenitors (Ehrendorfer, 1980; Grant, 1981; Levin, 2004; Morton, 1993; Otto & Whitton, 2000; Stebbins, 1985). Intraspecific variation in ploidy level is frequently observed in angiosperms (Kolář et al., 2015; Wood et al., 2009), and polyploidization constitutes one of the few speciation mechanisms that can operate in sympatry due to the immediate reproductive isolation that emerges between individuals of different ploidy levels (Husband & Sabara, 2003). Consequently, the geographic distribution of cytotypes provides valuable insights into the origin and maintenance of different ploidy levels (Baack, 2004; Kolář et al., 2009; Rieseberg & Willis, 2007; Segraves et al., 1999).

The perennial herb *Caltha palustris* inhabits mountain regions, valleys, marshlands, forests, streams, and grassy slopes from 600–4,000 m across the north temperate zone (Wang et al., 2001). Since its initial description by Linnaeus (1753), substantial morphological variability has been documented in this species, including variation in plant size, leaf shape and size, leaf margins, flowers, mature follicles, nodal rooting, tepal number and color, and seed color and symmetry (Smit, 1973; Kumar & Singhal, 2008). This morphological diversity has been attributed to environmental conditions (Blagojevic et al., 2013). The present study focuses on cytotype distribution within the *C. palustris* complex, which includes tetraploids (Wang et al., 2013; Yang, 2002; Yuan & Yang, 2006), hexaploids (Parfenov & Dmitrieva, 1985; Wang et al., 2013; Yang, 2002; Yuan & Yang, 2006), and octoploids (Wang et al., 2013; Yang, 2002; Yuan & Yang, 2006) (x = 8, Langlet, 1927). Molecular phylogenetic evidence indicates that *C. scaposa* is sister to *C. palustris* with 100% bootstrap support (Cheng & Xie, 2014; Schuettpelz & Hoot, 2004). *Caltha scaposa* is endemic to the Sino-Himalayan region, growing in wet alpine meadows and valleys from 2,800–4,100 m. Only two cytotypes have been detected: tetraploids (Wang et al., 2013) and octoploids (Wang et al., 2013; Yuan & Yang, 2006) (x = 8, Langlet, 1927). The existence of different cytotypes in these species suggests strong spatial segregation, potentially resulting from niche differentiation (Ehrendorfer, 1980; Lewis, 1980), reproductive exclusion (Levin, 1975; Van-Dijk & Bakx-Schotman, 1997), historical factors (Ančev, 2006), and differential reproductive success (Munoz-Pajares et al., 2017). By conducting a novel analysis of previous cytotype distribution data, we present a cytogeographical study of *C. palustris* and *C. scaposa* in China with two primary objectives: (1) to assess the geographic distribution of different cytotypes and propose a scenario of dispersal events, and (2) to determine the major driving forces of speciation in these taxa.

Materials and Methods

1.1 Taxon Sampling

We sampled six *C. palustris* accessions and four *C. scaposa* accessions for this study . From each population, 15–20 plants were collected, with geographical

coordinates recorded in the field using GPS. Living plants were cultivated in a greenhouse, and voucher specimens were deposited in the herbarium at the Kunming Institute of Botany, Chinese Academy of Sciences. Cytogeographical analysis incorporated both these new accessions and previously reported data (Yang, 2002; Yuan & Yang, 2006; Table 2).

1.2 Chromosome Number

Root tips were collected from each individual and pretreated with 0.002 mol · L ^{1} 8-hydroxyquinoline at 20–21 °C for 4–5 h. After fixation in Carnoy' s solution (3:1 ethanol:acetic acid) at 4 °C for 50 min, root tips were dissociated in a 1:1 mixture of 1 N HCl and 45% acetic acid at 60 °C for 30 s, stained with 1% acetic orcein for 2–3 h, and squashed on glass slides (Wang et al., 2013). Chromosome numbers were determined from at least 50 cells from a minimum of two seedlings per accession through mitotic observation. Mitotic interphase nuclei and prophase chromosome preparations followed Tanaka (1971, 1977, 1987), centromeric position designation followed Levan et al. (1964), and karyotype asymmetry was classified according to Stebbins (1971).

1.3 Flow Cytometry and DNA Ploidy Level Determination

Flow cytometry (FCM) analysis with propidium iodide was performed on fresh leaf samples from greenhouse-grown plants. Approximately 0.5 cm² of leaf material was finely diced with a new razor blade in a Petri dish containing 1,500–2,000 µL of WPB nuclear isolation buffer (0.2 mol · L ^{1} Tris · HCl, 4 mmol · L ^{1} MgCl · 6H O, 2 mmol · L ^{1} EDTA Na · 2H O, 86 mmol · L ^{1} NaCl, 10 mmol · L ^{1} Na S O , 1% PVP-10, 1% [v/v] Triton X-100, pH 7.5) (Tian et al., 2011). The nuclear suspension was filtered through 30 µm disposable filters to remove cell debris and stained with 150 µL propidium iodide (50 µg · mL ^{1}; including RNAse [500 µg · mL ^{1}]) for 10 min. Samples were analyzed on a CyFlow Space (Partec, Münster, Germany) flow cytometer equipped with a 488 nm blue laser, with at least 5,000 nuclei measured per sample. Histograms were analyzed using FlowMax ver. 2.82. Ploidy levels were estimated by comparison with a known tetraploid standard (yyp04) using the formula: Ploidy level of sample = (mean of sample peak/mean of standard peak) × ploidy level of the standard species (Tian et al., 2011).

Results

2.1 Chromosome Counts and DNA Ploidy Level Determination

Our study identified 13 tetraploid, one hexaploid, and nine octoploid accessions of *C. palustris*, along with seven tetraploid and three octoploid accessions of *C. scaposa* . These specimens were collected from Gansu (one accession), Yunnan (16 accessions), Sichuan (four accessions), Tibet (one accession), Guizhou

(one accession), and Qinghai (one accession). Metaphase chromosomes for eight representative accessions are illustrated in [Figure 1: see original paper]. Flow cytometry successfully estimated ploidy levels for two *C. palustris* accessions (yyp09 and yyp10) as 4x and 8x, respectively [Figure 2: see original paper].

2.2 Cytogeography

The ploidy distribution patterns for both species were mapped based on currently available data [Figure 3: see original paper]. All *C. palustris* accessions were single-cytotype, though sampling was limited for some populations. Secondary constrictions were observed in three *C. palustris* accessions . Tetraploids were more common than hexaploids or octoploids, and exhibited considerable karyotypic variation among accessions. In Yunnan' s Diqing Prefecture, all three cytotypes (tetraploid, hexaploid, and octoploid) co-occurred, while Lijiang (Yunnan) harbored both tetraploids and octoploids. Single cytotypes were found in Tewo (Gansu), Dali (Yunnan), Gongshan (Yunnan), Hongyuan (Sichuan), Nayong (Guizhou), and Zuogong (Tibet).

All *C. scaposa* accessions were single-cytotype, with tetraploids being most common. Both tetraploids and octoploids occurred in Sichuan, while single cytotypes were found in Tibet, Qinghai, and Yunnan. Only one contact zone between different cytotypes was detected [Figure 3: see original paper], where the ranges of 4x *C. palustris* and 8x *C. scaposa* overlapped in the Xiaozhongdian accession.

Discussion

Flow cytometry provides a rapid and precise method for identifying taxa of different ploidy levels, enabling fine-scale mapping of ploidy distributions within populations (Suda et al., 2004). This approach has been successfully applied in ploidy analyses of *Ranunculus* (Ranunculaceae) (Cires et al., 2010) and *C. leptosepala* s.l. (Wefferling et al., 2017). In our study, FCM confirmed ploidy levels for two accessions (yyp09 and yyp10). The results reveal that *C. palustris* functions as a polyploid complex with distinct cytotype distribution patterns. Polyploidy represents a prevalent phenomenon in the chromosomal evolution of extant species and genera (Otto & Whitton, 2000), potentially contributing to the origin of flowering plants (De Bodt et al., 2005). Consequently, plant systematists recognize that polyploid lineages may maintain complex relationships with each other and their diploid ancestors, complicating species concept applications (Soltis et al., 2007, 2009).

The *C. palustris* polyploid complex exhibits varied cytotype distribution. No diploids and only rare hexaploids were detected, while tetraploid and octoploid cytotypes were common and widespread. Similarly, *C. scaposa* showed common tetraploids and octoploids but lacked diploids and hexaploids. Such distribution patterns typically reflect cytotype-specific adaptive responses to environmental

heterogeneity (Lewis, 1980). With the exception of accessions from Guizhou and Gansu, all samples originated from extreme habitats, particularly alpine regions of the Qinghai–Tibetan Plateau. Polyploidy is especially common in plants from cold climates with harsh, stressful environments (Grant, 1981; Löve & Löve, 1949, 1967), explaining the high frequency of polyploidy observed here. Ancestral diploids likely inhabited this region during glacial periods and colonized surrounding areas at the end of glacial cycles, but were subsequently replaced by other ploidy levels with greater fitness in changing environments (Cui et al., 2008).

Chromosome counts in the *C. palustris* complex demonstrate that ploidy changes have been evolutionarily significant, often showing marked differences among accessions within a single species. Our analysis suggests the Hengduan Mountains may represent a polyploid complex comprising diploids, tetraploids, and hexaploids. Symmetrical karyotypes are widely considered more primitive than asymmetrical ones (Stebbins, 1971). In our combined dataset , accessions from Zhongdian (Yunnan) exhibited the highest asymmetric tendencies, with different karyotype categories (3B, 3C), asymmetry indices (11.39, 7.34, 5.96), and secondary constrictions. We therefore propose two potential evolutionary trends: one from Gansu to Yunnan, and another from Tibet to Yunnan.

Molecular phylogenetic studies have demonstrated the close relationship between *C. scaposa* and *C. palustris* (Cheng & Xie, 2014; Schuettpelz & Hoot, 2004). However, the cytogeography of *C. scaposa* remains incompletely characterized due to our limited sample of only 10 populations. Notably, *C. scaposa* possesses smaller chromosomes than *C. palustris*. Since chromosome size is subject to evolutionary change and tends to decrease over time (Martel et al., 2004), smaller chromosomes may represent a derived evolutionary character. Future studies incorporating additional samples will be necessary to fully elucidate the cytogeography of *C. scaposa*.

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Tables

Table 1. Voucher information of *Caltha palustris* and *C. scaposa* in this study

Table 2. Cytological characteristics of *Caltha palustris* and *C. scaposa* in this study

Note: LC: longest chromosome length; SC: shortest chromosome length; CL: mean chromosome length.

Figures

Figure 1. Mitotic nuclei and metaphase chromosomes of *C. palustris* and *C. scaposa* [Figure 1: see original paper]. (A) *C. palustris* (yyp04); (B) *C. palustris* (yyp06); (C) *C. palustris* (yyp07); (D) *C. palustris* (yyp08); (E) *C. scaposa* (yyp01); (F) *C. scaposa* (yyp02); (G) *C. scaposa* (yyp03); (H) *C. scaposa* (yyp05). Scale bar = 5 μm.

Figure 2. Flow cytometry (FCM) histograms of populations yyp09 and yyp10 [Figure 2: see original paper]. (A) G /G peak of standard (yyp04); (B) G /G peak of sample (yyp09); (C) G /G peak of sample (yyp10); (D) Peaks 1 and 2 at G /G phase of samples yyp09 and yyp10.

Figure 3. Distribution of cytotypes of *C. palustris* and *C. scaposa* [Figure 3: see original paper]. Black and gray circles indicate *C. palustris* and *C. scaposa*, respectively.

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