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Disentangling the Subalpine Marshmarigold Species Complex: the Cytogeography, Phylogeography, and Systematics of Caltha Biflora DC., C. Chionophila Greene, and C. Leptosepala DC. (Ranunculaceae)

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DISENTANGLING THE SUBALPINE MARSHMARIGOLD SPECIES COMPLEX: THE CYTOGEOGRAPHY, PHYLOGEOGRAPHY, AND SYSTEMATICS OF *Caltha biflora* DC., *C. chionophila* Greene, and *C. leptosepala* DC. (Ranunculaceae)

by

Keir Michael Wefferling

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biological Sciences at The University of Wisconsin-Milwaukee May 2018
ABSTRACT

DISENTANGLING THE SUBALPINE MARSHMARIGOLD SPECIES COMPLEX: THE CYTOGEOGRAPHY, PHYLOGEOGRAPHY, AND SYSTEMATICS OF Caltha biflora DC., C. chionophila Greene, and C. leptosepala DC. (Ranunculaceae)
by
Keir Wefferling
The University of Wisconsin-Milwaukee, 2018
Under the Supervision of Professor Sara B. Hoot

Subalpine marshmarigolds (Caltha biflora DC., C. chionophila DC., and C. leptosepala Greene, Ranunculaceae) are herbaceous perennials that grow in western North American mountainous and subarctic regions, from Alaska and Yukon in the north to California, Arizona, and New Mexico in the south. Variation in morphology across the range has generally led to recognition of a single species, though some have described up to nine species in the complex. In this dissertation, I describe our approaches to disentangling reticulate evolution across the geographical range of the subalpine marshmarigold complex, including chromosome counts (Chapter II), genome size estimates (Chapter III), morphology (Chapters III and IV), taxonomic circumscription (Chapter IV), and a dated phylogeographic reconstruction of divergence, migration, and allopolyploidization (Chapter V). This work delineates three species in the complex, including two hexaploid species (C. biflora in the Cascades, Sierra Nevada, and Coastal Ranges, and C. chionophila in the Rockies), a rare allononaploid (C. leptosepala in a single population in the Northern Rockies), and a widespread allododecaploid (C. leptosepala more or less throughout the range excluding the southern Rockies). The hexaploids are estimated to have diverged in the Upper Miocene to Upper Pliocene, persisted to the south (and possibly west) of Last Glacial Maximum icesheets, formed allododecaploids multiple times in the late Cenozoic, and recolonized deglaciated regions in multiple waves during the Pleistocene.
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Chapter I: Introduction to the subalpine marshmarigolds


1.1 Introduction

The subalpine marshmarigolds (*Caltha biflora* DC., *C. chionophila* Greene, and *C. leptosepala* DC.) exhibit a broad ecological tolerance within the subalpine to subarctic zones, occurring across a range of latitudes and altitudes to the north, south, interior and coastal regions of western North America. For pollination, subalpine marshmarigolds apparently follow an opportunistic “strategy” appropriate to the short subalpine summer, attracting butterflies (Lepidoptera), flies (Diptera) and bees (Hymenoptera). Despite these generalist patterns that allow subalpine marshmarigold to thrive in a diversity of habitats, local adaptations to a particular set of environmental and biotic variables manifest themselves in very tangible forms that are visible to those who visit this diverse lineage across its range.

A markedly beautiful plant, broad-leaved marshmarigold (*Caltha biflora* DC.) appears in the North Cascades growing in high meadows and bogs of the Alpine Lakes Wilderness under yellow cedars (*Chamaecyparis nootkatensis*), interspersed with round-leaved sundews (*Drosera rotundifolia*) and butterwort (*Pinguicula vulgaris*). Its bluish buds emerge from between tightly folded reniform leaves early in the subalpine spring. Elsewhere in the western Cascades, it occurs in massive colonies along shallow lake edges and in rich soil along streambanks, growing with skunk cabbage (*Lysichiton americanus*) and bog orchids (*Platanthera* spp.), often shaded by red huckleberries (*Vaccinium parvifolium*), fool’s huckleberry (*Menziesia ferruginea*), mountain hemlock (*Tsuga mertensiana*) and Douglas fir (*Pseudotsuga menziesii*). In the south, robust
diplophyllous populations of broad-leaved marshmarigold grow in serpentine fens of the Trinity Alps of northern California under Jeffrey pines (*Pinus jeffreyi*) and surrounded by pitcher-plants (*Darlingtonia californica*), Sierra shooting-star (*Dodecatheon jeffreyi*), primrose monkeyflowers (*Mimulus primuloides*), and diverse rushes (*Juncus* spp.) and sedges (*Carex* spp.).

To the east, elkslip marshmarigold (*Caltha chionophila* Greene) dominates high meadows above tree-line in the Snowy Range (Medicine Bow Mountains, Southern Rockies) of Wyoming. Its oblong, sagittate leaves share wet scree slopes with kingscrown (*Rhodiola integrifolia*) and saxifrages (*Saxifraga* spp.), or at slightly lower elevations grow under willows (*Salix* spp.) with white globeflower (*Trollius albiflorus*) and water-plantain buttercup (*Ranunculus alismifolius*).

Far to the north, in the Chugach Mountains of the Pacific Coast Range of Alaska, the northern mountain marshmarigold (*Caltha leptosepala* DC.) appears in wet subalpine seeps near tangles of Sitka alder (*Alnus viridis* ssp. *sinuata*) alongside Indian hellebore (*Veratrum viride*), arrowleaf senecio (*Senecio triangularis*), Nelson’s saxifrage (*Micranthes nelsoniana*), and coltsfoot (*Petasites frigidus*).

The subalpine marshmarigold complex exhibits adaptability to diverse montane, alpine, and subarctic biomes, precocious flowers reaching toward the sun and attracting pollinators while its fibrous sub-rhizomatous roots mine the substrate for nutrients and establish an anchor in the steep wet habitats to which it is so well suited. Contemporary genetic and morphological diversity attest to a dynamic history of isolation, reestablished contact, and gene flow in the mountain marsh-marigold species complex.
2.0 Chapter II: Cytology of *Caltha leptosepala* DC. (Ranunculaceae)


“*Caltha leptosepala* ssp. howellii” = *Caltha biflora* DC.; “*Caltha leptosepala* ssp. leptosepala” = *Caltha chionophila* Greene; “*Caltha leptosepala*” remains *Caltha leptosepala* DC.

2.1 Introduction

*Caltha* L. is a relatively small genus, comprising 10–12 species widely distributed in both Northern and Southern Hemispheres, in maritime to subalpine habitats (Smit, 1973; Hoffmann, 1999; Schuettpez and Hoot, 2004; Cheng and Xie, 2014). Polyploidy is proposed to have played an important role in diversification of the genus *Caltha* (Gregory, 1941), and may be an important mechanism driving divergence within our study group, the *Caltha leptosepala* species complex. *C. leptosepala* s.l. presents unique taxonomic challenges. While Greene (1899) segregated the species complex into nine species based on morphology, Ford (1997) groups all these taxa into a single species with no subtaxa recognized. Most authors regard the species complex as comprising two subspecies of *Caltha leptosepala*, most often as *C. leptosepala* DC. ssp. *leptosepala* and *C. leptosepala* DC. ssp. *howellii* (Huth) P.G. Sm., and sometimes as two species, *Caltha leptosepala* DC. and *C. biflora* DC. We here follow the nomenclature of Smit (1973) based on the work of Smit and Punt (1969), who described two subspecies and a “hybrid swarm” of *C. leptosepala*, citing observed differences in leaf, flower, and pollen morphology, and corresponding with three major geographic regions in western North America: “narrow-leaved” or “elkslip marsh-marigold” (*C. leptosepala* ssp. *leptosepala*) occurring in the southern Rocky Mountains; “broad-leaved” or “twin-flowered marsh-marigold” (*C. leptosepala* ssp.
howellii) occurring in the Sierra, Cascade and Klamath Mountain ranges; and morphologically intermediate C. leptosepala s.l. in the northern Rocky and Coastal Mountains, with an apparently broad region of sympatry of these three entities in the Pacific Northwest.

Previous cytological work in the species complex has found some chromosome number variation, including hexaploids ($6x$) and dodecaploids ($12x$; based on $x = 8$; Gregory, 1941): using somatic material, Langlet (1932) found $2n = 48$ from *Caltha leptocephala* [sic] (material of an unknown source); Taylor and Mulligan (1968) counted $2n = 48$ from *C. biflora* (= *Caltha leptosepala* ssp. *howellii*; three specimens from Queen Charlotte Islands, British Columbia, Canada); Smit and Punt (1969) found $2n = 96$ (from a Grenoble, France, botanical garden, wild source unknown), $2n = 96$ (from Winnemucca Lake, California, U.S.A.), and $2n = 48$ (from Kangaroo Lake, California, U.S.A.), all material listed as “*C. leptosepala* coll.”; Löve et al., (1971) counted $2n = 48$ from *C. leptosepala* (= *C. leptosepala* ssp. *leptosepala*?; from Bear Lake and Niwot Ridge, Colorado State, U.S.A.). Using gametic material, Morris (1971) found $n = 24$ in *C. leptosepala* (= *C. leptosepala* ssp. *leptosepala*?; from Mirror Lake, Utah State, U.S.A.). All the above data cast doubt on the aneuploid counts by Wiens and Halleck (1962; $n = 22$ for material from Niwot Ridge) and Mosquin (1968; $2n = 46$ for material from SW of Bear Tooth Pass, Wyoming State, U.S.A.; also see Morris [1972] for discussion invalidating the aneuploid counts).

### 2.2 Materials and methods

Mitotic chromosomes were counted in metaphase cells from root tips harvested from seedlings (*KMW 276-6, MM1-1, JNS1-1a, 216-4, WNPS3-1, 180-1, and AB1-1*) or field-collected plants (*KMW 212lepto, 299-3, CR1, CR2, 212rotA, CR3, CR5, and JS6*) raised in a growth chamber or windowsill. Root tips were pretreated in 1°C distilled water for ca. 24 h, then fixed in
a mixture of 95% ethanol and glacial acetic acid (3:1) for 30 min at room temperature (ca. 20°C), then ca. 24 h in a 4°C refrigerator. Pretreated and fixed root tips were stored in a -20°C freezer in 70% ethanol until squashing, as follows: Root tips were hydrolyzed in 60°C 1N hydrochloric acid for 10 min, rinsed for 15 min in 95% ethanol, then transferred to Wittman’s (1962) aceto-iron hematoxylin [50 mL 45% acetic acid, 2 g hematoxylin, 0.5 g ferric ammonium sulfate] ca. 45 min to 1 h, destained ca. 5–15 min in glacial acetic acid. The cells were broken apart and spread with a brass rod in Hoyer’s solution (Anderson, 1954; 25 mL distilled water, 15 g gum arabic, 100 g chloral hydrate, 10 g glycerol) diluted to 50% strength with distilled water, and covered with a number 1.5 coverslip. The slide was placed on a slide warmer set to 45°C for 30 sec, then squashed by hand, and the edges of the coverslip were sealed with clear nail polish.

Slides were inspected on a Nikon Eclipse 80i light microscope and images of countable spreads were captured using a Nikon Plan Apo VC 100x/1.40 oil-immersion lens (except Figs. 2C and 2D, captured with a Nikon Plan Apo 40x/0.95 lens) with a QImaging Retiga 2000R Fast 1394 digital camera and Q-Capture Pro v7 software. Images were cropped, and brightness and contrast applied uniformly to the entire image using Adobe Photoshop version 5.5.

2.3 Results

As in previous cytological studies of the *Caltha leptosepala* species complex, hexaploids and dodecaploids were found, and all countable spreads had a base number of \( x = 8 \) chromosomes. Nonaploids were also encountered, providing the first such count for the species. In this study we document six hexaploid *C. leptosepala* ssp. *leptosepala*, three hexaploid *C. leptosepala* ssp. *howellii*, two nonaploid *C. leptosepala*, and six dodecaploid *C. leptosepala*. 
The records (chromosome counts and localities of original collections) are organized by subspecies below:

**Caltha leptosepala DC. ssp. leptosepala**

2n = 48. U.S.A., Idaho State, Custer County, east side Sawtooth Range, Sawtooth National Recreation Area, Elk Meadows, 44°16’0.11”N, 115°5’41.83”W, 2060 m, 27 Feb 2015, K. Wefferling 212lepto (UWM) [Fig. 2.1A]; U.S.A., Idaho State, Custer County, Sawtooth National Recreation Area, south end of Decker Flat, south of junction of FR 315 and FR 037,
44°1’8.56”N, 114°51’ 32.01”W, 2090 m, 27 Feb 2015, K. Wefferling 276-6 (UWM) [Fig. 2.1B];
U.S.A., Idaho State, Custer County, Challis National Forest, Pioneer Mountains, at pass between
Summit Creek and Right Fork Kane Creek, 43°48’8.89”N, 114°12’11.43”W, 2890 m, 16 Apr
2015, K. Wefferling 299-3 (UWM) [Fig. 2.1C]; U.S.A., Colorado State, Grand County, Arapaho
National Forest, on trail to Lost Lake, 40°17’50.06”N, 105°57’42.74”W, 2970 m, 16 Apr 2015,
K. Wefferling MM1-1 from seeds collected by M. Majack (UWM) [Fig. 2.1D].

*Caltha leptosepala* DC. ssp. *howellii* (Huth) P.G. Sm.

2n = 48. U.S.A., Washington State, Skamania County, Gifford-Pinchot National Forest,
Takhlakh Meadow, 46°16”10.94”N, 121°35’19.23”W, 1400 m, 27 Feb 2015, K. Wefferling CR1
(UWM) [Fig. 2.1E]; U.S.A., Oregon State, Hood River County, Mount Hood Wilderness, NW
end of Elk Meadows, 45°20’42.9”N, 121°37’13.90”W, 1570 m, 13 May 2015, K. Wefferling
CR2 (UWM) [Fig. 2.1F]; U.S.A., Oregon State, Jackson County, Klamath National Forest,
Mount Ashland, Pacific Crest Trail, 42°4’35.76”N, 122°43’4.8”W, 2012 m, 5 May 2015, K.
Wefferling JNS1-1a from seeds collected by J. Anderson (UWM) [Fig. 2.1G].

*Caltha leptosepala* DC.

2n = 72. U.S.A., Idaho State, Custer County, east side Sawtooth Range, Sawtooth
National Recreation Area, Elk Meadows, 44°16’0.11”N, 115°5’41.83”W, 2060 m, 15 Jun 2015,
K. Wefferling 212rotA (UWM) [Fig. 2.1H]; U.S.A., Idaho State, Custer County, east side
Sawtooth Range, Sawtooth National Recreation Area, Elk Meadows, 44°16’0.11”N,
115°5’41.83”W, 2060 m, 6 Aug 2015, K. Wefferling 216-4 (UWM) [Fig. 2.11].
Figure 2.2. Mitotic metaphases. A, Caltha leptosepala (K. Wefferling WNPS3-1), 2n = 96; B, Caltha leptosepala (K. Wefferling CR3), 2n = 96; C, Caltha leptosepala (K. Wefferling CR5), 2n = 96; D, Caltha leptosepala (K. Wefferling JS6), 2n = 96; E, Caltha leptosepala (K. Wefferling 180-1), 2n = 96; F, Caltha leptosepala (K. Wefferling AB1-1), 2n = 96. Scale bars = 10µm.

2n = 96. U.S.A., Washington State, Kittitas County, Wenatchee Mountains, 47°25’12.0”N, 120°56’24.0”W, 1187 m, 13 May 2015, K. Wefferling WNPS3-1 (UWM) [Fig. 2.2A]; U.S.A., Washington State, Skamania County, Gifford-Pinchot National Forest, Takhlakh Meadow, 46°16”10.94”N, 121°35’19.23”W, 1400 m, 13 May 2015, K. Wefferling CR3 (UWM) [Fig. 2.2B]; U.S.A., Oregon State, Hood River County, Mount Hood Wilderness, NW end of Elk Meadows, 45°20’42.9”N, 121°37’13.90”W, 1570 m, 15 Jan 2015, K. Wefferling CR5 (UWM) [Fig. 2.2C]; U.S.A., Idaho State, Valley County, Bear Valley Road (FS579), Boise National Forest, old burned forest along small creek south of Bruce Meadows, 44°22’1.42”N, 115°16’28.67”W, 2184 m, 9 Jun 2015, K. Wefferling JS6 from plants collected by J. Smith (UWM) [Fig. 2.2D]; U.S.A., Idaho State, Boise County, Salmon River Mountains, Boise National Forest, Canyon Creek at pullout along ID21, 44°17’18.14”N, 115°13’37.17”W, 2060 m, 6 Aug 2015, K. Wefferling 180-1 (UWM) [Fig. 2.2E]; U.S.A., Idaho State, Idaho County,
Square Mountain, edge of Gospel Hump Wilderness, 45°35’45.43”N, 115°52’58.33”W, 2260 m, 5 May 2015, K. Wefferling AB1-1 from seeds collected by A. Bradshaw (UWM) [Fig. 2.2F].

2.4 Discussion

For the present study, sampling of subspecies and putative hybrids in the taxonomically challenging species complex was geographically and morphologically diverse; 15 specimens were counted from 11 populations. For the first time, sympatry of cytotypes was documented: hexaploid and dodecaploid cytotypes in the Cascades of southern Washington State; hexaploids and dodecaploids in the Cascades of northern Oregon State; and hexaploids and nonaploids (9x; the nonaploid cytotype documented here for the first time) in the central Rockies of southwest Idaho State. Using molecular data in a genus-wide study of *Caltha*, both Schuettpelz and Hoot (2004) and Cheng and Xie (2014) resolved the two subspecies of *C. leptosepala* as paraphyletic to one another or unresolved, and sister to a clade of Southern Hemisphere species (*Caltha* section *Psychrophila*). The present cytological study is intended to lay the groundwork for further phylogenetic and cytogeographical work in the *C. leptosepala* species complex.
2.5 References


Cytological aspects of the vascular plants. Research Branch, Canada Department of Agriculture Monograph No. 4, part 2.


3.0 Chapter III: Cytogeography of the subalpine marshmarigold polyploid complex
(Caltha leptosepala s.l., Ranunculaceae)


3.1 Introduction

Polyploidy, the duplication of entire sets of chromosomes, is a central feature in the evolution and diversification of vascular plants, driving genomic novelty and acting as a mode of immediate and sympatric speciation (Otto and Whitton, 2000; Hegarty et al., 2013; Husband et al., 2013). Genome duplication may be accompanied by a shift in phenology, ecology, and morphology, but these changes are not always obvious or discrete (Otto and Whitton, 2000; Soltis et al., 2010; Thompson et al., 2014). Importantly, unrecognized cytotype variation can lead to an underestimation of species richness (Soltis et al., 2007, 2010) and a misleading delineation of ecological breadth and geographic range. In order to further our understanding of natural polyploid complexes, it is imperative to delineate the geographic extent of cytotypes (Suda et al., 2007) and, if possible, to identify morphological characters associated with the different ploidy levels. Without such basic cytogeographical data, taxonomic conclusions in a given system are tentative, and genetic approaches to species delimitation are difficult or impossible as they may be confounded by cryptic (and often reticulate) speciation (Grusz et al., 2009). Hence, more cytogeographical studies of species or species complexes across their entire
geographic range are needed to detect and understand patterns of cytotype formation, establishment, and migration.

The present study examines cytotype distribution and contact zones in the subalpine marshmarigold polyploid complex (*Caltha leptosepala* s.l., Ranunculaceae), comprising hexaploids (*2n* = 6*x* = 48 chromosomes), dodecaploids (*2n* = 12*x* = 96), and rare nonaploids (*2n* = 9*x* = 72; Langlet, 1932; Taylor and Mulligan, 1968; Smit and Punt, 1969; Löve et al., 1971; Morris, 1971; summarized in Wefferling et al., 2015) (*x* = 8; Gregory, 1941). The perennial sub-rhizomatous herb *Caltha leptosepala* s.l. grows in wet tundra, subalpine meadows and forests, seepage slopes, and alpine turf in western North America from ~3900 m above sea level in the south to near sea level in the north (Welsh et al., 1987; Wilken, 1993; Ford, 1997). While various floras define the taxa and delineate their distribution differently, we provisionally follow the taxonomy and biogeography of Smit and Punt (1969) and Smit (1973; Fig. 1). In the southern portion of its range, *C. leptosepala* s.l. is differentiated into two morphologically distinct, putatively hexaploid subspecies: to the west, “broad-leaved marshmarigold” (*C. leptosepala* DC. ssp. *howellii* (Huth) P.G. Sm. [= *C. biflora* DC.]) occurs in the Sierra Nevadas, Klamath Ranges, Siskiyous, Oregon Coast Range, Cascades, Olympic Ranges, Vancouver Island, British Columbia Coast Ranges, and Haida Gwaii (Figs. 3.1–3.3); it is characterized by leaves that are reniform to orbicular and ≤ 1× as long as broad, single- or double-flowered inflorescences, and pollen grains pantoporate to pantocolpate (Smit and Punt, 1969; Smit, 1973). To the east, “elkslip marshmarigold” (*C. leptosepala* DC. ssp. *leptosepala*) inhabits portions of the Colorado Plateau and the Rocky Mountains; ssp. *leptosepala* has leaves that are oblong-ovate and > 1× as long as broad, single-flowered inflorescences, and pollen grains tricolpate (Smit and Punt, 1969; Smit, 1973). Under the above definitions, the two hexaploid
subspecies do not overlap in range (Fig. 3.1).

![Diagram](image)

**Figure 3.1.** Distribution of the *Caltha leptosepala* polyploid complex. Dashed lines show approximate distribution of taxa as defined in Smit and Punt (1969). Abbreviations: ssp. *howellii* = *C. leptosepala* ssp. *howellii*; ssp. *leptosepala* = *C. leptosepala* ssp. *leptosepala*. Population points (1-99, this study) are numbered sequentially from south to north for reference; points C1-C8 refer to chromosome counts obtained from the literature (C1, C5: Smit and Punt, 1969; C2, C3: Løve et al., 1971; C4: Morris, 1971; C6–C8: Taylor and Mulligan, 1968). Population numbers in parentheses (pops. 12, 46, 48, and 77) indicate where putative aneuploids were detected in sympatry with the indicated cytotype. Dotted-line box indicates the region shown in Figure 3.2.

In the north of the distribution range, continuous morphological variation between the two subspecies makes their subdivision difficult (Ford, 1997; Hoffmann, 1999; Schuettpelz and Hoot, 2004). Smit and Punt (1969) referred to the northern material (with varied leaf shapes and pollen grains 4- to 6-colpate) simply as *Caltha leptosepala* DC.; we refer to this polymorphic entity as “Northern *Caltha leptosepala*” (Fig. 3.1). A lack of discrete morphological breaks between forms led Ford (1997), writing in the Flora of North America North of Mexico, to group all entities within *C. leptosepala* with no recognition of subordinate taxa. Notably, most authors of regional floras implicitly equate Northern *Caltha leptosepala* with *C. leptosepala* ssp. *leptosepala* (Anderson, 1959; Hitchcock et al., 1964; Hultén, 1968; Hitchcock and Cronquist,
If grouping *C. leptosepala* ssp. *leptosepala* with Northern *Caltha leptosepala* is justified, it indicates a very broad range for this entity as well as sympatry of *C. leptosepala* ssp. *howellii* with *C. leptosepala* ssp. *leptosepala*. Additionally, as mentioned above, different cytotypes have been detected: while hexaploids include both *C. leptosepala* ssp. *howellii* and ssp. *leptosepala*, dodecaploids and nonaploids (the latter are apparently rare) comprise “Northern *Caltha leptosepala*” (Wefferling et al., 2015). The mode of formation (i.e., of allopolyploid or autopolyploid origin) is presently unknown for all of these cytotypes.

![Diagram](image)

**Figure 3.2.** Detail of the *Caltha leptosepala* s.l. cytotype contact zone, including submaritime Pacific Northwest and Northern Rockies. Abbreviations: ssp. *howellii* = *Caltha leptosepala* ssp. *howellii*; ssp. *leptosepala* = *C. leptosepala* ssp. *leptosepala*. Population numbers in parentheses (pops. 46, 48, and 77) indicate where putative aneuploids were detected in sympatry with the indicated cytotype.

Smit and Punt (1969) proposed that the heterogeneous Northern *Caltha leptosepala* (Alaska, Alberta, British Columbia, northwestern Idaho, northeastern Oregon, and northern
Washington; Fig. 3.1) is of hybrid origin, though they did not specify the likelihood of homoploid versus polyploid reticulation. In their scenario, populations with a formerly continuous distribution were restricted to reciprocally isolated last glacial maximum (LGM) refugia in the southern Rocky Mountains and Sierra Nevadas (Fig. 3.3; Smit and Punt, 1969).

Figure 3.3. Map of northwestern North America, showing approximate extent of ice sheets at the last glacial maximum (redrawn from Dyke, 2004), names of mountain ranges, and other features referred to in text, and state, province, and international boundaries. Abbreviations: LGM = Last Glacial Maximum (~18 kya); R. = River. State and Province abbreviations: AB = Alberta; AK = Alaska; AZ = Arizona; BC = British Columbia; CA = California; CO = Colorado; ID = Idaho; MT = Montana; NM = New Mexico; NV = Nevada; NWT = Northwest Territories; OR = Oregon; UT = Utah; WA = Washington; WY = Wyoming; YT = Yukon Territories.

These lineages are proposed to have diverged in allopatry, then migrated north with the glacial retreat and hybridized upon secondary contact in the region of the Snake and Columbia Rivers (Smit and Punt, 1969). In contrast, Hultén (1937) regarded *Caltha leptosepala* as having a center of origin in Alaska, in a Beringian refugium to the north of LGM ice sheets (Fig. 3.3). This scenario implies the presence of a hexaploid (or lower) and therefore potentially ancestral ploidy level in the north, with divergence between eastern and western forms in the south of the
range. To date, no chromosome counts are available from the northern part of the range of *C. leptosepala* s.l. These contrasting hypotheses of persistence in northern (Hultén, 1937) versus southern (Smit and Punt, 1969) refugia can be tested at least in part with cytotype distribution data.

We here present the first cytogeographical study of the geographically widespread *Caltha leptosepala* polyploid complex, wherein cytotype variation is known to occur, but for which almost no cytotype distribution data exist (Wefferling et al., 2015). Our goals in the present study were to: (1) delineate the geographic distribution of the different cytotypes and hexaploid subspecies in order to evaluate how cytogeography informs biogeographic history and evolution in the polyploid complex; (2) identify cytotype contact zones for more targeted population-level studies in the future; and (3) investigate whether the different cytotypes are recognizable using macro-morphological characters and whether morphology supports recognition of existing taxa or further subdivision of the complex. To achieve these objectives, we undertook a large-scale screening covering most of the distribution range of the *Caltha leptosepala* polyploid complex, assessing DNA ploidy level and leaf morphology.

### 3.2 Materials and methods

**Sampling and mapping of cytotypes**—The species has a western North American distribution, from Arizona, New Mexico, and California in the south to Alaska and the Yukon Territories in the north (Figs. 3.1, 3.3; Smit and Punt, 1969; Smit, 1973; Ford, 1997). Our sampling encompasses all major regions inhabited by the species, and more specimens were collected in areas where high morphological variability was observed (suggesting potential cytotype or morphotype contact zones). During the summers of 2012–2015 specimens were
collected in the field and dried either on silica gel (individual leaves) or in a plant press (whole plant herbarium vouchers) for flow cytometric analyses and morphological measurements. Photographs were taken of each sampled plant before harvest. In each population, from one to 28 samples were collected (Appendix A). Specimens were not selected randomly within a population, but targeted to capture extant morphological diversity; populations with higher morphological diversity were sampled more heavily, especially within or near the putative cytotype contact (or hybrid) zones in both the western, subarctic Pacific Northwest and in the Northern Rockies (Figs. 3.1–3.3). Mature seeds were collected whenever possible and whole plants were transported to the greenhouse for root meristem chromosome counts; additional seeds or living plants were procured from colleagues in order to expand our sampling as much as possible for chromosome counts. Herbarium vouchers (from ALA, ASC, DAV, ID, NYBG, OSC, RBCM, RM, SRP, UWM, and WTU; Appendix A) spanning the years 1991 to 2015 were also analyzed, mainly with the aim of expanding our geographic sampling.

All individuals sampled were mapped in ArcGIS v10.1 (Environmental Systems Research Institute, Redlands, California, USA) by importing collection points (in decimal degree format) as X, Y coordinates. Population markers in close proximity were combined on the maps in order to improve visibility; this was only performed where merging populations did not change the geographical patterns of cytotype occurrence (Appendix A).

**Chromosome counts**—Chromosome counts were made to ascertain DNA ploidy levels based on the estimates of genome size obtained using flow cytometry. The chromosome count protocol followed Wefferling et al., (2015). Briefly, field-collected seeds were briefly soaked, cold stratified (from 2–6 months at 4°C), and sown, or whole plants were transplanted from the
field to a growth chamber. Actively growing root tips were harvested and pre-treated in 1°C distilled water and fixed in 95% ethanol and glacial acetic acid in a ratio of 3:1. Root tips were stained using Wittman’s aceto-iron hematoxylin (Wittman, 1962), de-stained in glacial acetic acid, and squashed under a glass cover slip in a diluted (to 50% with distilled water) Hoyer’s solution (Anderson, 1954). Chromosome spreads were observed using a Nikon Eclipse 80i light microscope (Tokyo, Japan) and photographed using a Nikon Plan Apo VC 100×/1.40 oil-immersion lens (Tokyo, Japan) with a QImaging Retiga 2000R Fast 1394 digital camera (Surrey, British Columbia, Canada) and Q-Capture Pro v.7 (Surrey, British Columbia, Canada). Images were cropped, and brightness and contrast applied uniformly to the entire image using Adobe Photoshop v.5.5 (San Jose, California, USA).

**Flow cytometry and determination of DNA ploidy levels**—Propidium iodide flow cytometry (FCM) analysis was performed using either silica-dried or herbarium voucher leaves. To assess the reliability of our FCM estimates from dried samples, 29 specimens were used to measure genome size using both fresh and dried leaf samples from the same plant. Herbarium vouchers were used to assess the DNA ploidy level only, while silica-dried material was also used as a proxy of genome size (Table 3.1). Silica-dried specimens were usually analyzed within a year of harvest, with the exception of samples collected in 2012 (n = 37). Nuclei were isolated in a Petri dish containing 1 mL of WPB buffer (Loureiro et al., 2007) following the procedure of Galbraith et al., (1983) in which 0.5 cm² of leaf tissue of *Caltha leptosepala* s.l. was co-chopped simultaneously with an equal amount of fresh leaf tissue of an internal standard using a sharp razor blade. *Vicia faba* ‘Inovec’ (2C = 26.90 pg; Bureš et al., 2003) was used as internal standard in all samples, except in seven individuals of Northern *Caltha leptosepala* in
which the peaks of the sample and standard overlapped slightly, affecting the estimates. In these individuals, *Pisum sativum* ‘Ctirad’ (2C = 9.09 pg; Doležel et al., 1998) was used instead. The suspension of nuclei was filtered through a 50 µm nylon mesh and stained with 50 µg mL⁻¹ of propidium iodide (PI; Fluka, Buchs, Switzerland); 50 µg mL⁻¹ of RNase (Fluka, Buchs, Switzerland) were also added to avoid staining of double stranded RNA. After incubation at room temperature for 5 min, the relative fluorescence intensities of at least 1300 particles per G1 peak were analyzed in a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH, Münster, Germany) using FloMax software (Partec GmbH, Görlitz, Germany).

Histograms were evaluated, retaining only samples with a coefficient of variation (CV) below 8% (herbarium material often generated histograms of lower quality); samples with higher CV values were discarded and a new sample was prepared. Histograms were generally of reasonable to high quality with a mean sample CV of 3.25% (range 2.08–7.28%). In order to accommodate more samples and encompass a greater geographical region in our sampling with the addition of herbarium specimens, we decided to follow a cut off value of 8% (usually used in clinical cell cycle analyses; Kron and Husband, 2012), higher than the often recommended 5% used for genome size analysis (Doležel and Bartoš, 2005; Doležel et al., 2007; Bainard et al., 2011). Herbarium specimen data were only used to infer cytotype, and are not included in holoploid genome size estimates (Table 3.1). Also, further caution was taken whenever samples presented CV values higher than 5%, and these samples were only considered if the holoploid genome size value fell within a DNA ploidy level category comprised of samples with low CVs (i.e., ≤ 5%). In some cases, even with low CV values the genome estimates were outliers and did not fall within any DNA ploidy level. In these instances at least two replicates were made to
confirm the results. We failed to obtain histograms from 11 samples (including fresh, silica-dried, and herbarium material), and another 7 samples (silica-dried and herbarium material) were excluded as they had a CV ≥ 8%.

A proxy of the holoploid genome size (2C) was calculated using the following equation:

\[
\text{Caltha leptosepala s.l. 2C nuclear DNA content (pg)} = \left( \frac{\text{Caltha leptosepala s.l. } G_1 \text{ peak mean}}{\text{reference standard } G_1 \text{ peak mean}} \right) \times \text{genome size of the reference standard.}
\]

The 15 specimens (sourced from 11 populations) that yielded the chromosome counts in Wefferling et al., (2015) and the five specimens used for the new counts performed in this study were also analyzed by FCM, which enabled the assignment of a DNA ploidy level to nearly every individual analyzed (Appendix A). Specifically, genome size estimates were sorted from the smallest to the largest value for each taxon and outliers were identified (i.e., discontinuous points in the distribution of values). The range of variation in genome size was than calculated excluding the outliers and using the mean value ± 2 × s.d. for each taxon and ploidy level (the latter determined based on chromosome counts) for the entire distribution area.

**Morphology**—Leaf length-to-width ratios and pollen morphology are cited as diagnostic characters in the species (Smit and Punt, 1969; Smit, 1973). Several authors examined leaf margins, plant height, follicles (stipitate or sessile), number of flowers per inflorescence, width of filaments, and sepal shape, but found these characters to be uninformative (Smit and Punt, 1969; Smit, 1973; Brayshaw, 1989; Ford, 1997; Douglas et al., 1999; K. Wefferling, personal observation). We tested the usefulness of leaf blade length-to-width ratios in identifying taxa and/or cytotypes. The length (including leaf auricles) and width of three (rarely two) leaf blades per specimen were measured; mean length-to-width ratio (L:W) and mean leaf blade “area”
were calculated for each specimen, except that seedlings were only used for L:W as leaf sizes were in general smaller in cultivation. A total of 144 (of 278) specimens were used for morphological data collection, representing 95 (of 99) populations.

Statistical analyses—The effect of the drying process in genome size estimates was assessed using a paired t-test with type of material (fresh versus silica-dried) as factors. The comparison revealed significant differences between fresh and silica-dried estimates ($t_{28} = -7.56$, $P < 0.001$), with the estimates of genome size for silica-dried specimens 4.6% larger on average than for fresh samples. Acknowledging the effects that the drying process has on the genome size estimates, the values obtained in this study were used only as a proxy for this trait and our statistical approach considered only silica-dried material (silica-dried material comprised the bulk of our samples; $n = 220$) so that all the samples were subjected to the same conservation method. Herbarium vouchers were used solely for DNA ploidy level estimates.

Kruskal-Wallis one-way ANOVA on ranks was used to test for differences in genome size (2C) estimates (based on silica-dried material only), leaf L:W, and leaf L×W among taxa and cytotypes (factor defined as: 6x ssp. howellii, 6x ssp. leptosepala, 9x Northern Caltha leptosepala, and 12x Northern Caltha leptosepala), since normality and homoscedasticity were not achieved even after transformation. Multiple comparisons were performed using Dunn’s test. All statistical analyses were performed using JMP Pro 12 (SAS Institute, Cary, North Carolina, USA).

3.3 Results
Chromosome counts, flow cytometry, and DNA ploidy level determination—Material for which we were able to obtain precise chromosome counts comprised three 6x *Caltha leptosepala* ssp. *howellii*, seven 6x *C. leptosepala* ssp. *leptosepala*, three 9x Northern *Caltha leptosepala*, and seven 12x Northern *Caltha leptosepala* individuals (Appendix A). These include the counts from Wefferling et al. (2015), where four populations from the Cascade-Sierra axis and seven populations from the Rockies were sampled. The new chromosome counts were consistent with previous observations: 6x individuals with 2n = 48 chromosomes, 9x individuals with 2n = 72, and 12x individuals with 2n = 96 (Figs. 2.1, 2.2).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Ploidy</th>
<th>n (Pop)</th>
<th>G.s. (pg DNA mean ± s.d., n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssp. <em>howellii</em></td>
<td>6x</td>
<td>83 (46)</td>
<td>13.33 ± 1.61, 75</td>
</tr>
<tr>
<td></td>
<td>an.§</td>
<td>3 (1)</td>
<td>17.47 ± 0.52, 3</td>
</tr>
<tr>
<td>ssp. <em>leptosepala</em></td>
<td>6x</td>
<td>64 (17)</td>
<td>8.40 ± 0.59, 57</td>
</tr>
<tr>
<td></td>
<td>an.†</td>
<td>2 (1)</td>
<td>12.91 ± 0.29, 2</td>
</tr>
<tr>
<td>Northern <em>Caltha</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leptosepala</td>
<td>12x</td>
<td>116 (38)</td>
<td>20.44 ± 1.41, 78</td>
</tr>
<tr>
<td>9x</td>
<td></td>
<td>8 (1)</td>
<td>14.38 ± 0.62, 7</td>
</tr>
<tr>
<td>an.§ *</td>
<td>2 (2)</td>
<td></td>
<td>17.96, 27.46†, 2</td>
</tr>
</tbody>
</table>

Table 3.1. Ploidy levels inferred using flow cytometry for 278 individuals grouped by taxon according to Smit and Punt (1969) and Smit (1973). Abbreviations: ssp. *howellii* = *Caltha leptosepala* ssp. *howellii*; ssp. *leptosepala* = *C. leptosepala* ssp. *leptosepala*. 6x, hexaploid; 9x, nonaploid; 12x, dodecaploid; an., aneuploid. Number of individuals (n) and populations (Pop) is provided for each taxon. The proxy of genome size (2C G.s., in pg DNA, based on silica-dried samples only) is provided as mean and standard deviation of the mean, followed by sample size. Information on putative aneuploids is provided based on all the available estimates (i.e., not only silica-dried material as above): § G.s. calculated from air-dried/herbarium and silica-dried material; † G.s. calculated from air-dried/herbarium material only; * due to the presence of disparate values, data for aneuploid Northern *Caltha leptosepala* estimates are listed (separated by commas) rather than averaged.
We were able to successfully analyze 278 individuals from 99 populations using flow cytometry, including 183 silica-dried field-collected plants, 37 silica-dried seedlings, and 58 herbarium vouchers (including both our own collections and other herbarium specimens). Most samples were characterized as 6x (n = 147) or 12x (n = 116), with few individuals (n = 8) characterized as 9x. We also observed individuals (n = 7) with intermediate or outlier estimates, possibly indicating aneuploidy (Table 3.1, Appendix A).

Significant differences in genome size estimates were observed among taxa and cytotypes ($H_3 = 190.82, P \leq 0.001$), with the mean estimates ranging from 8.40 and 13.33 pg/2C DNA in the 6x taxa to 20.44 pg/2C in the 12x individuals (Fig. 3.4, Table 3.1). Interestingly, significant differences were also observed between the two 6x subspecies, with the interior 6x ssp. leptosepala presenting significantly lower genome size estimates than the submaritime 6x ssp. howellii ($P < 0.05$; Figs. 3.1, 3.4A). The 9x Northern Caltha leptosepala had estimates overlapping those of (allopatric) 6x ssp. howellii ($P > 0.05$), but significantly different and intermediate estimates were obtained when compared with the 6x ssp. leptosepala and 12x Northern Caltha leptosepala entities growing in sympathy with the 9x Northern Caltha leptosepala ($P < 0.05$; Fig. 3.4B).

**Cytogeography**—The large-scale screening covering most of the distribution range of the Caltha leptosepala complex enabled the characterization of cytotype distribution patterns (Fig. 3.1, Appendix A). Most populations were single-ploidy (n = 96), although our sampling was very limited in most populations (mean = 2.8 samples per population). Some mixed-ploidy populations were also found (n = 3; Table 3.1). Also, FCM analysis of 10 of the surveyed populations (ssp. howellii: pop. 30; ssp. leptosepala: pops. 13, 14, 40, 42; Northern Caltha
leptosepala: pops. 47, 52, 59, 69; Fig. 3.1) revealed that seedlings presented the ploidy level described for the population or taxon except in the mixed-ploidy population of the Northern Rockies (pop. 46): one family of seedlings from a parent of unknown ploidy collected in this locality yielded both 9x and 12x seedlings. The 9x Northern Caltha leptosepala (both seedling and field-collected) were found only in this population.

![Flow cytometric histograms](image)

**Figure 3.4.** Flow cytometric histograms of relative fluorescence intensity obtained after simultaneous analysis of propidium iodide stained nuclei isolated from Caltha leptosepala s.l. and Vicia faba cv. Inovec (2C = 26.90 pg of DNA, the internal reference standard): A hexaploids (6x) C. leptosepala ssp. leptosepala and C. leptosepala ssp. howellii; B hexaploid C. leptosepala ssp. leptosepala, nonaploid (9x) Northern Caltha leptosepala and dodecaploid (12x) Northern Caltha leptosepala.

Putative aneuploids were distributed as follows: three plants with the morphology of 6x ssp. howellii, including both air-dried and silica-dried material, gave 2C estimates approaching those of 12x Northern Caltha leptosepala (from pop. 77 in the Olympic Range of Washington, sympatric with 6x ssp. howellii; Figs. 3.1, 3.2, Table 3.1, Appendix A). Two additional specimens (the only samples from pop. 12 in the southern Rockies of Colorado) from a single herbarium sheet yielded 2C estimates intermediate to those of 6x ssp. leptosepala and 9x Northern Caltha leptosepala. In one case (pop. 46 in the Northern Rockies, sympatric with 6x ssp. leptosepala, 9x Northern Caltha leptosepala, and 12x Northern Caltha leptosepala), an
intermediate value between $9x$ and $12x$ samples was obtained from silica-dried material. Finally, one individual originally identified as Northern *Caltha leptosepala* (pop. 48) presented an outlier $2C$ estimate from herbarium material, much greater than any values obtained in this study (Figs. 3.1, 3.2, Table 3.1, Appendix A).

The two $6x$ subspecies are distributed allopatrically: about 530 km separate the closest populations of $6x$ ssp. *leptosepala* in the Sawtooth Range in the Northern Rockies (pop. 46) from $6x$ ssp. *howellii* growing on Mt. Hood in the Oregon Cascades (pop. 57; Fig. 3.2). The $6x$ ssp. *leptosepala* populations are distributed south through the Southern Rockies, while $6x$ ssp. *howellii* occurs only in the Cascade-Sierra axis. Almost exclusively, $6x$ individuals are present in the southern parts of both Cascade-Sierra and Rocky Mountain Ranges (Figs. 3.1–3.3). All samples from British Columbia and Alaska are $12x$, with the exception of three $6x$ chromosome counts from coastal British Columbia (C6–C8; Taylor and Mulligan, 1968; Figs. 3.1, 3.3). Disjunct populations of $12x$ Northern *Caltha leptosepala* also occur in the Blue Mountains of northeast Oregon (pop. 53), the eastern Cascades of south-central Oregon (pop. 31), and in the central Sierra Nevada of California (pop. C1; Smit and Punt, 1969; Figs. 3.1, 3.3).

In addition, two main contact areas between different cytotypes were detected (Figs. 3.1, 3.2). In the Cascades of Oregon and Washington, a region of overlap between the ranges of $6x$ ssp. *howellii* and $12x$ Northern *Caltha leptosepala* was observed. Two Cascade Range populations yielded both $6x$ ssp. *howellii* and $12x$ Northern *Caltha leptosepala*: Mount Hood, Oregon (pop. 57, with 11 $6x$ and 13 $12x$ individuals sampled), and Takhlakh Meadow, Washington (pop. 61, with eight $6x$ and 13 $12x$ individuals sampled). In the Sawtooth Range of the Northern Rockies, $6x$ ssp. *leptosepala* and $12x$ Northern *Caltha leptosepala* occur in parapatry, with the former to the southeast and the latter to the northwest (Fig. 3.2). A single
mixed-ploidy population (pop. 46) divides the range of the two cytotypes. This mixed-ploidy population was composed of 6x (13 samples), 9x (eight samples), and 12x (six samples) individuals.

**Morphology**—The comparison of leaf length-to-width ratio (L:W) showed significant differences between some of the taxa or cytotypes ($H_3 = 117.61$, $P < 0.0001$; Fig. 3.5, Table 3.1). *Caltha leptosepala* ssp. *howellii* (6x) in the Cascade-Sierra axis have reniform to orbicular leaves $0.78 – 1.01 \times$ as long as broad (median, mean ± s.d.: 0.88, 0.87 ± 0.05), with sinus open or closed (i.e., with overlapping basal leaf auricles). In the Rockies, 6x *C. leptosepala* ssp. *leptosepala* have oblong-ovate leaves $1.16 – 2.24 \times$ as long as broad (1.83, 1.77 ± 0.29).

Northern *Caltha leptosepala* (12x) have oblong to obovate leaves $1.03 – 1.41 \times$ as long as broad (1.21, 1.22 ± 0.12), and a shallow (usually open) sinus, while 9x Northern *C. leptosepala* have...
similar leaves 1.13 – 1.51× as long as broad (1.33, 1.36 ± 0.17). L:W was significantly different between all cytotypes and taxon pairs ($P < 0.05$) except between 9x and 12x Northern C. leptosepala and between 6x ssp. leptosepala and 9x Northern C. leptosepala ($P > 0.05$; Fig. 3.5). A comparison of mean leaf length by width (L×W) showed large differences, but also considerable variation and high overlap between taxa (Table 3.1). Significant differences were also observed for mean L×W ($H_3 = 36.26$, $P < 0.0001$), with all pairs differing ($P < 0.05$) except the 9x Northern C. leptosepala and any other taxon or cytotype, and 6x ssp. howellii and 12x Northern C. leptosepala ($P > 0.05$).

3.4 Discussion

The Caltha leptosepala polyploid complex presents clear patterns of cytotype distribution at the large scale, with hexaploids in the south, dodecaploids in the north, and nonaploids encountered in a single population in the Northern Rockies. There are also clear differences in morphology and genome size between the hexaploid subspecies–C. leptosepala ssp. howellii in the Cascade-Sierra axis and C. leptosepala ssp. leptosepala in the Rockies. A lack of discrete differences in macro-morphology between hexaploid C. leptosepala ssp. leptosepala and dodecaploid Northern Caltha leptosepala has historically obscured the geographic distribution of cytotypes in the polyploid complex and misled previous delineations of the taxa. The present study, using flow cytometry to estimate genome size and infer DNA ploidy levels across the range of C. leptosepala s.l., has revealed that subalpine marshmarigold may be best viewed as a polyploid complex, with cryptic differences between cytotypes in the Rockies. Our present study of the cytogeography of C. leptosepala s.l. informs its biogeography, evolutionary history, and taxonomy, and lays the groundwork for further investigations of the
complex.

Table 3.2. Leaf blade length-to-width ratios (L:W), and leaf length multiplied by width (L×W, roughly leaf blade size in cm\(^2\)), provided as mean and standard deviation of the mean (and range values), grouped by taxon according to Smit and Punt (1969) and Smit (1973). Abbreviations: ssp. howellii = Caltha leptosepala ssp. howellii; ssp. leptosepala = C. leptosepala ssp. leptosepala. 6x, hexaploid; 9x, nonaploid; 12x, dodecaploid; an., aneuploid. Number of individuals (n) and populations (Pop) is provided for each taxon. Due to the presence of disparate genome size values, data for aneuploid Northern Caltha leptosepala estimates are listed (separated by commas) rather than averaged.

**Cytotype distribution, refugia, and vicariance**—Hexaploid Caltha leptosepala s.l. occur almost exclusively to the south of the LGM southern limit of the Cordilleran ice sheets, with the notable exception of the Haida Gwaii populations (C6–C8 in Fig. 3.1), while the range of dodecaploids stretches from south of the LGM boundary to the far north of the polyploid complex’s range (Figs. 3.1, 3.3). Similar patterns—diploids or lower-ploidy cytotypes restricted
to less extensively glaciated regions and higher-ploidy cytotypes in both unglaciated and in
disturbed, heavily glaciated areas–have been found in cytogeographical studies of *Senecio
carniolicus* (Asteraceae) in the European Alps (Suda et al., 2007; Sonnleitner et al., 2010,
2013), *Dianthus broteri* (Caryophyllaceae) on the Iberian Peninsula (Balao et al., 2009), and
*Heuchera cylindrica* (Saxifragaceae) in the Pacific Northwest (Godsoe et al., 2013), although
the opposite pattern has also been observed (e.g., *Chamerion angustifolium*, Onagraceae) in
North America with diploids mainly occupying formerly glaciated regions (Husband and
Schemske, 1998; Thompson et al., 2014).

Smit and Punt (1969) proposed that during the Pleistocene (2.6 mya–11.5 kya) a
previously continuous distribution of *C. leptosepala* s.l. was restricted to two separate refugia to
the south of the LGM ice sheets, with ancestors of ssp. *howellii* persisting in the Sierra Nevada
and ancestors of ssp. *leptosepala* in the southern Rockies. Vicariant divergence between
lineages in interior and subarctic regions, as suggested by Smit and Punt (1969), is partially
supported in our study, though further sampling and other (e.g., molecular, phylogeographic)
data are needed for a more robust test of this hypothesis. Intriguingly, the two cytotype contact
zones detected here (Figs. 3.1–3.3) occur in or near regions reported to be refugia for other taxa:
the Cascades (Soltis et al., 1997; Hewitt, 2004; Latch et al., 2009) and the Northern Rockies
(Brunsfeld et al., 2001; Brunsfeld and Sullivan, 2005).

The most notable exception to the north-south distribution of cytotypes is the presence of
apparently disjunct populations of hexaploid *C. leptosepala* ssp. *howellii* in the islands of Haida
Gwaii (C6–C8 in Fig. 3.1; Taylor and Mulligan, 1968). These islands (or the currently
submerged portions of the surrounding continental shelf) at the western margin of the
Cordilleran ice sheet either remained unglaciated during the LGM or were one of the earliest
regions to become free of ice (Fig. 3.3; Dyke, 2004; Lacourse et al., 2005, 2012). However, this apparent disjunction for *C. leptosepala* ssp. *howellii* is likely not real but simply an artifact of sampling for the present study, as ssp. *howellii* is known to grow in coastal British Columbia (Brayshaw, 1989; Douglas et al., 2002; Pojar and MacKinnon, 2013). Nevertheless, it remains unknown whether this northward extension of hexaploid ssp. *howellii* is a relict of a coastal LGM refugium (e.g., Soltis et al., 1997) or is descended from the earliest colonizing lineages. These populations were also apparently missed in the geographic delineations of taxa in Smit and Punt (1969; Fig. 3.1).

At present, it is impossible to estimate with any precision the timing of divergence between the allopatric subspecies, which may have begun much earlier than during the LGM as suggested by Smit and Punt (1969). For example, orogeny of the Cascades ~5–2 mya (Graham, 1999, 2011) could have driven divergence between ancestral *C. leptosepala* lineages. This mountain uplift event drove xerification of the high plateaus to the east, essentially isolating mesic and alpine components of the northern Rocky Mountains (Brunsfeld et al., 2001, 2007; Carstens et al., 2005) and created the opportunity for allopatric divergence between populations with a formerly continuous distribution (Daubenmire, 1975). In *Caltha leptosepala* s.l., allopatry between the hexaploid subspecies paired with clear morphological and genome size differences (Figs. 3.1, 3.4A, 3.5, Table 3.1) is consistent with incipient speciation between lineages (Loureiro et al., 2010) and also mirrors phylogeographic patterns found in some other coastal-interior disjunct entities (e.g., the conifer *Pseudotsuga menziesii*; Brunsfeld et al., 2001; the amphibians *Ascaphus, Dicamptodon, and Plethodon*; Carstens et al., 2005). If possible, careful molecular/fossil dating of the divergence between the two *C. leptosepala* subspecies would address the potential role of orogeny- vs. climate-induced speciation.
**Cytotype contact zones**—In a review of the influence of ploidy on species range focused on angiosperm genera in North America, Martin and Husband (2009) found greater overlap between the ranges of diploids and congeneric polyploids than between sister diploid species, perhaps due to phenotypic divergence and reproductive isolation between cytotypes (Husband et al., 2013). Similarly, in the present study the hexaploid subspecies of *Caltha leptosepala* are fully allopatric in distribution, and cytotype contact zones are disjunct. Limited sympatry occurs between cytotypes in both western and eastern regions.

Therefore, what can the cytogeographic patterns in subalpine marshmarigolds tell us about the potentially very different evolutionary histories and dynamics in the two contact zones? In the North Cascades a diffuse contact zone exists between hexaploids to the south and dodecaploids to the north, with sympatry or parapatry between cytotypes across much of the Cascades in Washington and northern Oregon (Figs. 3.1, 3.2). In the Cascade-Sierra axis we found only two populations (near the Columbia River at the Oregon-Washington border) where hexaploid and dodecaploid cytotypes occur in direct sympatry, and further north the hexaploids occupy the wetter western flank of the North Cascades while the dodecaploids are located at higher and drier elevations further east (Fig. 3.2, Appendix A). We observed the potential for ecological differentiation at the microhabitat scale in the Cascade Range mixed-cytotype populations, and found that hexaploids occupy wetter and shadier sites than dodecaploids (K. Wefferling, personal observation). Spatial segregation of cytotypes has been observed in diploid and tetraploid *Ranunculus adoneus* (Ranunculaceae) in the Rockies (Baack, 2004), where only two mixed-cytotype populations were found across the entire range of the taxon. Baack (2004) found triploid hybrids at very low frequencies (~1.6%) in the diploid-tetraploid contact zone,
and suggested that minority cytotype exclusion is an important driver of cytotype segregation in the polyploid R. adoneus. In Caltha, we did not detect evidence for hybridization in the Cascades, even in the two mixed-ploidy populations, suggesting a lack of intracytotype gene flow mediated by intermediates (i.e., nonaploids) in the Cascade-Sierra axis. However, gene flow among cytotypes may be occurring through the multiple independent origins of polyploids (e.g., Sigel et al., 2014; Servick et al., 2015), as discussed below (“Secondary Contact Hypothesis and mode of origin” subsection). Rare cytotypes may also remain unsampled, and the presence of nonaploids in the Cascade-Sierra axis cannot be ruled out with our limited sampling. As an example, Senecio carniolicus s.l. in the European Alps and Carpathians was once believed to be almost exclusively hexaploid, but FCM analysis of 402 individuals from 82 populations revealed five cytotypes (2x, 4x, 5x, 6x, and 7x), three of which (4x, 5x, and 7x) were previously undetected (Suda et al., 2007). By sampling 5033 individuals from 100 populations, Sonnleitner et al., (2010) discovered three additional cytotypes, such that all ploidy levels from diploids (2x) through nonaploids (9x) are now known. Additional targeted sampling in the C. leptosepala complex will be crucial for detecting cryptic cytotype contact zones and additional rare cytotypes.

In contrast to the more extensive Caltha leptosepala s.l. cytotype contact zone described for the Cascades, sampling in the Northern Rockies yielded a single sympatric population of hexaploid, nonaploid, and dodecaploid individuals, dividing dodecaploids to the northwest and hexaploids to the southeast (Figs. 3.1, 3.2). Notably, this is the only population where nonaploids were found. No microhabitat differences were apparent here, but flowering occurred later in hexaploids than in nonaploids or dodecaploids (K. Wefferling, personal observation), possibly mediating some temporal isolation between cytotypes. In our single Northern Rockies
mixed cytotype population, the presence of an intermediate cytotype at possibly high proportions (eight nonaploid individuals out of 27 total; note that nonaploids and dodecaploids are indistinguishable and therefore our collections of these cytotypes should be unbiased) and detection of nonaploid and dodecaploid seedlings from the same wild parent suggest a high potential for gene flow between cytotypes where they overlap.

Putative aneuploids (unknown cytotypes based on outlier FCM genome size estimates from both herbarium and silica-dried material; Table 3.1, Appendix A) were detected in four populations: in the Southern Rockies (pop. 12) far from any cytotype contact zone; on the Olympic Peninsula, isolated from any documented cytotype contact zones but sympatric with hexaploid ssp. howellii; and two individuals from two populations in the Northern Rockies near or in the hexaploid (ssp. leptosepala)-nonaploid-dodecaploid (Northern Caltha leptosepala) mixed cytotype population (Figs. 3.1, 3.2). Unfortunately, no chromosome counts are available for these individuals. However, given that the genome size estimates are derived from both herbarium and silica-dried material and results are consistent between putative aneuploids from a given population (Appendix A), differences in genome size are most likely real. In recently formed polyploids, aneuploidy occurs at higher rates than in established polyploids or diploids (Ramsey and Schemske, 2002). Aneuploids could be expected to form especially in the 6x-9x-12x contact zone of the Northern Rockies (Figs. 3.1, 3.2) where inter-cytotype crosses with chromosomally unbalanced gametes may occur at non-negligible rates.

Natural mixed ploidy populations may persist due to mating barriers between the cytotypes (Levin, 1975; Baack, 2005; Godsoe et al., 2013). Alternatively, post-mating isolation could drive stable contact zones, as observed in a mixed-ploidy population of Senecio carniolicus, where heteroploid crosses involving diploids had lower fitness than crosses
between polyploids (Sonnleitner et al., 2013). Population-level screening for minority cytotypes (i.e., performed at multiple life stages including seeds, seedlings, and adults) should be performed in contact zones of *C. leptosepala* s.l. In a study of *Aster amellus* (Asteraceae) in Europe, fine-scale screening of all individuals in a mixed-ploidy population of diploids and hexaploids found no intermediate (i.e., tetraploid) adults or seedlings, but notably found tetraploid (and other ploidy level) seeds from both diploid and hexaploid parents (Castro et al., 2011, 2012). Small-scale analysis of mixed-cytotype populations would inform our understanding of micro-geographic cytotype distribution, production of unreduced gametes, gene flow within and among ploidy levels, and relative fertility of the different cytotypes, all contributing to an understanding of the factors involved in cytotype coexistence (Petit et al., 1999; Castro et al., 2012; Husband et al., 2016).

**Secondary Contact Hypothesis and mode of origin**—A prevalence of polyploid plants in arctic and alpine regions has been attributed to refugial history and secondary contact of diverged lineages (Stebbins, 1984, 1985). Stebbins (1984) proposed the “Secondary Contact Hypothesis” to explain the higher incidence of polyploids in previously glaciated regions of western North America. This hypothesis invokes recurrent cycles of allopatric divergence of populations in glacial refugia followed by mixing of ancestral genomes in hybrid or contact zones after deglaciation (Stebbins, 1984, 1985; Murray, 1995; Brochmann et al., 2004). Hybridization in contact zones between genetically differentiated populations of the same species or between divergent diploid species may drive the formation of polyploids (Parisod et al., 2010). Positive correlations between degree of Pleistocene glaciation and polyploidy have been proposed and observed in some floras and taxa, e.g., North American Poaceae (Stebbins,
1984), high arctic endemics (Brochmann et al., 2004), and arctic-alpine Primula (Primulaceae; Guggisberg et al., 2009). The clustering of Caltha leptosepala s.l. cytotype contact zones near the southern limit of LGM ice sheets (Figs. 3.1, 3.3) is consistent with Stebbins’ (1984, 1985) Secondary Contact Hypothesis, as only southern and possibly coastal refugia are supported by the distribution of the C. leptosepala polyploid complex.

Whereas polyploid plants in general might have an adaptive advantage over diploids in recolonizing deglaciated terrain (Levin, 2002), large glacial refugia likely harboured both low- and high-ploidy cytotypes and species through glacial maxima (Brochmann et al., 2004). The cytotype distribution of Caltha leptosepala s.l. suggests that through dispersal from refugia, dodecaploids have tracked the retreat of glacial ice in a leading edge effect (Hewitt, 1993; Soltis et al., 1997) from the south. The establishment and predominance of dodecaploids in the north could have come about in a number of ways. After long-distance dispersal from the ice sheet margins, dodecaploids may have competitively excluded (and continue to exclude) conspecific hexaploid entities from invading otherwise suitable habitats. Competitive exclusion could conceivably occur whether dodecaploids are of auto- or allopolyploid origin due to genomic buffering (gene redundancy leading to sub- or neofunctionalization of duplicated genes), biochemical diversity, and, in the case of allopolyploids, higher allelic diversity in fixed-heterozygous individuals (Stebbins, 1985; Brochmann et al., 2004; Adams and Wendel, 2005; Parisod et al., 2010; te Beest et al., 2012). Alternatively, through minority cytotype exclusion (i.e., frequency-dependent selection against rare cytotypes due to chromosome number incompatabilities; Levin, 1975; Baack, 2005), hexaploids may be unable to establish in regions dominated by dodecaploids. Any inter-cytotype matings (6x × 12x) would result in the production of nonaploids or more dodecaploids (if the hexaploid produces unreduced gametes).
Furthermore, nonaploids, if viable, may produce extremely variable gametes (Ramsey and Schemske, 1998; Costa et al., 2014). Backcrossing with parental cytotypes might in turn give rise to more nonaploids and dodecaploids or to other odd ploidy levels, creating a dynamic contact zone with recurrent polyploid formation. Trials of parental cytotypes and their hybrids in common-garden or reciprocal transplant experiments for assessment of inter-cytotype mating compatibilities, phenology, and fitness (e.g., Husband, 2000; Castro et al., 2011; Husband et al., 2016) are needed to discern adaptive from non-adaptive processes responsible for the extant cytogeography of *C. leptosepala* s.l.

Based almost exclusively on herbarium studies, Smit and Punt (1969) concluded that the highly variable *Caltha leptosepala* in the north of the species range (our Northern *Caltha leptosepala*) was of hybrid origin, but did not address ploidy level in their hypothesis. A hybrid origin of Northern *C. leptosepala* is consistent with the current cytogeographic patterns of the complex: the extant distribution of *C. leptosepala* s.l. cytotypes provides evidence for LGM survival only to the south and possibly west of the Laurentide and Cordilleran ice sheets (Figs. 3.1, 3.3). Due to a lack of hexaploids in the far north, survival of *C. leptosepala* in a Beringian refugium, as proposed by Hultén (1937), is not supported, although ancestral hexaploids could have been displaced by dodecaploids after their emergence. Preliminary nuclear ribosomal DNA sequence data (K. Wefferling and S. Hoot, unpublished data) support an allopolyploid origin for at least some dodecaploid Northern *C. leptosepala*. Whether the mode of origin proves to be autopolyploid or allopolyploid (or both) in different regions, a history of multiple independent origins of higher-level polyploids should be considered, as such a scenario has been documented in an ever-growing number of cases (Brochmann et al., 2004; Guggisberg et al., 2009; Soltis et al., 2010; Hegarty et al., 2013; Sigel et al., 2014; Servick et al., 2015; Laport et al., 2016). Given
their geographic spread and morphological variation, dodecaploid Northern *Caltha leptosepala*
likely have formed recurrently, incorporating genetic diversity from divergent populations of
hexaploid progenitors. Ongoing analyses of nuclear and chloroplast molecular sequence data
will address the mode and number of origins of the dodecaploids and nonaploids.

Decrypting the mode of origin for the hexaploids would provide a fuller picture of the
*Caltha leptosepala* polyploid complex, but will require broader taxonomic sampling within
*Caltha*, increased screening of chromosome numbers across the genus, and integration of data
from a number of nuclear loci. *Caltha leptosepala* s.l. is resolved as basal within the otherwise
entirely southern hemisphere and hexaploid 

### Morphology, range, and taxonomic implications

*Extensive morphological variation among taxa and populations in the Caltha leptosepala* polyploid complex has led to contrasting
taxonomic treatments, from their subdivision into as many as nine species (Greene, 1899) to
their grouping into a single taxon with no recognition of subspecies or varieties (Ford, 1997). In
the Cascade-Sierra axis, field identification of hexaploid ssp. *howellii* and dodecaploid Northern *Caltha leptosepala* is clear and, with few exceptions, leaf length-to-width ratio is sufficient for this task. Northern *Caltha leptosepala* is characterized by oblong-ovate leaves with a cordate base and a narrow, open sinus; it is broadly sympatric with hexaploid ssp. *howellii*, though Northern *Caltha leptosepala* tends to be more continental and grows at higher elevations, ranging from the central Sierra Nevada of California sporadically north through the Cascade and Olympic ranges and Vancouver Island, and becoming increasingly common in the Canadian Rockies and alpine parts of the Coast Ranges of British Columbia to coastal Alaska and the Chugach Ranges of Alaska (Klinka et al., 1989). In the Rockies, identification of Northern *Caltha leptosepala* and its separation from spp. *leptosepala* based on morphology alone is difficult and may require examination of micro-morphological characters such as pollen. While in most of the distribution range the mean leaf length-to-width ratios are significantly different between hexaploid *C. leptosepala* ssp. *leptosepala* and dodecaploid Northern *Caltha leptosepala*, these ratios greatly overlap in the US Rockies. In the Northern Rockies of Idaho (and possibly in the Bitterroot Mountains of Montana), nonaploid and dodecaploid Northern *Caltha leptosepala* are often larger, more robust plants with broader leaves and more sinuate leaf margins than hexaploid ssp. *leptosepala*. Given the morphological similarities between Rocky Mountain Range cytotypes, it is not surprising that previous workers grouped hexaploid *C. leptosepala* ssp. *leptosepala* with dodecaploid Northern *Caltha leptosepala*, leading to a misleadingly broad range inferred for ssp. *leptosepala*. However, our results suggest a northern and western limit of *C. leptosepala* ssp. *leptosepala* in the Northern Rockies of Idaho (Figs. 3.1–3.3).

The two hexaploid subspecies as defined by Smit and Punt (1969) and Smit (1973) are
easily differentiated from one another by both morphology and geography: broad leaves and a Cascade-Sierran distribution characterize *C. leptosepala* ssp. *howellii*, while *C. leptosepala* ssp. *leptosepala* occur only in the Rockies and have narrow leaves. These subspecies are fully allopatric in distribution and, given the present data, separated by over 500 km of the intervening arid to semi-arid Oregon Highlands. However, this region includes several mountain ranges where *Caltha leptosepala* s.l. occurs (Ochoco, Steens, Blues) but cytotype information is mostly lacking (dodecaploid Northern *C. leptosepala* is found in the Blue Mountains; Figs. 3.1–3.3). This distribution contrasts with ranges described in most floras of the region that apparently conflate hexaploid *C. leptosepala* ssp. *leptosepala* with dodecaploid Northern *Caltha leptosepala* (e.g., Anderson, 1959; Hitchcock et al., 1964; Hultén, 1968; Hitchcock and Cronquist, 1973; Welsh, 1974; Welsh et al., 1987; Turner and Gustafson, 2006; Pojar and MacKinnon, 2013).

### 3.5 Conclusion

Our exploratory study provides the first look at cytotype distribution in the *Caltha leptosepala* polyploid complex and identifies cytotype contact zones. We have found support for persistence of *C. leptosepala* s.l. to the south and perhaps west of LGM ice sheets, likely in separate (submaritime and continental) refugia. Range delimitations for the cytotypes and the hexaploid subspecies (*C. leptosepala* ssp. *howellii* and *C. leptosepala* ssp. ssp. *leptosepala*) as found here are radically different from most local floras or wildflower field guides and should inform regional botanists and floras. Although sampling is still limited, our data suggest genome size variation between and within cytotypes, morphological differentiation among cytotypes, and a paucity of intermediate cytotypes except at a single mixed-ploidy population in the
Sawtooth Range of the Northern Rockies. The cytogeography, morphology, and genome size estimates presented here allow for key insights into taxon ranges and cytotype distributions (northern dodecaploids and southern hexaploids) and identify disjunct and evolutionarily distinct contact zones in submaritime and interior regions of the Pacific Northwest.
3.6 References


4.0 Chapter IV: Species circumscription of the *Caltha leptosepala* polyploid complex (Ranunculaceae) based on molecular and morphological data


4.1 Introduction

The subalpine marshmarigold polyploid complex, *Caltha leptosepala* Candolle (1818: 310) *sensu lato* (Ranunculaceae), is a heterogeneous group of herbaceous plants growing in mountainous regions of western North America. A recent cytogeographical study showed that hexaploids (2\(n = 6x = 48\)) are more southerly in distribution, growing mainly in the southern Rockies, Cascade-Sierra axis, and Coast Ranges (Wefferling et al., 2017). Dodecaploids (2\(n = 12x = 96\)) generally occupy the north of the range, from the Northern Rockies and Cascades in the south to the Alaska Range in the north (Wefferling et al., 2017). Known nonaploids (2\(n = 9x = 72\)) are limited to a single population in the Northern Rockies of Idaho, U.S.A., in a contact zone between hexaploids and dodecaploids (Wefferling et al., 2017). The Pacific Northwest of North America is an important intersection of three major lineages: two hexaploids and a morphologically intermediate dodecaploid (Smit and Punt, 1969, Wefferling et al., 2017). The present study reviews the taxonomic history of the complex, examines molecular, biogeographic, cytological, and morphological evidence for species-level recognition of taxa that are currently subsumed within *Caltha leptosepala*, and provides an updated taxonomy and key for the *Caltha leptosepala* polyploid complex. A brief history of the species that were, at one time or another, subsumed in *Caltha leptosepala* is given.
In 1818, Candolle described two species of *Caltha* in western North America: *C. biflora* Candolle (1818: 310) (Fig. 4.1) and *C. leptosepala* Candolle (1818: 310) (Fig. 4.2). Lawson (1884) recognized only one of Candolle’s (1818) two species, relegating *C. biflora* to *C. leptosepala* var. *biflora* (Candolle) Lawson (1884: 69), though with reservations about this placement. Huth (1892) also recognized only one species: *C. leptosepala* with two varieties, *C. leptosepala* var. *rotundifolia* Huth (1892: 68) and *C. leptosepala* var. *howellii* Huth (1892: 68) (Fig. 4.3). Huth (1892) questioned whether Candolle’s original description of *C. biflora* was an accurate description of the type specimen (Greene, 1899), pointing out that Candolle (1818) referred to the leaves as having a “sinu latissimo”, or a very broad sinus at the leaf base. Indeed, in the holotype specimen for *C. biflora* (Fig. 4.1), if not prevented by the mounting and drying process, the sinuses of at least some of the leaves would be closed, with overlapping leaf auricles (Huth 1892, Greene 1899). Regional variation in characters led Greene (1899) to segregate the complex into nine species: *C. biflora, C. malvacea* Greene (1899: 75), *C. leptosepala, C. macounii* Greene (1899: 77), *C. chelidonii* Greene (1899: 78), *C. howellii* (Huth) Greene (1899: 79), *C. rotundifolia* (Huth) Greene (1899: 80), *C. chionophila* Greene (1899: 80) (Fig. 4.4), and *C. confinis* Greene (1899: 76; though *C. confinis*, based on a single incomplete specimen [US 270276, collected by *J. T. White s.n.*], is almost certainly *C. palustris* Linneaus [1753: 558]; Smit and Punt 1969). Rydberg (1900), working on the Flora of Montana and Yellowstone National Park, recognized *C. rotundifolia*, and described *C. uniflora* Rydberg (1900: 474). Davis (1900) recognized a combination of species described by Candolle (1818), Greene (1899), and Rydberg (1900), and additionally described two varieties: *C. biflora, C. chionophila, C. leptosepala, C. confinis, C. chelidonii, C. uniflora, C. rotundifolia var. howellii* (Huth) Davis (1900: 15), and *C. leptosepala var. macounii* (Greene) Davis (1900: 16). Abrams (1944) recognized *Caltha biflora*

By integrating molecular, biogeographic, cytological, and morphological data, we aim to build on earlier work and provide an updated taxonomy and key for the Caltha leptosepala polyploid complex.
Figure 4.3. Neotype of *Caltha leptosepala* var. *howellii*, collected by R. M. Austin, July 1896. “Colby, Butte County, Northern (U.S.A.), California”. Housed at the Greene-Nieuwland Herbarium, Notre Dame, U.S.A. (NDG17325). Inset photo shows narrow filaments; magnified 5×.
Figure 4.4. Isolectotype of *Caltha chionophila*, collected by C. F. Baker, 1899. “Near Pagosa Peak, 11,000 ft., Colorado” (U.S.A.). Housed at the Greene-Nieuwland Herbarium, Notre Dame, U.S.A. (NDG17369). Inset photo shows broad filaments; magnified 5×.
4.2 Materials and methods

Sampling:—The sampling of Schuettpelz and Hoot (2004) served as a starting point, and included nine outgroup species of *Caltha*. From the *Caltha leptosepala* polyploid complex, we included two accessions each of the hexaploid taxa, from either the Cascades of Washington and Oregon in the United States (identified as “*Caltha leptosepala* subsp. *howellii*” in Wefferling et al., 2017) or from the Rockies of Colorado and Idaho (identified as “*C. leptosepala* subsp. *leptosepala*” in Wefferling et al., 2017), and four accessions of dodecaploid *C. leptosepala* from the Coastal Range in southeast Alaska, the North Cascades of Washington, the Blue Mountains of northeastern Oregon, and the Northern Rockies of Idaho (identified as “Northern *Caltha leptosepala*” in Wefferling et al., 2017) (Appendix B).

Genomic DNA was extracted from silica-dried or herbarium leaf material using either the DNeasy Plant Mini Kit (Qiagen, Valencia, California, U.S.A.) or the GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) following manufacturers’ protocols after homogenization in a mortar and pestle under liquid nitrogen or in 2 mL tubes with a tungsten bead and lysed in a TissueLyser II (Qiagen) bead mill for two 30 sec cycles at 20 Hz.

Molecular data collection:—For PCR amplification and Sanger sequencing, we targeted both nuclear ribosomal internal transcribed spacer (ITS) and chloroplast (cpDNA) regions in order to track hybridization events and the direction of crosses. Molecular data included ITS, cpDNA intergenic spacer *rpL32–trnL*\textsuperscript{UAG} (*rpL32–trnL*), and *trnL*\textsuperscript{UAA}–*trnF*\textsuperscript{GAA} (*trnL–trnF*) (Table 1). PCR was performed in 20 μL reactions as follows: 7 μL GoTaq Green Master Mix (Promega Corporation), 10 μL water, and 1 μL each of 10 μM upstream and downstream primers. Initial ITS amplification primers were based on those of Nickrent et al. (1994) and then redesigned.
based on preliminary sequence data (Table 4.1). The cpDNA \textit{rpL32-trnL} primer sequences were obtained from Shaw et al. (2007) then modified as necessary (Table 4.1). CpDNA \textit{trnL-trnF} primers were from Taberlet et al. (1991) and Azuma et al. (2011) (Table 4.1). PCR conditions for all reactions were as follows: 5 min at 94º C, followed by 41 amplification cycles (1 min at 94º C 1 min at 52º C 1 min 30 sec at 72º C), a final extension step of 7 min at 72º C, then cooled to 4º C. After checking for successful amplification on a 1% TBE agarose gel with ethidium bromide, 7 µL of each PCR amplicon were treated with 4 units of Exonuclease I (Thermo Fisher Scientific) and 1 unit of FastAP thermosensitive alkaline phosphotase (Thermo Fisher Scientific) in a total volume of 10 µL at 37º C for 30 min and 80º C for 15 min. Amplicons were then sent to the University of Chicago comprehensive cancer center DNA sequencing and genotyping facility (http://cancer-seqbase.uchicago.edu/) for Sanger sequencing of both DNA strands on an Applied Biosystems (Foster City, California, U.S.A.) 3730XL 96-capillary automated DNA sequencer using the amplification primers. When direct sequencing of ITS amplicons resulted in polymorphic chromatograms (putative hybrids with insertions or deletions among ribotypes), the mixed PCR product was column-purified using the QIAquick Gel Extraction Kit (Qiagen) and then cloned with the pGEM-T easy vector system and JM-109 competent \textit{E. coli} cells (Promega Corporation, Madison, Wisconsin, U.S.A.). Successfully transformed (white) colonies were picked and re-amplified using the RNA polymerase promoter primers SP6 (5’-TATTTAGGTGACACTATAG-3’) and T7 (5’-TAATACGACTCACTATAGGG-3’) with the following PCR conditions: 2 min at 94º C, followed by 30 cycles of 15 sec at 94º C 15 sec at 40º C, 45 sec at 72º C, a final extension step of 5 min at 72º C, then cooled to 4º C. Amplicons were sequenced in one direction with the SP6 primer. After initial sequences were compared from
hexaploids and dodecaploids, repeat-specific primers (Rauscher et al., 2002) were designed to separate ribotypes in putative hybrids (Table 4.1).

**Data analysis:**—Alignments (Table 4.2) were partitioned by gene region, codon, and spacer regions (8 cpDNA partitions, 3 ITS partitions). Partitionfinder 1.1.1 (Lanfear et al., 2012) determined the optimal partitioning scheme as comprising 3 partitions for cpDNA regions, and no partitioning of ITS (including ITS1, 5.8S, and ITS2). The nuclear and concatenated chloroplast datasets (Table 2) were analyzed separately using Bayesian Markov chain Monte Carlo (MCMC), maximum likelihood (ML), and maximum parsimony (MP) approaches. Posterior probability (PP) values ≥ 95% and bootstrap (BS) values of ≥ 70% (Hillis and Bull 1993, Alfaro et al., 2003) were considered moderate to strong support.

Bayesian MCMC phylogenetic inference was conducted using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012), with datasets partitioned as described above, using reversible jump MCMC (Huelsenbeck et al., 2004). Four chains (three heated) were run, sampling trees every 500 generations, until reaching a conservative convergence diagnostic of average standard deviation of split frequencies ≤ 0.005 (i.e., much lower than the 0.1 default value in MrBayes 3.2; Ronquist et al., 2012). Additionally, stationarity and convergence of runs were assessed visually by monitoring trace plots of parameters using Tracer v1.6 (Rambaut et al., 2014) and checking that effective sample sizes for all parameters were ≥ 200 (Drummond and Bouckaert, 2015). After discarding 25% of steps, a 50% majority rule consensus tree was generated. RAxML 7.2.8 (Stamatakis, 2006, implemented through Geneious 7.1.6, Kearse et al., 2012) was used for ML analysis. We used the GTR GAMMA model of sequence evolution (as recommended for trees with < 50 taxa in the RAxML manual v8.2.X, Stamatakis, 2016) with rapid bootstrapping, a search for the best-scoring ML
tree, and 1,000 BS replicates. PAUP* version 4.0b10 (Swofford, 2002) was used for MP analysis. To assess branch support, BS analyses were performed using a full heuristic search, with 500 replications of 20 random additions each; maxtrees were set to 5,000, and a 50% majority rule consensus tree was generated.

Nuclear ribosomal internal transcribed spacer (ITS)

<table>
<thead>
<tr>
<th>Primer Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1830F (Nickrent et al., 1994) forward</td>
<td>AACAAGGTTCCTCCGTAAGGTGA</td>
</tr>
<tr>
<td>25R (Nickrent et al., 1994) reverse</td>
<td>TATGCTTAAAYTCAAGCCGGT</td>
</tr>
<tr>
<td>364F (Hoot lab primer) internal forward</td>
<td>ATCGATGAGGAAGCGTACGC</td>
</tr>
<tr>
<td>390R (Hoot lab primer) internal reverse</td>
<td>CAATTCACACCAAGTATGCC</td>
</tr>
<tr>
<td>*Wr13 (this study) ribotype specific reverse</td>
<td>CTGGGTTCCAGG</td>
</tr>
<tr>
<td>*Er14 (this study) ribotype specific reverse</td>
<td>CCTGGGTTCCAGA</td>
</tr>
<tr>
<td>*Eo19 (this study) ribotype specific forward</td>
<td>TGTTGGATTTGTAAATCT</td>
</tr>
<tr>
<td>*Wf21 (this study) ribotype specific forward</td>
<td>GCAAAGATAGGGTACCAAGC</td>
</tr>
</tbody>
</table>

Table 4.1. Primers used in this study. Nucleotide sequences read 5’ to 3’. Polymorphic nucleotide sites are designated using IUPAC ambiguity codes. * ribotype specific primers, designed to amplify a single subgenome/ribotype.

Morphology:—Morphological and ecological data were gleaned from herbarium specimens and our own collections and observations of Caltha in the field. Herbarium specimens were borrowed from the following herbaria (using abbreviations as in the Index Herbariorum):

ALA, ASC, CIC, CSU, DAV, HSU, ID, MO, MONTU, NYBG, OSC, RBCM, RM, RSA, SRP, US, USFS/RM, UWM, V, and WTU. Digitized type specimens of Caltha were examined from BM, CAN, GH, K, NDG, NY, US, and WTU (the ! symbol is used to denote type specimens that were seen by the first author, albeit in their digital form; see descriptions in Taxonomic treatment section below). Morphological characters were examined using a stereomicroscope, measured,
and scored for samples from each of the cytotypes and putative taxa encompassing the entire geographic range (for a total of 140 specimens). Particular attention was given to the following characters: ratio of leaf blade length (including the leaf auricles) to width; form of auricles or basal leaf lobes (closed: large and overlapping to cover leaf sinus; diplophyllous: upturned over the leaf blade; or open: auricles not covering the sinus); number of flowers per stem (1–4); filament width relative to anther width (filamentous: much narrower than anthers; intermediate: approximately the same width as anthers; or broad: wider than anthers); attachment of carpels or follicles to peduncle (sessile, subtipitate, or stipitate); and shape of stylar beak (straight, curved, or rolled into tight fiddlehead form).

<table>
<thead>
<tr>
<th>Gene region</th>
<th>Aligned length</th>
<th>Taxa/homeologues</th>
<th>% missing or ambiguous data</th>
<th>% taxon coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>646</td>
<td>15</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>trnL-trnF</td>
<td>837</td>
<td>15</td>
<td>1.5</td>
<td>100</td>
</tr>
<tr>
<td>rpL32-trnL</td>
<td>952</td>
<td>8</td>
<td>48.5</td>
<td>53.3</td>
</tr>
</tbody>
</table>

Table 4.2. Statistics for gene regions used in molecular dataset.

Scanning electron microscopy (SEM) of 48 pollen samples was also performed, and number and shape of apertures (porate or colpate) were scored. For SEM, anthers were collected from herbarium specimens, dried overnight in a 50º C oven, then gently tapped over or rolled on stubs with ultra smooth carbon adhesive tabs (Electron Microscopy Sciences, Hatfield, PA, U.S.A.), coated with iridium (6 nm deposition at a 90º angle, followed by 5 nm at ~30º angle), and examined using a Hitachi S-4800 field emission scanning electron microscope at 3.0 kV. We examined at least 30 pollen grains of each specimen, and for publication we selected pollen.
grains that were “typical” of the specimen. Image processing and capture functions were made through the Hitachi PC-SEM software.

All figures were prepared (brightness and contrast, cropping, etc.) using Adobe Illustrator CS5 and Adobe Photoshop CS5.

Figure 4.5. Bayesian MCMC phylogram of Caltha species based on concatenated cpDNA data (rpL32–trnL and trnL–trnF). Posterior probability and bootstrap (ML and MP) support is given for each node. — indicates branch was not found. Dashed branches indicate less than moderate support for at least one approach (PP ≤ 0.95, BS ≤ 70). Psychrophila group and Caltha leptosepala complex indicated with vertical bars to right. Ploidy level (x = 8) and collection site is given for each sample. AK = Alaska, CO = Colorado, ID = Idaho, OR = Oregon, WA = Washington (all U.S.A.).

4.3 Results

The phylogenetic tree topologies derived from different approaches (Bayesian MCMC, ML, or MP) were all similar or identical when comparing a single dataset (cpDNA or ITS alone; Figs. 4.5, 4.6), but were discordant between datasets. Well-supported clades, by all measures (PP, MLBS, and MPBS) and in both datasets (cpDNA and ITS), included the “Psychrophila group” (Schuettpelz and Hoot, 2004), Caltha chionophila (ID, CO) + C. leptosepala (ID, OR), and C. biflora (OR, WA) + C. leptosepala (AK, WA) (Figs. 4.5, 4.6). In contrast, the C.
leptosepala complex as a whole was either monophyletic (cpDNA; Fig. 4.5) or paraphyletic (ITS; Fig. 4.6). Each C. leptosepala individual yielded two ITS ribotypes, one of which grouped with C. biflora, the other with C. chionophila, both with strong support (Fig. 4.6). Additionally, cpDNA from 12x C. leptosepala specimens either grouped with C. biflora or C. chionophila with strong support (Fig. 4.5).

**Figure 4.6.** Bayesian MCMC phylogram of Caltha species based on nuclear ribosomal DNA (ITS1, 5.8S, and ITS2). Posterior probability and bootstrap (ML and MP) support is given for each node. – indicates branch was not found. Dashed branches indicate less than moderate support for at least one approach (PP ≤ 0.95, BS ≤ 70). Dashed lines connect ribotypes from a single allododecaploid individual. Psychrophila group and Caltha leptosepala complex indicated with vertical bars to right. Ploidy level (x = 8) and collection site is given for each sample. AK = Alaska, CO = Colorado, ID = Idaho, OR = Oregon, WA = Washington (all U.S.A.).

Of the morphological characters examined, leaf length to width ratio (Wefferling et al., 2017), filament width, and pollen morphology (number and shape of pori or colpi) (Table 4.3) were most consistent with molecular data (Wefferling and Hoot, unpublished data) and cytological determination (Wefferling et al., 2017). However, pollen characters were not entirely consistent with leaf macromorphology or molecular data (Table 4.3; Figs. 4.7–4.10; Wefferling
and Hoot, unpublished data). Pollen from *C. biflora* ranged from pantoporate to pantocolpate (Fig. 4.7). *Caltha chionophila* was almost always tricolpate (Fig. 4.8) with some notable exceptions (Fig. 4.8H, I). *Caltha leptosepala* ranged from tricolpate (Fig. 4.9G) to pantoporate (Fig. 4.9I). Several specimens had malformed, variably sized, and apparently inviable pollen, including some hexaploids (Fig. 4.8I), dodecaploids (Fig. 4.9I), hybrid or non-hybrid “aneuploids” (based on flow cytometry data, Wefferling et al., 2017; Fig. 4.10A, B), and the single nonaploid specimen (Fig. 4.10C).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>ploidy (inferred or measured)</th>
<th>leaf length:width</th>
<th>leaf auricles</th>
<th>number of flowers/stem</th>
<th>filament: anther width</th>
<th>pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caltha biflora</em></td>
<td>$2n = 6x = 48$</td>
<td>0.7–1</td>
<td>large, often overlapping or diplophyllous</td>
<td>1–3</td>
<td>&lt; 1</td>
<td>9–12-porate (rarely -colpate with short colpi)</td>
</tr>
<tr>
<td><em>Caltha chionophila</em></td>
<td>$2n = 6x = 48$</td>
<td>1.2–3</td>
<td>small, rarely overlapping, often diplophyllous</td>
<td>1–3</td>
<td>&gt; 1</td>
<td>usually 3-colpate; rarely malformed</td>
</tr>
<tr>
<td><em>Caltha leptosepala</em></td>
<td>$2n = 12x = 96$ (rarely 9x = 72)</td>
<td>1–1.7</td>
<td>variable; sometimes overlapping, sometimes diplophyllous</td>
<td>1–3</td>
<td>≥ 1</td>
<td>(3–)4–8(12)-colpate (rarely -porate)</td>
</tr>
</tbody>
</table>

Table 4.3. Key morphological characters for discriminating among *Caltha biflora*, *C. chionophila*, and *C. leptosepala*. 


Figure 4.10. Pollen of putative aneuploid and nonaploid Caltha, determined by morphology and molecular data (Wefferling and Hoot, unpublished data). † †ploidy level determined by chromosome counts (Wefferling et al., 2015); † †ploidy level estimated by flow cytometry (Wefferling et al., 2017). A. Hybrid with morphology of C. biflora, with larger genome size (aneuploid?) than any other sampled C. biflora (Wefferling et al., 2017); U.S.A.: Washington, Hunter, G. LR5† (UWM). B. Non-hybrid with morphology of C. chionophila, with larger genome size (aneuploid?) than any other C. chionophila (Wefferling et al., 2017); U.S.A.: Colorado, Townesmith, A., G. Gust, and L. Nye 202† (UWM). C. Allononaploid (9x) C. leptosepala; U.S.A.: Idaho, Wefferling, K.M. 212rotA†† (UWM).


All members of the species complex can be described as follows: fleshy hairless herbs arising 5 to 40 cm from thick caudices or short rhizomes; simple petiolate leaves basal (to cauline), with dentate, crenate, or subentire margins; leaf bases cordate to sagittate, or auricles overlapping (sometimes upturned and covering part of the lamina; i.e., diplophyllous); plants with 1 to 6 perfect, hypogynous flowers, apetalous with 5 to 12 (to 18) white (maturing to
yellow-white) linear to oblong petaloid sepals (abaxially blue- or green-tinted); many (up to 50) stamens with filiform to broad and flattened filaments, the filaments covered with trichomes; 4 to 12 (to 32) sessile to stipitate carpels with stigma and style straight to slightly curved, carpels maturing to many-seeded follicles; ovules anatropous, seeds dark, with slight “checkerboard” texturing on seed coat, endosperm present, and embryo small relative to mature seed (Hitchcock and Cronquist, 1973; Morris, 1973; Smit, 1973; Ford, 1997).

4.4 Discussion

The present study includes more specimens of the Caltha leptosepala complex than previous phylogenetic work on the genus and provides novel insights into relationships among members of the polyploid complex. In particular, examining the phylogeny in light of ploidy level variation (Wefferling et al., 2015, 2017) and morphology (Wefferling et al., 2017, present study) allows for a better understanding of the biological diversity present in the group. Examination of type specimens (Figs. 4.1–4.4) and 140 additional accessions from across the geographical range of the complex supports the recognition of three species in the polyploid complex: hexaploid C. biflora, hexaploid C. chionophila, and allododecaploid C. leptosepala.

The discordance between nuclear and chloroplast phylogenies (Figs. 4.5, 4.6) is consistent with some earlier work on the genus (Schuettpelz and Hoot, 2004; Cheng and Xie, 2015), and the topologies are similar (Cheng and Xie, 2015; Liu et al., 2016) or identical (Schuettpelz and Hoot, 2004) to previous work. Notably, we did not combine nuclear and chloroplast data as in previous studies due to the presence of multiple nuclear ribotypes in the dodecaploid Caltha leptosepala. The discordant topologies between nuclear and chloroplast datasets could be explained by longer coalescent times in nuclear genomes due to a larger
effective population size than in chloroplast genomes paired with incomplete lineage sorting (Rautenberg et al., 2010). Alternatively, introgressive hybridization could explain the discordance (Hardig et al., 2000; Yoo et al., 2002; Rautenberg et al., 2010), though it is not clear which populations or lineages would be involved in such crosses.

Through the use of ribotype-specific primers and/or cloning of ITS PCR product, we were able to demonstrate a likely hybrid origin of the sampled dodecaploids between ancestral Caltha biflora and C. chionophila (Fig. 4.6). Further, in the cpDNA dataset allododecaploid C. leptosepala groups with either C. biflora or C. chionophila (Fig. 4.5), providing evidence for reciprocal origins of allododecaploid C. leptosepala.

Individual specimens are most easily grouped by leaf blade length to width ratio, filament width, and shape and number of colpi (Table 4.3). These characters, along with geography, are almost always sufficient for discriminating among Caltha biflora, C. chionophila, and C. leptosepala. However, pollen can be misleading in differentiating among taxa, despite reports of the informativeness of such characters (Smit and Punt, 1969). For example, C. biflora almost always has pantoporate pollen (Fig. 4.7), as found by Smit and Punt (1969), but some specimens have colpate pollen (Fig. 4.7I). Similarly, C. chionophila displays tricolpate pollen (Fig. 4.8), but sometimes diverges from this morphology, with colpi merged (Fig. 4.8H) or very poorly developed (4.8I). Notably, the previous exceptional cases occur in mixed-ploidy populations in the Pacific Northwest of North America (Wefferling et al., 2017). Pollen from allododecaploid C. leptosepala is usually 4–8-colpate (Fig. 4.9), but quite variable, with pantoporate (Fig. 4.9H), tricolpate (Fig. 4.9I), or malformed and apparently inviable (Fig. 4.9G) grains. Some rare plants, such as nonaploids and putative aneuploids (Wefferling et al., 2017), showed variable and malformed (Fig. 4.10C) or tricolpate pollen (Fig. 4.10B). One putative aneuploid with the
morphology of *C. biflora* (Wefferling et al., 2017) is apparently a hybrid (Wefferling and Hoot, unpublished data) with malformed to pantocolpate pollen (Fig. 4.10A).

In conclusion, some difficulties remain in discriminating between *Caltha chionophila* and *C. leptosepala* in the Northern Rockies (Wefferling et al., 2017) where the two are sometimes morphologically very similar. There are also some specimens that morphologically match *C. biflora* on the Olympic Peninsula (Washington, U.S.A.) and Vancouver Island (British Columbia, Canada), but have putatively aneuploid genomes (Wefferling et al., 2017) and hybrid origins (Wefferling and Hoot, unpublished data). Nevertheless, a combination of leaf and anther characters should allow for field identification in almost all cases. The few exceptions we have seen to the overall molecular, cytogenetic, and morphological patterns among members of the marshamarigold polyploid complex should be addressed through studies sampling a larger number of individuals from across the range.

### 4.5 Taxonomic treatment


Type:—CANADA. British Columbia: Northwest coast of North America, inland behind Banks Island (between Haida Gwaii and mainland), 1792, *Menzies s.n.* (holotype BM!). Fig. 1.

Type:—U.S.A. California: near Colby, Butte County, 1896, R.M. Austin s.n. (neotype, designated here, NDG!). Fig. 3.

_Caltha malvacea_ Greene (1899: 75).

Type:—U.S.A. Washington: Cascade Mountains, 1838–1842, _Wilkes Expedition 484_ (Lectotype US!).

**Morphology and cytology:**—Leaf blades 0.7–1 × as long as wide, reniform to orbicular (rarely emarginate), often diplophyllous (especially in the southern part of the range), margins crenate (in the north) to subentire (in the south) (Figs. 4.1, 4.3); flowers (1–)2(–3) per stem; filaments filiform, 0.1–0.2(–0.3) mm wide (Giblin et al., in press), narrower and often longer than anthers (Figs. 4.1, 4.11A); pollen pantoporate to pantocolpate (Fig. 4.7); follicles with stylar beak 0.1–0.2 mm in length (Giblin et al., in press); hexaploid (2n = 6x = 48, Wefferling et al., 2015; holoploid genome size ca. 10–18 pg/2C, Wefferling et al., 2017).

**Taxonomic notes:**—Regarding the original type specimen of _Caltha leptosepala_ var. _howellii_, collected by J. Howell in 1882 in the Cascade Mountains and deposited in “HGB” (HGB was Howell’s abbreviation for “Herbarium generale Berolinense” at Berlin, The Botanic Garden and Botanical Museum Berlin-Dahlem, current acronym B; B. Hellenthal, Museum of Biodiversity and Greene-Nieuwland Herbarium, pers. comm.): “…If the specimen has ever been part of our herbarium [B] then it was probably lost in WW2” (R. Vogt, Botanischer Garten und Botanisches Museum Berlin-Dahlem, pers. comm.). Another specimen collected by Howell (NDG17323) would be an ideal neotype, but lacks clear morphology of the carpels, among other important features. Therefore, NDG17325 (Fig. 4.3), collected by R.M. Austin near Colby, Butte Co. in 1896 is selected as neotype as (1) it is mentioned by Greene (1899), (2) the leaves bear resemblance to those drawn in Huth’s (1892) treatment (his figure 8), and (3) it is a complete
specimen showing stamens, nearly mature and immature carpels, etc. The diplophyllous nature of the leaves (Huth, 1892) is not so clear in this specimen, but one upturned auricle can be seen in the leftmost leaf.

**Geographic distribution:**—Lowland to subalpine in and west of Coast Ranges and Cascade-Sierra axis; from southeast Alaska south through coastal British Columbia, Washington, Oregon, California, and western Nevada (Wefferling et al., 2017; Giblin et al., in press).

**Additional specimens examined** are listed in Appendix C.

*Caltha chionophila* Greene (1899: 80).

Type:—U.S.A. Colorado: near Pagosa Peak, 11,000 ft., 1899; *Baker s.n.* (isoelectotypes BM! K! NDG!). Fig. 4.

*Caltha uniflora* Rydberg (1900: 474).

Type:—U.S.A. Montana: Haystack Peak, 3000–3300 m., August 1899, *Koch s.n.* (holotype NY!).

**Morphology and cytology:**—Leaf blades 1.2–2.5(–3) × as long as wide, +/- sagittate or auriculate, the generally short auricles little overlapping or not at all, rarely diplophyllous (upturned over the leaf blade), margins mostly sinuate-dentate to subentire (Fig. 4.4); flowers 1(–3) per stem; filaments strongly flattened, 0.5–l(–1.2) mm wide (Giblin et al., in press), wider and generally shorter than anthers (Figs. 4.4, 4.11B); pollen tricolpate (rarely misshapen) (Fig. 4.8); follicles with ± curved stylar beak 0.5–1.2 mm in length (Giblin et al., in press); hexaploid (2n = 6x = 48, Wefferling et al., 2015; holoploid genome size ca. 7–10 pg/2C, Wefferling et al., 2017).

**Taxonomic notes:**—The specimen pictured in Figure 4.4 (NDG17369) is at the Greene-Nieuwland Herbarium, determined by E.L. Greene and labeled with his own hand (B. Hellenthal,
pers. comm.). Smit (1973) designated Baker 322 as lectotype, but Greene did not indicate a collection number; the collector, collection locality, and date match his 1899 description of *Caltha chionophila*.


**Additional specimens examined** are listed in Appendix D.

*Caltha leptosepala* Candolle (1818: 310).

Type:—U.S.A. Alaska: Prince William Sound, 1792, *Menzies s.n.* (holotype BM!). Fig. 2.


*C. biflora* var. *rotundifolia* (Huth) Hitchcock (1964: 335).

Type:— U.S.A. Rocky Mountains, 1872, *A. Gray s.n.* (isotype GH!).

*Caltha chelidonii* Greene (1899: 78).

Type:—CANADA. Alberta: Yellowhead Pass, Rocky Mountains on Alberta-British Columbia boundary, Jasper National Park, 13 July 1898, *Spreadborough 19250* (holotype CAN!).

*Caltha macounii* Greene (1899: 77). *C. leptosepala* var. *macounii* (Greene) Davis (1900: 16).

Type:— CANADA. British Columbia: Mount Queest, 28 July 1889, *Macoun 1255* (syntype US!).

*Caltha leptosepala* var. *sulfurea* Hitchcock (1964: 337).

Type:—U.S.A. Idaho: Custer County, Mount Borah, Rock Creek, 12 August 1944, *Hitchcock and Muhlick 10942* (isotypes US! WTU!).

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**Morphology and cytology:**—Leaf blades 1–1.4(–1.7) × as long as wide, +/- sagittate to cordate to obovate, auricles variable (diplophyllous or not, sinus open or closed), margins crenate or dentate (more so near base, tending toward entire near the apex) (Fig. 4.2); filaments broadly filiform to flattened, 0.2–0.7(–0.9) mm wide (Giblin et al., in press), slightly narrower to as wide as anthers (Figs. 4.2, 4.11C); pollen (3–)4–8(–12) colpate (rarely -porate) or misshapen (Fig. 4.9); follicles with curved stylar beak ~0.1 mm in length (Giblin et al., in press); allododecaploid (2n = 12x = 96, Wefferling et al., 2015; holoploid genome size ca. 18–25.5 pg/2C, Wefferling et al., 2017), rarely allononaploid (2n = 9x = 72, Wefferling et al., 2015; holoploid genome size ca. 13.8–16.8 pg/2C, Wefferling et al., 2017); apparently formed (bidirectionally) through hybridization of *Caltha biflora* and *C. chionophila*.

**Taxonomic notes:**—Candolle (1818) designated as holotype the Menzies collections from Prince William Sound. This part of the complex’s range is well outside the ranges of *Caltha biflora* or *C. chionophila*; all specimens that we have seen from north of Prince of Wales Island, Alaska (near Haida Gwaii, British Columbia) are apparently allododecaploids. In 1970, P. G. Smit annotated the holotype (Fig. 4.2), indicating that the pollen was colpate.

**Geographic distribution:**—Montane to subalpine in central Alaska and Yukon south through British Columbia to Olympic Mountain Range and Cascades of Washington and Oregon, Sierran California, east to western Alberta, central Idaho, northeastern and southeastern Oregon, and western Montana (Wefferling et al., 2017; Giblin et al., in press).

**Additional specimens examined** are listed in Appendix E.

### 4.6 Key to *Caltha leptosepala* polyploid complex

1. Leaves as broad as leaf length or broader; filaments filiform (i.e., narrower than anthers) and generally longer than carpels; in Coastal Ranges (British Columbia and Alaska), Cascades
(Washington, Oregon, California), Klamath - Siskiyous (Oregon, California), or Sierra Nevada (California, western Nevada) … C. biflora

- Leaves longer than broad…………………………………………………………………………………………………… 2

2. Filaments broader and generally shorter than carpels; in U.S.A. Rockies or Basin and Range (Steens, Ruby Mtns.) ……………………………………………………………………………… C. chionophila

- Filaments no broader than anthers (broadly filiform to about as wide as anthers) and generally longer than carpels; in all regions except southern Rockies …………………… C. leptosepala
4.7 References


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Chapter V: Dated phylogeography of western North American subalpine marshmarigolds (Caltha spp., Ranunculaceae)

5.1 Introduction

In western North America, Cenozoic mountain uplift events and a fluctuating climate drove massive and repeated range contractions and expansions, resulting in allopatric speciation, genetic divergence in last glacial maximum (LGM) refugia, post-LGM recolonization, and interactions among divergent lineages in contact zones (Stebbins, 1984; Brunsfeld et al., 2001; Shafer et al., 2010). Dynamic geologic and climatic changes helped to shape the species composition and genetic diversity of the region (Hewitt, 2004). In this study, we seek to understand how specific historical events (e.g., orogenies, Pleistocene glaciations) affected the evolution and distribution of a widespread subalpine polyploid species complex by integrating molecular, ploidy level, morphological, and geographic data in a fossil-calibrated phylogeographic framework.

In the Pacific Northwest of North America, there are many disjunctions within species—or among sister taxa—between submaritime (e.g., Cascades) and interior (Northern Rockies) regions (Brunsfeld et al., 2001; Björk, 2010; Gavin, 2010; Fig. 5.1). Within a given taxon or sister species pair, genetic divergence between these regions may be traced to one of at least three time periods, detailed below. (1) Orogeny of the Cascade and Sierra Nevada Ranges, near
the Miocene-Pliocene boundary ca. 5.3 Mya, cast a rainshadow to the east and drove xerification of the Columbia Plateau and Oregon highlands (Daubenmire, 1975; Graham, 2011). These young mountain ranges changed drainage patterns and increased habitat heterogeneity, isolating the more sessile biota between the Cascade-Sierra axis and the Rockies and allowing speciation to occur in allopatry (Daubenmire, 1975; the Ancient Vicariance Hypothesis of Brunsfeld et al., 2001). (2) Early Pleistocene glaciations and the first major encroachment of icesheets in North America outside of polar regions occurred about 1–0.8 Mya, potentially isolating lineages between regions (Ehlers and Gibbard, 2007). (3) A recent, post-LGM dispersal between regions, in the last 16,000–18,000 years, has been inferred in a number of taxa, suggesting refugial persistence in one region followed by recolonization (e.g., Brunsfeld et al., 2001; Albach et al., 2006; DeChaine et al., 2013a).

Northwestern North America is an important region of phylogeographic discontinuities (i.e., contact zone of closely related lineages), suggesting multiple dispersal events from Pleistocene refugia (Soltis et al., 1997; Swenson and Howard, 2005; Shafer et al., 2010). While biota respond to climatic shifts in a species- or population-specific manner (Taberlet et al., 1998; Gugger et al., 2010), some general patterns emerge from regional phylogeographic studies, supporting a Beringian refugium to the north of glacial ice (Abbott and Brochmann, 2003) and, to the south, the Pacific Northwest (Soltis et al., 1997). Additional smaller putative refugia or areas of early post-glacial recolonization supported by molecular data include parts of the exposed continental shelf (the Alexander Archipelago, Haida Gwaii, and Vancouver Island; Soltis et al., 1997; Beatty and Provan, 2010), western mountain ranges (the Cascades, Olympics, Klamath-Siskiyous, and Sierra Nevadas; Liston et al., 1992; Latch et al., 2009), and interior
regions of the Pacific Northwest (the Northern Rockies; Brunsfeld et al., 2001; Brunsfeld and Sullivan, 2005) (Fig. 5.1).

**Figure 5.1.** Map of western North America showing extent of icesheets (light gray) at the Last Glacial Maximum (LGM, Upper Pleistocene), approximately 17,000 years before present. State and Province names use standard abbreviations. LGM data are from Ehlers et al., (2011) and Lindgren et al., (2016). Map layers were projected using the WGS84 coordinate reference system.

This study focuses on the biogeography of the subalpine marshmarigold polyploid complex: hexaploid (6x = 48) *Caltha biflora* DC., hexaploid *C. chionophila* Greene, and allododecaploid (12x = 96)—rarely allononaploid (9x = 72)—*C. leptosepala* DC. (Ranunculaceae) (Wefferling and Hoot, 2017). These herbaceous perennials are found in wet seeps, open forests, and meadows in subarctic to subalpine regions of western North America, spanning the mountains of Alaska and Yukon in the North to California, Arizona, and New Mexico in the south (Figs. 5.1, 5.2). The hexaploid *C. biflora* and *C. chionophila* grow only in the southern and westernmost parts of the range, while dodecaploid *C. leptosepala* are present...
in every region except the southern Rockies, and are the only species in the complex to occupy the interior and far north; nonaploid \textit{C. leptosepala} have only been found in a single Northern Rockies population (Wefferling et al., 2017; Wefferling and Hoot, 2017) (Figs 5.1, 5.2).

![Subalpine marshmarigold key](image)

**Figure 5.2.** Species and cytotype distribution of specimens in western North America (see key for colors and symbols), showing hexaploids in the south and west and allododecaploids mainly in the north. Last Glacial Maximum icesheet data (in light gray) from Ehlers et al., (2011) and Lindgren et al., (2016).

The subalpine marshmarigold species complex has several attributes that make it an ideal study system for phylogeographic study. (1) The hexaploids display a classic disjunct distribution (Daubenmire, 1975; Brunsfeld et al., 2001) between the Cascades and Sierra Nevadas in the west (\textit{C. biflora}) and the Rockies in the east (\textit{C. chionophila}). (2) The range of the complex encompasses several putative Pleistocene refugia, including the Pacific Northwest, Northern Rockies, coastal British Columbia, southern Beringia, and the “ice-free corridor” of the Canadian Rockies (Figs. 5.1, 5.2). (3) The plants are cold tolerant (Forbis and Diggle, 2001) and
should be capable of persisting in northern and small refugia. (4) There is an apparent lack of homogenizing, concerted evolution of homoeologous ribotypes (Nieto Feliner and Rosseló, 2007) in Caltha allopolyploids (Wefferling and Hoot, 2017), allowing for the molecular identification of hybrid origins. (5) Fossil pollen of Caltha, along with other boreal or tundra-adapted taxa, was located in Haida Gwaii, in sediments dating to ~16,400 yr BP (Lacourse et al., 2005), and of Caltha biflora from ~12,000 to 5,000 yr BP (Lacourse et al., 2012; T. Lacourse, University of Victoria, personal communication). These records indicate that coastal populations of subalpine marshmarigolds persisted in, or were early recolonists of, the west coast. (6) Finally, previous researchers have proposed contrasting, testable hypotheses as to the origin or early diversification of the subalpine marshmarigolds. Smit and Punt (1969) hypothesized that ancestral subalpine marshmarigolds, persisting in southern Pleistocene refugia in the Sierra Nevadas and southern Rockies, diversified in allopatry and hybridized upon secondary contact in the Pacific Northwest (Figs. 5.1, 5.2). Cytogeographic data provided some evidence of multiple refugia in southern and western portions of the range (Wefferling et al., 2017). In contrast, Hultén (1937) proposed solely a Beringian origin of the complex based on phytogeographic patterns.

Our overarching aim in the present study is to use molecular data to better understand the distributional and genetic responses of montane plant lineages to the climate and geology of western North America during the Cenozoic. This study of the subalpine marshmarigold species complex has the following specific goals: (1) provide a new set of divergence time estimates for Ranunculales using multiple fossil-derived age priors; (2) estimate the divergence time between the hexaploid species C. biflora and C. chionophila; (3) infer the locations of major and cryptic LGM refugia; (4) reconstruct source regions and recolonization routes into deglaciated parts of
western North America; and (5) explore the major modes of polyploid formation (i.e., auto- and/or allo-), direction(s) of hybridization in allopolyploids, and possible independent polyploid origins within the complex. These data will allow us to address temporal and spatial aspects of diversification and reticulation within this polyploid complex.

5.2 Materials and methods

We used Bayesian Monte Carlo Markov chain (MCMC) approaches implemented in BEAST 2 (Bouckaert et al., 2014) to infer phylogenetic relationships and estimate divergence times among lineages at three different taxonomic levels (Table 5.1). (1) Using prior age distributions based on fossil records for Ranunculales and outgroups (Appendices F, G) we inferred a 98-taxon order-level chronogram under a relaxed lognormal clock (Appendix H). (2) Age estimates derived from the Ranunculales dataset were used to place age priors on a 15-taxon Caltha genus-level dataset (Appendices I, J). (3) Age and substitution rates from the genus-level dataset were used to place priors on a 161-accession intraspecific-level structured coalescent biogeographic reconstruction (Vaughan et al., 2014) of the subalpine marshmarigold polyploid complex, including *C. biflora*, *C. chionophila*, and *C. leptosepala* (Appendix K). We separately analyzed ITS for the same 161 accessions (222 ribotype sequences; Appendix K) under a relaxed lognormal clock (Drummond et al., 2006) and a coalescent exponential tree prior.

**Ranunculales phylogeny and divergence time estimates**

Molecular data for 11 taxa (obtained from GenBank) were added to the dataset of Hoot et al. (2015) for a total of 98 taxa at four loci: three chloroplast (cpDNA) loci (*atpB*, *matK*, and *rbcL*) and one nuclear ribosomal region (26S) (Table 5.1; Appendix H).
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<th>% taxon coverage</th>
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**Table 5.1.** Statistics for gene regions used in Ranunculales, *Caltha*, and subalpine marshmarigold phylogenetic datasets.

A total of 10 fossils (eight Ranunculales taxa, one outgroup, and the first records of eudicot pollen) were included as age priors (see Appendix F for a list of type specimens, age considerations, and justification for all phylogenetic placements including morphological synapomorphies). Fossils we included were: leaves, perianth parts, and rhizomes of *Nelumbites extenuinervis* from the Lower Cretaceous (Upchurch, Crane, and Drinnan, 1994); pollen of *Tricolpites crassimurus* from the Barremian/Aptian boundary in the Lower Cretaceous (Doyle,
Biens, Doerenkamp, and Jardiné, 1977); a flower of *Kajanthenus lusitanicus* from the Lower to Middle Cretaceous (Mendes, Grimm, Pais, and Friis, 2014); seeds of *Sargentodoxa globosa* from the Middle Eocene (Manchester, 1999); a partial endocarp of *Anamirta* sp. from the Lower Eocene (Jacques and DeFranchesi, 2005); a seed locule cast of *Tinospora excavata* from near the Paleocene-Eocene transition (Fairon-Demaret and Smith, 2002); an endocarp of *Eohypserpa parsonii* from the Lower Eocene (Jacques and DeFranchesi, 2005); endocarps of *Stephania palaeosudamericana* from the Middle to Upper Paleocene (Herrera et al., 2011); an endocarp of *Cocculus lottii* from the Middle Eocene (Collinson, Manchester, and Wilde, 2012); and leaves of *Mahonia* sp. from the Late Eocene (Manchester, 1999). Eleven other putative Ranunculales or allied fossils were considered but not included as calibrations in this study (discussed in Appendix G).

Molecular sequences were aligned in *Geneious* 7.1.6 (Kearse et al., 2012) and concatenated in *SequenceMatrix* v100.0 (Vaidya et al., 2011). The alignment was partitioned by gene region and codon position. The optimal partitioning scheme for model(s) of sequence evolution in each dataset was searched under the corrected Akaike Information Criterion in a full search of models in *PartitionFinder* 1.1.1 (Lanfear et al., 2012), which determined the best partitioning scheme as comprising nine partitions (eight cpDNA and one 26S). Partitioned alignments were imported into *Beauti* 2.4.4 (Bouckaert et al., 2014), and an xml file generated for analysis in *Beast* 2.4.4 (Bouckaert et al., 2014). Clocks were linked across all cpDNA partitions and trees were linked across all partitions. The bModelTest package (Bouckaert and Drummond, 2017) was used to estimate site model evolution of both linked partition group using reversible-jump Markov chain Monte Carlo (MCMC) and empirical frequencies from the data. A relaxed lognormal clock was employed (Drummond, Ho, Phillips, and Rambaut, 2006) for both
clock partition groups, with clock rates estimated. Based on published rates (Sanderson, 2002; Magallón, Hilu, and Quandt, 2013), starting values for clock rates were input as follows: 0.0001 substitutions per site per million years (subst./site/My) for 26S and 0.00092 subst./site/My for cpDNA. A birth death tree prior (unscaled) was used (Gernhard, 2008); priors were left at their defaults with the exception of the uncorrelated lognormal relaxed clock mean for both clock partitions, which were each given a uniform (0, 1) prior. Based on fossilized plant remains, 10 gamma-distributed probability age priors (Appendix A) were placed as most recent common ancestor (MRCA) priors (all constrained as monophyletic). Following the ages and associated uncertainties given in Cohen et al. (2013, updated 2016), priors were designed such that the 5% and 95% quantiles spanned the geological stage in which a given fossil was found (alpha shape parameters were all 2, and the beta parameter and offset were changed as needed to span a given geologic stage; Appendix A). Two MCMC chains were run in BEAST for 200 M generations each on the CIPRES Portal (Miller et al., 2010), and log files were visually checked using TRACER 1.6 (Rambaut et al., 2014) to ensure that each pair converged on the same posterior distribution and that effective sample sizes (ESS) were ≥ 200 for all parameters after a 10% burnin. We also ran the MCMC chain while sampling only from the prior (i.e., without data) in order to compare our user-specified priors, the marginal priors for those same parameters, and posterior distributions (Brown and Smith, 2017); data not presented. Treefiles from the two independent chains were combined and resampled at a frequency of 20,000 in LOGCOMBINER 2.4.4 (Bouckaert et al., 2014). From a sample of 18,000 trees, we searched for the maximum clade credibility tree and calculated median heights using TREEANNOTATOR 2.4.4 (Bouckaert et al., 2014).

*Caltha* phylogeny and divergence time estimates
A multilocus genus-level *Caltha* dataset of single species representatives, excluding known hybrids (Table 5.1; Appendix I), was created for nuclear ribosomal internal transcribed spacer regions (ITS1, 5.8S, and ITS2; ITS) and cpDNA spacer regions *rpl32–trnl* and *trnl*–*trnf* GAA. Regions were amplified and sequenced as in Wefferling and Hoot (2017); cloning or ribotype specific PCR primers (Rauscher et al., 2002) were used to isolate ITS homeologues from allopolyloid *C. leptosepala*. We followed the guidelines of Nieto Feliner and Rosselló (2007) for assessment of functional ITS1, 5.8S, and ITS2 ribotypes, and identified functional ITS2 copies through their secondary structure (Ankenbrand et al., 2015). We also compared our 5.8S sequences to characteristic pseudogene identification motifs (Jobes and Thien, 1997; Harpke and Peterson, 2008), and removed putatively nonfunctional copies before proceeding with analysis.

Alignments were partitioned by gene region, codon, and spacer regions, as in Wefferling and Hoot (2017). Using BEAUTI (Bouckaert et al., 2014), clocks and trees were linked across cpDNA partitions but estimated separately for cpDNA and ITS. Relaxed lognormal clocks were applied (Drummond et al., 2006), with clock rates estimated and unscaled birth death tree priors (Gernhard, 2008). Additional priors were left at their defaults with the exception of the uncorrelated lognormal relaxed clock mean for both clock partition groups, which were each given a gamma (alpha, beta = 0.001, 1000) prior. Four lognormal age priors (two outgroup, two ingroup, in real space) were placed on the *Caltha* trees (all constrained as monophyletic) based on the 95% highest posterior age probabilities for nodes estimated in the Ranunculales dataset (Table 5.2; Appendix J). Two MCMC chains were run for 40 M generations each in BEAST on the CIPRES Portal (Miller et al., 2010), sampling trees and log files every 6,000 steps. Again, we ran the MCMC chain while sampling only from the prior. We compared our user-specified
priors, the marginal priors for those same parameters, and posterior distributions. Convergence on the posterior and ESSs were checked as above. Each set of treefiles (cpDNA and ITS) from two independent chains was combined in LOGCOMBINER (Bouckaert et al., 2014), and from a sample of 12,000 trees each (for cpDNA and ITS), we searched for the maximum clade credibility tree and calculated median heights using TREEANNOTATOR (Bouckaert et al., 2014).

Subalpine marshmarigold phylogeographic datasets and analysis

Subalpine marshmarigold samples included a total of 39 specimens of *C. chionophila* (hexaploid or putative hexaploid), 61 *C. biflora* (hexaploid or putative hexaploid), 59 *C. leptosepala* (one nonaploid and the rest dodecaploid or putative dodecaploid), and two samples of undetermined ploidy level (one morphologically *C. chionophila* and one *C. biflora*) (Table 5.1; Appendices K, L). All plant collection locality data were determined using a Garmin etrex 30 handheld GPS or coordinates were transcribed from herbarium accessions. Approximately half (77) of the 161 total specimens were also included in the cytogeographical study of Wefferling et al. (2017) and have a ploidy level either estimated (via propidium iodide flow cytometric analysis, FCM; \( n = 67 \)) or cytologically determined \( (n = 10) \). Samples that were not previously analyzed via FCM or chromosome counts were tentatively identified based on macro-morphology and geography, then reassessed in light of the genetic data.

LGM icesheets (Figs. 5.1, 5.2, 5.4) were mapped in QGIS 2.14 (QGIS Development Team, 2012) using the World Geodetic System (WGS) 84 coordinate reference system and imported as layers from Ehlers et al. (2011), Lindgren et al. (2016), and A. Lindgren, Stockholm University (personal communication). The southern extent of the Cordilleran icesheet was more recent in
the Pacific Northwest than the global LGM (Clark et al., 2009; Gugger et al., 2010) and was used to delineate glaciated from unglaciated regions in our biogeographical reconstructions.

Molecular data for the phylogeographic datasets included four loci: three cpDNA intergenic spacers and nuclear ribosomal ITS. In addition to \( rpL32-trnL \) and \( trnL-trnF \), we included the cpDNA spacer region \( rps16-trnL^{UUG} \) (Table 5.1; Appendix K). Primers used to amplify \( rps16-trnQ \) were obtained from Shaw et al., (2007; forward primer “\( trnQ^{(uug)} \)”, reverse primer “\( rps16x1' \”) then modified as necessary for more efficient amplification (“\( trnQ6F' \)”: forward primer CGA GAA AGG TAT GAC TCG CA). For all other cpDNA and ITS primers, see Wefferling and Hoot (2017). A concatenated dataset of all cpDNA regions was partitioned by gene region, codon, and spacer region (11 cpDNA partitions in all). Four partitions were determined as the optimal scheme for sequence evolution model selection, under the corrected Akaike Information Criterion in a full search of models in PARTITIONFINDER (Lanfear et al., 2012). The concatenated alignment was imported into BEAUTI (Bouckaert et al., 2014) using the MultiTypeTree template (Vaughan et al., 2014). Phylogeny and biogeographical history were inferred simultaneously for the cpDNA dataset using the structured coalescent, implemented in the MultiTypeTree package (Vaughan et al., 2014) within BEAST (Bouckaert et al., 2014). Clocks and trees were all linked, and the bModelTest package (Bouckaert and Drummond, 2017) was used to estimate site model evolution of each partition. Tip locations were set to delineate regions that were covered by the Laurentide or Cordilleran icesheets at the local LGM (“Glaciated”) from those that were not (either “Cascade-Sierra axis” or “Rockies”). Due to very small sample size relative to the other three regions, several accessions collected in the Basin and Range of North America were scored as “Rockies” (three samples from Steens Mountain in southeastern Oregon and two samples from the Ruby Mountains in northeastern Nevada). A
strict molecular clock was used with the clock rate estimated from a starting value of 0.001 subst./site/My. Substitution model priors (estimated in bModeltest) were left at their defaults. The clock rate prior ("clockRate") was set to exponential with a mean of 0.0012 subst./site/My based on the median substitution rate estimated for *Caltha biflora* and *C. chionophila* (identical between these accessions) in the *Caltha* interspecific cpDNA dataset. A diffuse lognormal prior with a mean of -1 and standard deviation of 1 (mean in log space) was used for effective population sizes ("popSizes") and migration rate ("rateMatrix"; individuals migrating between regions per million years). For the "structuredCoalescent" prior, three demes were set with estimated population sizes of 0.3, 0.1, and 0.3 for the Cascade-Sierra, Glaciated, and Rockies regions, respectively. The migration rate matrix (of the "structuredCoalescent" prior) was set as follows (all are listed moving along branches backwards in time): Cascade-Sierra axis to Glaciated: 0.2; Cascade-Sierra axis to Rockies: 0.4; Glaciated to Cascade-Sierra axis: 2.0; Glaciated to Rockies: 1.0; Rockies to Cascade-Sierra axis: 0.4; Rockies to Glaciated: 0.2. A MRCA prior on the root of the tree (constrained as monophyletic), based on the 95% highest posterior age probability for the divergence between *Caltha biflora* and *C. chionophila* in the *Caltha* cpDNA chronogram was lognormally-distributed with a mean (in real space) of 3, standard deviation of 0.52, and zero offset. In *BEAST* (Bouckaert et al., 2014) implemented on CIPRES (Miller et al., 2010), two chains were run until ESSs for all parameters were ≥ 200 after a 10% burnin (for 60–73 M generations), sampling trees and log files every 12,000 steps. Chains were visually checked using *TRACER* (Rambaut et al., 2014) to ensure they converged on the same posterior. Again, we ran the MCMC chain while sampling only from the prior, comparing the resulting marginal priors with our user-specified priors and posterior distributions. Treefiles from two independent chains were combined in *LOGCOMBINER* (Bouckaert et al., 2014). From a
sample of 9,975 trees, we searched for the maximum clade credibility tree and calculated common ancestor heights using TREEANNOTATOR (Bouckaert et al., 2014). To assess the effect of priors on the tree topology and divergence time estimates, several priors were changed: the “structuredCoalescent” prior (where population sizes were originally set at 0.3, 0.1, and 0.3 for the Cascade-Sierra, Glaciated, and Rockies regions, respectively) was additionally tested at 0.03, 0.01, and 0.03; and also 3, 1, and 3 (data not presented). We varied the “clockRate” prior to double the rate of the original 0.0012. We only varied the clock to faster rates (not slower) due to the addition of \textit{rps16–trnQ} in the phylogeographic (subalpine marshmarigold) dataset, not included in the genus-level (\textit{Caltha}) dataset.

The intraspecific ITS dataset was analyzed independently. The optimal partitioning scheme for sequence evolution model selection as determined in PARTITIONFINDER (Lanfear et al., 2012) was two partitions for ITS (5.8S, ITS1 + ITS2.) The alignment was imported into BEAUTI (Bouckaert et al., 2014), and an xml file generated for analysis in BEAST (Bouckaert et al., 2014). Clocks and trees were linked and, due to estimated substitution rate differences between \textit{Caltha biflora} (median = 0.033) and \textit{C. chionophila} (median = 0.044) in the \textit{Caltha} interspecific analysis, we incorporated a relaxed lognormal clock (Drummond et al., 2006) with clock rates estimated. A coalescent exponential tree prior was used. Other priors were left at their defaults with the exception of the uncorrelated lognormal relaxed clock mean, which was given a lognormal prior (mean = 0.0045, standard deviation = 0.65, median = 0.0036). An age prior on the root of the tree, based on the 95% highest posterior age probability for the divergence between \textit{Caltha biflora} and \textit{C. chionophila} in the \textit{Caltha} ITS chronogram, was lognormally distributed with a mean (in real space) of 6.15, standard deviation of 0.44 and zero offset. In BEAST (Bouckaert et al., 2014), two chains were run for 40 M generations each, sampling trees
and log files every 8,000 steps. We ran the MCMC chain while sampling only from the prior and examined the results. Treefiles from two independent chains were combined in LOGCOMBINER (Bouckaert et al., 2014). From a sample of 9,000 trees we searched for the maximum clade credibility tree and calculated common ancestor heights using TREEANNOTATOR (Bouckaert et al., 2014).

**Phylogenetic and network analyses**

Bayesian MCMC phylogenetic inference was conducted in MRBAYES 3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012) as in Wefferling and Hoot (2017). Briefly, datasets were partitioned as described above, using reversible jump MCMC (Huelsenbeck et al., 2004) and allowing rates to vary across partitions (Ronquist et al., 2011). Four chains (three heated) were run, sampling trees every 500 generations, until reaching an average standard deviation of split frequencies < 0.01. Stationarity and convergence of runs were assessed visually by monitoring trace plots of parameters using TRACER (Rambaut et al., 2014), checking that effective sample sizes for all parameters were ≥ 200 (Drummond and Bouckaert, 2015). After discarding 25% of steps, a 50% majority rule consensus tree was generated. A TCS network (Clement et al., 2000) was inferred in POPART v1.7 (Leigh and Bryant, 2015) for *rpL32-trnL* (alignment of 857 nucleotide characters and 152 accessions), as this was the most complete cpDNA dataset (Table 5.1).

### 5.3 Results

**Ranunculales and *Caltha* datasets**
<table>
<thead>
<tr>
<th>Lineage</th>
<th>Anderson et al., 2005</th>
<th>Bell et al., 2010</th>
<th>Cheng and Xie, 2014</th>
<th>Magallón et al., 2015</th>
<th>Wang et al., 2016</th>
<th>present study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PL/NPRS point estimate</td>
<td>UCLN, exponential prior mean (95% HPD)/ln prior mean (95% HPD)</td>
<td>UCLN mean (95% HPD)</td>
<td>PL point (min–max)/UCLN median (95% HPD)</td>
<td>UCLN mean (95% HPD)</td>
<td>UCLN mean, median (95% HPD)</td>
</tr>
<tr>
<td>Ranunculales SG</td>
<td>120/122</td>
<td>129 (123–134)/129 (116–143)</td>
<td>NA</td>
<td>130.9 (130.3–132.0)/131.7 (129.7–133.4)</td>
<td>NA</td>
<td>116.3, 116.4 (110.9–121.4)</td>
</tr>
<tr>
<td>Ranunculales CG</td>
<td>114/121</td>
<td>100 (85–115)/108 (94–122)</td>
<td>NA</td>
<td>123.4 (120.9–126.2)/114.8 (112.1–123.2)</td>
<td>NA</td>
<td>112.2, 112.2 (106.2–118.2)</td>
</tr>
<tr>
<td>Ranunculaceae SG</td>
<td>90/104</td>
<td>67 (54–80)/72 (58–87)</td>
<td>NA</td>
<td>100.6 (96.0–105.0)/80.3 (68.2–95.9)</td>
<td>123.7 (123.3–124.3)*</td>
<td>95.7, 95.8 (87.3–104.2)</td>
</tr>
<tr>
<td>Ranunculaceae CG</td>
<td>73/87</td>
<td>55 (41–66)/59 (44–73)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>85.9, 86.1 (75.0–96.4)</td>
</tr>
<tr>
<td>Caltha SG</td>
<td>NA/53.45‡</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>76.18 (70.8–80.9)</td>
<td>45.0, 45.1 (32.2–57.4)</td>
</tr>
<tr>
<td>Caltha CG</td>
<td>NA/NA</td>
<td>NA</td>
<td>50.5 (37.1–63.9)</td>
<td>NA</td>
<td>37.7 (28.4–47.9)†#</td>
<td>cpDNA 17.6, 17.1 (9.8–27.0)/ITS 23.9, 23.3 (14.3–35.0)#</td>
</tr>
<tr>
<td>Caltha clade I/ clade II split</td>
<td>NA/5.78‡</td>
<td>NA</td>
<td>39.7 (27.7–53.8)</td>
<td>NA</td>
<td>7.4 (4.1–11.9)</td>
<td>14.7, 14.0 (6.1–24.8)</td>
</tr>
<tr>
<td>section Psychrophila Northern/ Southern hemisphere split</td>
<td>NA/NA</td>
<td>NA</td>
<td>26.2 (16.7–36.6)</td>
<td>NA</td>
<td>cpDNA 6.6, 6.3 (3.5–10.2)/ITS 10.3, 10.0 (5.7–15.5)#</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 Age estimates for Ranunculales, Ranunculaceae, Caltha, and the split between Caltha Clades I and II (sensu Cheng and Xie, 2014; i.e., all Caltha except C. natans) in millions of years (Mya). Dating approach is given beneath each study: NPRS, non-parametric rate smoothing (Sanderson, 1997); PL, penalized likelihood (Sanderson, 2002); UCLN, uncorrelated lognormal (Drummond et al., 2006); 95% HPD, 95% highest posterior density. *Fossil Leefructus (Sun et al., 2011) was used to calibrate this node in Wang et al., (2016); i.e., the age was not estimated independently. #Age estimate is based on a secondary age calibration within the same study. ‡Data from Wei Wang, Institute of Botany, Chinese Academy of Sciences, personal communication. §Data from Cajsa Anderson, personal communication. Estimates from the present study are based on the Ranunculales or Caltha datasets.

Tree topology and divergence time estimates of the Ranunculales BEAST analysis are presented in Table 5.2. Anemonopsis, Eranthis, Actaea, and Cimicifuga were well supported (posterior
probability [PP = 0.96] as sister to *Caltha* (PP values ≥ 95% were considered strong support; Alfaro et al., 2003). Ranunculaceae had a stem group (SG) age in the Middle to Upper Cretaceous, and a crown group (CG) age in the Upper Cretaceous. The *Caltha* SG age was Paleocene to Oligocene, and a CG age of “core” *Caltha* (i.e., all *Caltha* excluding *C. natans*) was dated to the Upper Oligocene to Miocene (Table 5.2). In the genus-level cpDNA dataset, *Caltha biflora* + *C. chionophila* were strongly supported (PP = 1.0) as forming a clade with a SG age in the Upper Miocene to Pliocene and a CG age in the Upper Miocene to Middle Pleistocene (Table 5.3). The genus-level ITS dataset moderately supported (PP = 0.94) the monophyly of *C. biflora* + *C. chionophila*, and gave older estimates than cpDNA, with a SG age in the Middle to Upper Miocene, and a CG age in the Upper Miocene to Pliocene (Table 5.3).

**Subalpine marshmarigold phylogeographic analyses**

In the cpDNA structured coalescent phylogeographic analysis of the subalpine marshmarigolds (Fig. 5.3), two clades were strongly supported (PP = 1.0): *Caltha biflora* + *C. leptosepala* (from the Sierra Nevada, Cascade, Coast, and Alaska Ranges), and *C. chionophila* + *C. leptosepala* (mainly from the US Rockies and Basin and Range). Of the two major clades, the *C. biflora* + *C. leptosepala* clade had greater substructure with several moderately- to well-supported subclades (Fig. 5.3). In contrast, the *C. chionophila* + *C. leptosepala* clade had only one small well-supported subclade (Fig. 5.3). SG age estimates for *C. biflora* and for *C. chionophila* were Pliocene to Pleistocene (Table 5.3). The *C. biflora* CG age was Lower to Middle Pleistocene, and the *C. chionophila* CG was Middle Pleistocene. The oldest well-supported *C. leptosepala* in the *C. biflora* clade (indicated by collapsing poorly supported branches, with PP ≤ 0.95, back in time to the youngest well-supported node) had a SG age of Lower to Middle Pleistocene and a
CG age of Middle Pleistocene. The oldest well-supported \textit{C. leptosepala} in the \textit{C. chionophila} clade had a SG age of Pliocene to Middle Pleistocene and a CG age of Middle Pleistocene (Table 5.3; Fig. 5.3).

<table>
<thead>
<tr>
<th>Lineage</th>
<th>SG median</th>
<th>SG 95% HPD</th>
<th>CG median</th>
<th>CG 95% HPD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caltha</em> cpDNA dataset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. biflora + C. chionophila</em></td>
<td>6.31</td>
<td>3.52–10.17</td>
<td>3.28</td>
<td>1.12–6.18</td>
</tr>
<tr>
<td><em>Caltha</em> ITS dataset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. biflora + C. chionophila</em></td>
<td>9.97</td>
<td>5.74–15.46</td>
<td>6.66</td>
<td>2.59–11.34</td>
</tr>
<tr>
<td><em>subalpine marsh-marigold complex (cpDNA) dataset</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. biflora</em></td>
<td>2.64</td>
<td>1.21–4.63</td>
<td>1.08</td>
<td>0.44–2.04</td>
</tr>
<tr>
<td><em>C. chionophila</em></td>
<td>2.64</td>
<td>1.21–4.63</td>
<td>0.40</td>
<td>0.15–0.82</td>
</tr>
<tr>
<td>oldest <em>C. leptosepala</em> (<em>C. biflora</em> haplotype)</td>
<td>1.08</td>
<td>0.44–2.04</td>
<td>0.77</td>
<td>0.31–1.48</td>
</tr>
<tr>
<td>oldest <em>C. leptosepala</em> (<em>C. chionophila</em> haplotype)</td>
<td>2.64</td>
<td>1.21–4.63</td>
<td>0.40</td>
<td>0.15–0.82</td>
</tr>
<tr>
<td><em>subalpine marsh-marigold complex (ITS) dataset</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. biflora</em></td>
<td>4.37</td>
<td>1.6–8.31</td>
<td>1.24</td>
<td>0.33–2.94</td>
</tr>
<tr>
<td><em>C. chionophila</em></td>
<td>4.37</td>
<td>1.6–8.31</td>
<td>2.06/1.13</td>
<td>(0.53–4.6)</td>
</tr>
<tr>
<td>‡<em>(0.53–4.6)</em></td>
<td></td>
<td></td>
<td>0.28–2.73</td>
<td></td>
</tr>
<tr>
<td>oldest <em>C. leptosepala</em> (<em>C. biflora</em> ribotype)</td>
<td>4.37</td>
<td>1.6–8.31</td>
<td>1.24</td>
<td>0.33–2.94</td>
</tr>
<tr>
<td>oldest <em>C. leptosepala</em> (<em>C. chionophila</em> ribotype)</td>
<td>4.37</td>
<td>1.6–8.31</td>
<td>1.13</td>
<td>0.28–2.73</td>
</tr>
</tbody>
</table>

\textbf{Table 5.3}. Age estimates of selected stem groups (SG) and crown groups (CG) in millions of years before present for *Caltha* and subalpine marshmarigold polyploid complex (*C. biflora*, *C. chionophila*, and *C. leptosepala*) datasets. Ages for the oldest *C. leptosepala* allopolyploids are estimated by collapsing poorly supported branches (posterior probability ≤ 0.95) back in time to the first well-supported node. ‡*C. chionophila* CG has two age estimates due to a poorly supported trichotomy in the ITS dataset.
We recovered two ITS ribotypes from each *C. leptosepala* specimen, and in every case one ribotype grouped with *C. biflora* and the other ribotype grouped with *C. chionophila*. The clade *C. biflora* + *C. leptosepala* was strongly supported, but had only a single well-supported subclade relationship. The relationship of *C. chionophila* + *C. leptosepala* as a whole had lower support (PP = 0.88), and was divided into two subclades. SG age estimates for *C. biflora* and *C. chionophila* were older than in the cpDNA dataset: Upper Miocene to Lower Pleistocene in age (Table 5.3). The CG age for *C. biflora* was Upper Pliocene to Middle Pleistocene. CG ages for *C. chionophila* were Lower Pliocene to Middle Pleistocene (complicated by a poorly supported trichotomy). The oldest well-supported *C. leptosepala* in the *C. biflora* clade was identical in age to *C. biflora* itself: Upper Pliocene to Middle Pleistocene. The oldest well-supported *C. leptosepala* in the *C. chionophila* clade was Upper Pliocene to Middle Pleistocene (Table 5.3).

**Tree topologies and network analysis**

**MRBAYES** analyses reconstructed similar relationships to those found using **BEAST** (data not shown). In the Ranunculales dataset, the position of *Caltha* was poorly resolved within a large Ranunculaceae polytomy. In the cpDNA *Caltha* dataset, *C. biflora* and *C. chionophila* formed a well-supported clade sister to Southern Hemisphere *Caltha* (*Psychrophila* group, comprised of *C. appendiculata*, *C. introloba*, *C. novae-zelandiae*, *C. obtusa*, and *C. sagittata*; Schuettpelz and Hoot, 2004). In the ITS *Caltha* dataset, *C. biflora* was sister to a strongly supported *C. chionophila* + *Psychrophila* group.

In the **MRBAYES** analysis of the cpDNA subalpine marshmarigold dataset, two major clades were well supported, but support and substructure was generally lower than with **BEAST**, particularly for backbone relationships (Fig. 5.3). The ITS subalpine marshmarigold dataset
showed higher support for subclades within both the *C. chionophila* + *C. leptosepala* and *C. biflora* + *C. leptosepala* clades than with BEAST.

The TCS network analysis (Fig. 5.4) identified 16 haplotypes in three major groups: (1) seven *Caltha chionophila* + *C. leptosepala* haplotypes from the Rocky Mountains, Basin and Range, and Cascade Range (each haplotype joined by one or two mutation steps; this group corresponds with the *Caltha chionophila* + *C. leptosepala* clade in the structured coalescent analysis), (2) one *C. biflora* haplotype from the central Sierra Nevada Range (corresponding with a small Sierra Nevada-Coastal Ranges subclade within the *C. biflora* + *C. leptosepala* clade in the structured coalescent analysis), and (3) eight *C. biflora* + *C. leptosepala* haplotypes from the Sierra Nevada, Cascade, Coast, and Alaska Ranges (each haplotype joined by a single mutation step; this group corresponds with the remaining members of the *C. biflora* + *C. leptosepala* clade; i.e., all but the Sierra Nevada-Coast Ranges subclade) (Figs. 5.3, 5.4). The three major haplotype groups in the TCS network were separated by 7, 9, or 14 mutation steps (Fig. 5.4).

### 5.4 Discussion

**Dating of Ranunculales, Ranunculaceae, and Caltha**

Our Ranunculales age estimates place both the stem group (SG) and crown group (CG) in the Lower Cretaceous Aptian to Albian Stages (Table 5.2). This is largely consistent with previous studies of angiosperms and “basal” eudicots (e.g., Anderson, Bremer, and Friis, 2005; Bell, Soltis, and Soltis, 2010; Magallón, Gómez-Acevedo, Sánchez-Reyes, and Hernández-Hernández, 2015). An Upper Cretaceous age for the diversification of Ranunculaceae (both SG and CG), as found here, is concordant with some published work (e.g., Anderson et al., 2005; Magallón et al., 2015). Younger (Bell et al., 2010) and older (Wang et al., 2016) estimates also exist (Table 5.2).
The disparity in timing is likely due to taxon sampling and choice of age priors; other than the first record of tricolpate pollen ~125 Mya there was little to no overlap in calibration points used in this and other studies (Appendices F, G).

Few studies have directly estimated the age of *Caltha*. Wang et al., (2016) included *Caltha* as part of a family-wide chronogram, finding an Eocene to Oligocene CG age that overlaps with our estimates of Upper Eocene to Miocene. In a phylogenetic and biogeographic study of *Caltha*, Cheng and Xie (2014) estimated a Paleocene to Eocene CG age for the genus, an older estimate that does not overlap with ours (Table 5.2); interspecific divergences in Cheng and Xie (2014) were consistently older than in our study, likely due to fossil choice (Appendix G).

**Divergence among subalpine marshmarigolds**

During the Cenozoic, orogeny of the Western Cordillera and a cooler post-Cretaceous climate created a diversity of habitats in western North America (Hewitt, 2004). Based on a Middle Miocene to Upper Pliocene SG age for the North American members of the *Psychrophila* group, the subalpine marshmarigolds (*Caltha biflora*, *C. chionophila*, and *C. leptosepala*) were on an independent evolutionary trajectory from other *Caltha*, surviving and diversifying in a colder and drier climate than earlier in the Cenozoic (Ehlers and Gibbard, 2007).
Figure 5.3. Structured coalescent chronogram (with common ancestor branch heights, inferred using the MultiTypeTree package in the BEAST framework) based on chloroplast data (\textit{rpl32-trnL}\textsubscript{UAG}, \textit{trnL}\textsubscript{UAA}-\textit{trnF}\textsubscript{GAA}, and \textit{rps16-trnQ}\textsubscript{UUG}) for 161 specimens in the subalpine marshmallow polyploid complex (\textit{Caltha biflora}, \textit{C. chionophila}, and \textit{C. leptosepala}). Branch colors show reconstructed regions through time (see key), and are colored only where the inferred region was reconstructed with a posterior probability (PP) \(\geq 0.95\); branches otherwise appear gray. Dashed branches indicate PP \(\leq 0.95\). Median age and 95% highest posterior probability range and bars are shown only at nodes that are well supported. Markers at branch tips indicate species and cytotypes (see key for colors and symbols, which match Figure 5.2). Labels to the right indicate regions from which the indicated specimens were collected.

Does the timing of divergence between the hexaploid sister species, \textit{Caltha biflora} and \textit{C. chionophila}, correspond with a specific geologic or climatic event? Chloroplast and nuclear estimates for the divergence between these two species overlap in the Upper Miocene to Upper Pliocene, consistent with allopatric speciation between lineages driven by orogeny of the Cascade and Sierra Nevada Ranges. Ancient vicariance (\textit{sensu} Brunsfeld et al., 2001), similarly driven by topographic changes in the region, has been supported in studies of other species, including various amphibians (e.g., tailed frogs, \textit{Ascaphus}; Pacific giant salamanders, \textit{Dicamptodon}; plethodontid salamanders, \textit{Plethodon}; Carstens et al., 2005), gray jays (\textit{Perisoreus canadensis}; van Els et al., 2012), Douglas-fir (\textit{Pseudotsuga menziesii}; Gugger and Sugita, 2010; Gugger et al., 2010), Constance’s bittercress (\textit{Cardamine constancei}; Brunsfeld and Sullivan, 2005), bluebells (\textit{Mertensia}; Nazaire et al., 2014), and kittentails (\textit{Synthyris}; Marlowe and Hufford, 2008). A Miocene-Pliocene orogeny-driven allopatric divergence between \textit{Caltha biflora} and \textit{C. chionophila} is supported by our data, contrasting with the hypothesis proposed by Smit and Punt (1969) of a more recent Pleistocene climate-driven divergence.

In our study, divergence time estimates are consistently older using nuclear ribosomal ITS than cpDNA spacer regions. This difference may be due to the larger effective population
sizes (and hence longer coalescent times) in nuclear than in organellar genomes and/or incomplete lineage sorting (ILS; i.e., retention of ancestral polymorphisms) of ITS ribotypes (Avise, 2000; Nieto Feliner and Rosselló, 2007; Rautenberg et al., 2010). The presence of ILS in *Caltha* is also suggested by a lack of monophyly of subalpine marshmarigold ribotypes, indicative of deep discordance with cpDNA phylogenies (Wefferling and Hoot, 2017). Other researchers have reported discrepancies in age estimates (in some cases non-overlapping) between nuclear ribosomal and organellar regions in both plants and animals (e.g., Hung et al., 2009; Houston et al., 2010; Rautenberg et al., 2010; Huang et al., 2011).

Figure 5.4. Haplotype distribution and TCS network of 16 haplotypes, in western North America, based on cpDNA data *(rpl32-trnL* UAG*) for 152 subalpine marshmarigold specimens, including *Caltha biflora*, *C. chionophila*, and *C. leptosepala*. Last Glacial Maximum icesheet data (in light gray) from Ehlers et al. (2011) and Lindgren et al. (2016).
Refugia and recolonization

In our intraspecific analyses, *Caltha biflora* radiated earlier than *C. chionophila* (Fig. 5.3; Table 5.3). *C. biflora* also has greater phylogenetic substructure and haplotype diversity than *C. chionophila* (Figs. 5.3, 5.4), and our data indicate that the Cascade-Sierra axis provided at least two refugia for ancestral *C. biflora*. This pattern is consistent with leaf morphology, e.g., a higher incidence of diplophyll (upturned leaf blade auricles) in the southern Cascades and Sierra Nevada (cf. type specimens of *C. howellii* Greene and *C. biflora* in Wefferling and Hoot, 2017). A small clade of Sierra Nevada and Coastal Range specimens that diverged from other *C. biflora* in the Lower to Middle Pleistocene (Fig. 5.3) corresponds with a unique haplotype (Fig. 5.4). This may indicate persistence in a Pleistocene refugium in the Sierra Nevada Range (Smit and Punt, 1969), though the presence of a single specimen with an identical haplotype from the Olympic Range in western Washington is noteworthy. A similarly disjunct “southern” haplotype was found in Haida Gwaii in a phylogeographic study of sword fern (*Polystichum munitum*; Soltis et al., 1997). In a meta-analysis of contact zones in North America, Swenson and Howard (2005) found support for the Sierra Nevada as a refugium for a number of plant and animal taxa. Similarly, relatively deep genetic divergence was found between Klamath and Sierra Nevada populations of foxtail pine (*Pinus balfouriana*; Eckert et al., 2008), between northern and southern Pacific coastal populations of arionid slugs (*Propyson coeruleum*; Wilke and Duncan, 2004), and among populations of *Datisca* to the north and south of the Transverse Ranges in southern California (Liston et al., 1992).

Within the *C. biflora* clade we detect two major waves of migration into LGM glaciated regions (Fig. 5.3). The first wave, in the Lower to Middle Pleistocene, consists mainly of hexaploids that appear to have recolonized the North Cascades and Coastal Ranges as far north
as the Alexander Archipelago (Figs. 5.1–5.4). A second period of migration occurred in the Middle to Upper Pleistocene, comprised mostly of allododecaploids that recolonized drier interior regions such as the Canadian Rockies (Figs. 5.1–5.4). Along coastal British Columbia at Haida Gwaii, there is evidence of tundra vegetation both at the last glaciation (the “Hecate Refugium”; Mathewes and Clague, 2017) and early post-LGM (~17.4–14.6 kya; Lacourse et al., 2005). There have been numerous collections of caribou dung, peat layers, and detrital wood in this region, all ¹⁴C-dated to Upper Pleistocene to Middle Holocene ages (Menounos et al., 2009; Osborn et al., 2012). An earlier appearance of Caltha along the coast than interior regions is consistent with Pleistocene persistence of coastal refugia and/or earlier suitability of the maritime region for tundra and cold-tolerant plants (Soltis et al., 1997; Gugger et al., 2010).

In the C. chionophila clade there is a lack of substructure in the cpDNA dataset (Fig 5.3). Recolonization of LGM glaciated regions from this clade was comparatively minor, with two incursions at the southern margin of the icesheet (Figs. 5.3, 5.4). Our analyses of ITS data point to further substructure in the C. chionophila clade, with two southern Rocky Mountain Range specimens well supported as sister to the remainder of the clade. This provides limited support for the southern Rockies acting as a Pleistocene refugium for subalpine marshmarigolds (Smit and Punt, 1969). In contrast to the C. biflora clade, within which we detected no migration between Rockies and Cascade-Sierra regions, allododecaploids from the C. chionophila clade apparently migrated from the Rockies into the Basin and Range and central Cascades during the Pleistocene (Figs. 5.3, 5.4). Pleistocene dispersal between the Rocky Mountains and the Cascade-Sierras has also been found in saxifragas (Saxifraga; DeChaine et al., 2013a), veronica (Veronica; Albach et al., 2006), and gray owls (Strix; Hull et al., 2010). Brunsfeld et al., (2001) list a number of taxa with shallow (i.e., recent) molecular divergence between the Northern
Rockies and Cascade-Sierras, suggesting LGM persistence in only one region (interior or coastal Pacific Northwest) followed by recent migration.

Future work may reveal the importance of submaritime refugia (e.g., Godbout et al., 2008) for the subalpine marshmarigold complex, including the Olympic Peninsula and Vancouver Island, and interior mountains such as the Northern Rockies (Fig. 5.1). That these regions may have acted as refugia for subalpine marshmarigolds is suggested by the cryptic morphology of several putatively homoploid hybrids and aneuploid individuals from these regions (Wefferling and Hoot, 2017).

**Recent radiations and bidirectional allopolyploidization**

Based on the combination of chromosome counts, flow cytometry, morphological data, and molecular analyses for a geographically broad sampling (Wefferling et al., 2015; Wefferling and Hoot, 2017; Wefferling et al., 2017; this study), bidirectional allopolyploidization between the hexaploid *C. biflora* and *C. chionophila* is well supported. The formation of allododecaploid *C. leptosepala* has resulted in two major lineages, identifiable by haplotype, each almost entirely restricted to either the Northern Rockies or the Cascade-Sierra axis and Coastal Ranges (Figs. 5.3, 5.4). We have not found evidence for autopolyploidy in the species complex.

Allododecaploid *C. leptosepala* with *C. biflora* haplotypes (“Western” *C. leptosepala*) have been more successful at recolonization of LGM glaciated regions than those with *C. chionophila* haplotypes (“Eastern” *C. leptosepala*) (Figs. 5.2–5.4), due either to ecological differences or location near recolonization routes. The lack of haplotype diversity within Western *C. leptosepala* LGM recolonists (Figs. 5.2–5.4) suggests a leading edge scenario (Soltis et al., 1997), whereby the first populations to recolonize a region were able to competitively
exclude later invasions (Wefferling et al., 2017). Western *C. leptosepala* has formed at least two times (Fig. 3), reinforcing the pattern of multiple origins found in an increasing number of polyploid taxa (e.g., Soltis and Soltis, 1999; Weiss-Schneeweiss et al., 2013). Bidirectional allopolyplidization (and multiple origins within the *Caltha biflora* lineage) supports the hypothesis of secondary contact between lineages that diverged in allopatry before coming into contact during the advance and retreat of Pleistocene glaciation (Stebbins, 1984; Murray, 1995; Brochmann et al., 2004; Guggisberg et al., 2010; Wefferling et al., 2017), though the precise location of this contact zone, somewhere in the subalpine marshmarigolds.

Further cytological and molecular work, especially at the population level in and around contact zones of the Pacific Northwest, may reveal additional independent allopolyplid origins, the presence of rare cytotypes, and unbalanced genomic contributions (and/or gene and chromosome loss) in hybrid and polyploid individuals. While further work on the complex should provide greater insight into their evolution, we believe that our sampling strategy—including ploidy level, nuclear and chloroplast sequence data, and wide geographic sampling—captures the major biogeographical patterns in the subalpine marshmarigolds.

**Regional perspective**

How does the subalpine marshmarigold complex add to our knowledge of broad phylogeographic patterns in western North American plant lineages? In a review of northwestern North American plants, Soltis et al., (1997) found north-south differentiation of haplotypes among diverse plant taxa, including sword fern, Sitka spruce (*Picea sitchensis*), and several herbaceous and woody angiosperms. In contrast to the broad concordance found among plant
lineages found by Soltis et al., (1997), other reviews of the regional phylogeographic literature (Brunsfeld et al., 2001; Jaramillo-Correa et al., 2009; Shafer et al., 2010; Roberts and Hamann, 2015) revealed clearly contrasting patterns among the surveyed taxa, with complex and seemingly idiosyncratic responses to past geologic and climatic changes. More recent phylogeographic studies of western North American plants (see below) further support the conclusion that species (or population)-specific responses are the rule rather than the exception (Taberlet et al., 1998).

As noted by other researchers, the phylogeographic patterns we infer are limited by our geographic and molecular sampling; in many cases, there are undersampled portions of a given species’ range that may harbor genetic diversity and unique histories (Winkler et al., 2012; Eidesen et al., 2013). Furthermore, relying only on organellar molecular sequence data while lacking information on, e.g., hybrid origins, ploidy level, and breeding system can potentially mislead historical inference (Eidesen et al., 2007). Of course, the shallower patterns identified in many phylogeographic studies are overlaid on deeper histories of migrations, allopatric divergences, genetic bottlenecks, genome duplications, hybridizations, and repeated contact among more or less divergent lineages. With these caveats—that our understanding of the region’s biogeography is subject to revision over time—we here illustrate four of the more common patterns of biotic, evolutionary, patterns in montane to arctic-alpine plants attributed to the LGM in western North America.

(1) Persistence south of the icesheets: Plant lineages that survived the LGM only to the south of icesheets (as found here with Caltha) include Douglas-fir, which apparently migrated out of multiple refugia in both the Cascade-Sierra axis and Rockies (Fig. 1) while closely tracking glacial retreat (Gugger et al., 2010), a conclusion corroborated by the fossil record
Similarly, *Sibbaldia procumbens* recolonized arctic-alpine western North America from refugia in the Sierra Nevadas and southern Rockies (Allen et al., 2015); given the broad extant range of this alpine-arctic species, the authors were surprised at the lack of evidence for survival in a northern refugium. High clonal (apomictic) diversity was found in polyploid rosy pussytoes (*Antennaria rosea*)—indicating multiple origins from sympatric sexual diploids—near the southern margins of the icesheets (Bayer, 1991). In western redcedar (*Thuja plicata*), a slow post-LGM rate of migration from a single, probably coastal, refugium was inferred to the south of the current range (Glaubitz et al., 2000; Roberts and Hamann, 2015).

(2) **Beringia:** Plants that persisted or are inferred to have a center of origin in Beringia include purple mountain saxifrage (*Saxifraga oppositifolia*; Abbott and Comes, 2004; DeChaine, 2008); the fossil record corroborates the lineage persisting in Beringia during the late Pleistocene (ca. 21.5 kya; Goetcheus and Birks, 2001), although more recent studies failed to find genetic evidence for this (Winkler et al., 2012). A broad molecular study of circumpolar Arctic plants (both woody and herbaceous, including *Chamerion, Dryas, Rubus*, and *Vaccinium*) provided support for refugia in both Siberia and Beringia, but with only Beringia acting as an important source region for subsequent recolonization of deglaciated regions (Eidesen et al., 2013).

(3) **Multiple refugia, both north and south of Cordilleran icesheets:** Some lineages likely persisted in refugia both to the north and south of LGM icesheets, such as bog bilberry (*Vaccinium uliginosum*; Alsos et al., 2005; Eidesen et al., 2007, 2013; DeChaine, 2008). White spruce (*Picea glauca*) and black spruce (*Picea mariana*) appear to have persisted both north and south of the LGM icesheets; in both of these cases, southern populations recolonized deglaciated terrain more successfully than northern, Beringian, populations (Anderson et al., 2006; Gérardi et al., 2010). Sexual diploid Easter daisies (*Townsendia hookeri*) are distributed disjunctly in
Yukon and the southern Rockies, with exclusively apomictic polyploids in the intervening, formerly glaciated, areas (Thompson and Whitton, 2006). *Packera pauciflora* has a disjunct distribution between the northern Rockies, Quebec-Labrador, and the Sierra Nevadas, with the northern lineage contributing more to recolonization of previously glaciated regions (Bain and Golden, 2005). Other examples for which both northern and southern refugia were inferred include locoweed (*Oxytropis*; Jorgensen et al., 2003), rockcress (*Arabidopsis lyrata*; Schmickl et al., 2008), harebell (*Campanula*; DeChaine et al., 2014), and moss campion (*Silene acaulis*; Gussarova et al., 2015).

(4) Cryptic refugia: Molecular data provide evidence for cryptic refugia (refugia undetected with fossil evidence), for example within the limits of the LGM icesheets (Shafer et al., 2010) in nunataks (Birks, 2008) or the ice-free corridor between the Laurentide and Cordilleran icesheets (Beatty and Provan, 2010; Gugger and Sugita, 2010; Allen et al., 2012), or in the high arctic (e.g., Tremblay and Schoen, 1999; Shafer et al., 2010) (Fig. 5.1). Lodgepole pine (*Pinus contorta*) apparently recolonized North America from four or five refugia north, south, and west of the Cordilleran icesheet, with each sublineage contributing significantly to extant populations (Godbout et al., 2008). The Pacific Northwest acted as both a refugium and contact zone for one-sided wintergreen (*Orthilia secunda*); the species persisted in multiple refugia, possibly surviving the LGM in the putative ice-free corridor between the Laurentide and Cordilleran icesheets along the Alberta-British Columbia border (Beatty and Provan, 2010). Further examples of widespread arctic-alpine species providing evidence for cryptic refugia include western rosroot (*Rhodiola integrifolia*; DeChaine et al., 2013b; Guest and Allen, 2014), mountain-sorrel (*Oxyria digyna*; Marr et al., 2008; Allen et al., 2012), and alpine bistort (*Bistorta vivipara*; Marr et al., 2013).
These various phylogeographic histories demonstrate some common themes, but also serve to remind us that similar extant distributions are not predictive of similar responses to geologic or climatic change (Taberlet et al., 1998). A deeper understanding of the biogeography of the subalpine marshmarigold complex, as presented here, supports the idea of individualistic responses to Quaternary changes among co-occurring species. Notably, Shafer et al., (2010) and Roberts and Hamann (2015) found that larger extant ranges are positively correlated with the number of inferred refugial populations at the LGM. The subalpine marshmarigolds provide a counterexample to this overall trend: despite a currently broad distribution in western North America, we conclude that subalpine marshmarigolds found climate refugia in the Rockies and two or more regions in the Cascade-Sierra axis, but we found no evidence of survival in Beringia, in nunataks, or the ice-free corridor.
5.5 References


Friis, E.M., Crane, P.R., and Pedersen, K.R. (2011) *Early Flowers and Angiosperm Evolution*. University of Cambridge, Cambridge, Massachusetts, USA.


Appendix A. *Caltha* specimens sampled in cytogeographic study.

For each locality: population ID number (in bold italics), “taxon” sampled, origin of material (state or province), voucher information (including collector(s), collection number, herbarium, and catalog number), geographical coordinates in decimal degree format, altitude (m above sea level), estimated ploidy level, genome size estimates based on silica-dried leaves or herbarium vouchers - each followed by sample size in parenthesis (n), chromosome counts from literature (\(^1\) Wefferling et al., 2015) and obtained in this study (\(^2\)), followed by the genome size estimates in parenthesis obtained in the same plants. Mixed-ploidy population numbers are marked with an asterisk. Abbreviations: ssp. *howellii*, *Caltha leptosepala* ssp. *howellii*; ssp. *leptosepala*, *Caltha leptosepala* ssp. *leptosepala*; 6x, hexaploid; 9x, nonaploid; 12x, dodecaploid. Herbaria: *ALA*, University of Alaska Museum of the North Herbarium, Fairbanks, Alaska, USA; *ASC*, Deaver Herbarium, Northern Arizona University, Flagstaff, Arizona; *DAV*, University of California Davis Center for Plant Diversity, Davis, California; *ID*, University of Idaho Herbarium, Moscow, Idaho; *NYBG*, New York Botanical Garden, Bronx, New York; *OSC*, Oregon State University, Corvallis, Oregon; *RCBM*, Royal British Columbia Museum, Victoria, British Columbia; *RM*, Rocky Mountain Herbarium, University of Wyoming, Laramie, Wyoming; *SRP*, Snake River Plains Herbarium, Boise State University, Boise, Idaho; *UWM*, University of Wisconsin-Milwaukee-Milwaukee, Milwaukee, Wisconsin; *WTU*, University of Washington Herbarium, Seattle, Washington.

1, ssp. *howellii*, California, Wefferling 27 (*UWM s.n.*), 37.27, -119.12, 2600m, 6x, 15.71 (1); 2, ssp. *howellii*, California, Wefferling 28 (*UWM s.n.*), 37.29, -119.14, 2700m, 6x, 15.26 (1); 3, ssp. *howellii*, California, Wefferling 23 (*UWM s.n.*), 37.29, -119.17, 2780m, 6x, 16.76 (1); 4, ssp. *howellii*, California, Wefferling 22 (*UWM s.n.*), 37.29, -119.17, 2780m, 6x, 16.81 (1); 5, ssp. *howellii*, California, Wefferling and Woo 24 (*UWM s.n.*), 37.29, -119.16, 2635m, 6x, 15.89 (1); 6, ssp. *howellii*, California, Wefferling 26 (*UWM s.n.*), 37.30, -119.10, 2780m, 6x, 15.14 (1); 7, ssp. *howellii*, California, Wefferling 929 (*UWM 65343*), 37.30, -119.10, 2743m, 6x, 15.53 (1); 8, ssp. *leptosepala*, Colorado, Rink 2273 (*ASC 75161*), 37.62, -107.32, 3140m, 6x, 8.91 (1); 9, ssp. *leptosepala*, Colorado, Hall, Jacobs, and Morgan 1565 (*NYBG s.n.*), 38.52, -106.32, 3200m, 6x, 7.05 (1); 10, ssp. *howellii*, California, Helm kamp and Helm kamp 10749 (*DAV 171547*), 38.83, -120.26, 2000m, 6x, 18.03 (1); 11, ssp. *leptosepala*, Utah, Holmgren and Holmgren 16196 (*NYBG 01208739*), 39.22, -111.50, 3040m, 6x, 7.02 (1); 12, ssp. *leptosepala*, Colorado, Townesmith, Gust and Nye 202 (*UWM 65105*), 39.32, -106.13, 3606m, aneuploid, 12.91 (2); 13, ssp. *leptosepala*, Colorado, Majack 2 (*UWM s.n.*), 40.08, -105.58, 3158m, 6x, 7.78 (3); 14, ssp. *leptosepala*, Colorado, Majack 1 (*UWM s.n.*), 40.30, -105.96, 2970m, 6x, 7.45 (3), 2n = 6x = 48\(^1\) (7.39pg); 15, ssp. *howellii*, California, Wefferling and Woo 29 (*UWM 65344*), 40.41, -121.53, 1972m, 6x, 16.28 (1); 16, ssp. *howellii*, California, Meyer and Townesmith 145 (*DAV 19205*), 40.50, -121.13, 1920m, 6x, 15.90 (1); 17, ssp. *leptosepala*, Utah, Holmgren and Holmgren 15424 (*NYBG 01057127*), 40.74, -110.87, 3030m, 6x, 8.74 (1); 18, ssp. *howellii*, California, Wefferling 32 (*UWM s.n.*), 41.21, -122.50, 1745m, 6x, 14.15 (1); 19, ssp. *howellii*, California, Wefferling and Woo 37 (*UWM s.n.*), 41.21, -122.51, 1870m, 6x, 14.37 (1); 20, ssp. *howellii*, California, Wefferling and Woo 931 (*UWM 65345*), 41.21, -122.52, 1904m, 6x, 15.10 (1); 21, ssp. *howellii*, California, Wefferling and Woo 31 (*UWM s.n.*), 41.21, -122.51, 1870m, 6x, 14.60 (1); 22, ssp. *howellii*, California, Wefferling and Woo 35 (*UWM s.n.*), 41.23, -122.51, 1890m, 6x, 14.80 (1); 23, ssp. *howellii*, California, Wefferling and Woo 34 (*UWM s.n.*), 41.24, -122.51, 1936m, 6x, 14.07 (1); 24, ssp. *howellii*, California, Wefferling and Woo 36 (*UWM s.n.*), 41.24, -122.51, 2015m, 6x, 14.80 (1); 25, ssp. *howellii*, California, Wefferling and Woo 981 (*UWM s.n.*), 41.24, -122.51, 1997m, 6x, 15.48 (1); 26, ssp. *leptosepala*, Wyoming, Lukas 2286 (*USFS/RM 880425*), 41.34, -106.37, 3050m, 6x, 7.40 (1); 27, ssp. *leptosepala*, Wyoming, Wefferling 927 (*UWM 65341*), 41.34, -106.33, 3496m, 6x, 9.37 (1); 28, ssp. *leptosepala*, Wyoming, Wefferling 20 (*UWM s.n.*), 41.36, -106.24, 3115m, 6x, 8.44 (1); 29, ssp. *howellii*, Oregon, Wefferling and Woo 933 (*UWM 65347*), 42.08, -122.73, 2000m, 6x, 15.44 (1); 30, ssp. *howellii*, Oregon, Anderson JNS1 (*UWM s.n.*), 42.08, -122.72, 2012m, 6x, 13.89 (4), 2n = 6x = 48\(^1\) (14.44pg); 31, Northern *Caltha leptosepala*, Oregon, Legler, Gage, Gibble, Goff, Birks, and Davis 1890 (*WTU 367680*), 42.60, -120.78, 2073m, 12x, 20.13 (1); 32, ssp. *howellii*, Oregon, Wefferling 41 (*UWM s.n.*), 42.92, -122.59, 1610m, 6x, 14.22 (1); 33, ssp. *howellii*, Oregon, Wefferling 40 (*UWM s.n.*), 42.92, -122.59, 1610m, 6x, 15.28 (1); 34, ssp. *howellii*, Oregon, Wefferling and Woo 978 (*UWM s.n.*), 42.92, -122.59, 1605m, 6x, 16.37 (1); 35, ssp. *howellii*, Oregon, Wefferling and Woo 934 (*UWM 65348*), 42.94, -122.55, 1790m, 6x, 17.20 (1); 36, ssp. *howellii*, Oregon, Wefferling and Woo 935 (*UWM 65350*), 43.13, -122.16, 1586m, 6x, 15.08 (1); 37, ssp.
75, ssp. howellii, Washington, Hunter BUI-6 (UWM s.n.), 47.97, -123.75, 1400m, 6x, 11.80 (5), 12.87 (1); 76, ssp. howellii, Washington, Hunter BLI-6 (UWM s.n.), 47.98, -123.70, 680m, 6x, 11.63 (5), 11.61 (1); 77*, ssp. howellii, Washington, Hunter LR2-6 (UWM s.n.), 48.05, -123.50, 336m, 6x, 11.80 (3), ssp. howellii, Washington, Hunter LR2-6 (UWM s.n.), 48.05, -123.50, 336m, aneuploid, 17.45 (2), 17.51 (1); 78, ssp. howellii, Washington, Wefferling 49 (UWM s.n.), 48.07, -121.78, 730m, 6x, 13.00 (1); 79, ssp. howellii, Washington, Wefferling 938 (UWM 65353), 48.07, -121.78, 740m, 6x, 13.49 (1); 80, Northern C. leptosepala, Washington, Knoke 267 (WTU 354477), 48.46, -121.04, 1407m, 12x, 22.29 (1); 81, Northern C. leptosepala, Washington, Tribsch and Schönswetter AP-44 (WTU 354479), 48.48, -121.05, 1830m, 12x, 20.82 (1); 82, Northern C. leptosepala, Washington, Rodeman 06-30 (WTU 366883), 48.48, -120.66, 1951m, 12x, 22.88 (1); 83, Northern C. leptosepala, Washington, Binov MPI-5 (UWM s.n.), 48.50, -120.77, 2042m, 12x, 18.84 (5); 84, ssp. howellii, Washington, Shrum SM3-5 (UWM s.n.), 48.51, -121.62, 700m, 6x, 11.96 (3); 85, Northern C. leptosepala, British Columbia, Marr, Hebda, and MacKenzie 06-0523 (V/RBCM V196823), 54.51, -128.45, 1199m, 12x, 22.17 (1); 86, Northern C. leptosepala, British Columbia, Hebda, Marr, and Forsyth KM5613 (V/RBCM V189433), 54.89, -120.94, 1650m, 12x, 23.91 (1); 87, Northern C. leptosepala, British Columbia, Marr, Hebda, and MacKenzie 06-0013 (V/RBCM V196348), 56.22, -129.44, 1752m, 12x, 22.68 (1); 88, Northern C. leptosepala, British Columbia, Hebda, Marr, and MacKenzie KM4777 (V/RBCM V189253), 56.41, -123.50, 1750m, 12x, 22.39 (1); 89, Northern C. leptosepala, British Columbia, Hebda and Forsyth KM4857m (V/RBCM V189360), 56.56, -125.26, 1700m, 12x, 22.31 (1); 90, Northern C. leptosepala, British Columbia, Marr, Hebda, and MacKenzie 09-0810 (V/RBCM V205521), 57.22, -127.43, 1877m, 12x, 20.58 (1); 91, Northern C. leptosepala, British Columbia, Marr, Hebda, and Berger 05-0652 (V/RBCM V195051), 57.24, -128.91, 1751m, 12x, 23.83 (1); 92, Northern C. leptosepala, Alaska, Parker 17964 (ALA V170049), 57.30, -153.29, 685m, 12x, 19.06 (1); 93, Northern C. leptosepala, Alaska, Parker and Studebaker 17293 (ALA V165181/H1046210), 58.41, -152.70, 15m, 12x, 23.83 (1); 94, Northern C. leptosepala, British Columbia, Marr, Hebda, and MacKenzie 07-1648 (V/RBCM V201979), 58.72, -128.26, 1685m, 12x, 23.31 (1); 95, Northern C. leptosepala, Alaska, Loomis and Larsen 1256 (ALA V153033/H1133866), 60.17, -141.41, 933m, 12x, 19.81 (1); 96, Northern C. leptosepala, Alaska, Wefferling 971 (UWM s.n.), 60.20, -149.43, 390m, 12x, 20.09 (1), 22.64 (2); 97, Northern C. leptosepala, Alaska, Wefferling 970 (UWM s.n.), 61.00, -149.57, 629m, 12x, 21.02 (1); 98, Northern C. leptosepala, Alaska, Wefferling 972 (UWM s.n.), 61.11, -149.66, 620m, 12x, 20.58 (1), 21.00(2); 99, Northern C. leptosepala, Alaska, Larsen and Cook 01-0092 (ALA V138664), 62.65, -150.96, 838m, 12x, 21.34 (1).
Appendix B. List of specimens included in recircumscription study phylogeny

Species name, inferred ploidy level (for *Caltha leptosepala* sensu lato specimens only; † indicates specimens with flow cytometry genome size estimates and/or chromosome counts), voucher information (for new sequences only), GenBank accession number. Gene regions are listed in the following order: internal transcribed spacer region (ITS; two GenBank numbers given for allododecaploids: *biflora/chionophila* ribotypes), *trnL–F*, *rpL32-trnL*. — indicates missing sequence data. Numbers with prefix AY- are from Schuettpelz and Hoot (2004).

**Ingroup:** *Caltha leptosepala* DC. (12x) (1), St. Elias Mountains, Chilkat River headwaters, Alaska, U.S.A., *Parker, Batten, and Blank 9523* (UWM63842): MF168897/MF168847, MF168834, MF168804.  
*Caltha introloba* F. v. M., AY365387, AY365368, —.  

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<th>Voucher information</th>
<th>Accession numbers</th>
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<td><strong>Ingroup:</strong> <em>Caltha</em>*</td>
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<tr>
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<td>Okanogan National Forest, Washington</td>
<td>MF168897/identical to MF168847, MF168838, identical to MF168804.</td>
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<td><em>leptosepala</em> DC. (12x†)</td>
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<td>Moses Butte area, Idaho</td>
<td>MF168902/MF168857, MF168834, MF168811.</td>
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<tr>
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<td>Wallowa Whitman National Forest,</td>
<td>MF168915/MF168862, identical to AY365370, MF168817.</td>
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<td><em>biflora</em> DC. (6x†)</td>
<td>(1)</td>
<td>Cascade Range, Willamette National</td>
<td>AY365395, AY365369, MF168803.</td>
</tr>
<tr>
<td><em>biflora</em> DC. (6x†)</td>
<td>(2)</td>
<td>North Cascades near Mt. Gardner,</td>
<td>MF168899, MF168836, MF168809.</td>
</tr>
<tr>
<td><em>chionophila</em> Greene</td>
<td>(6x)</td>
<td>Roosevelt National Forest, Niwot</td>
<td>AY365394, AY365370, MF168802.</td>
</tr>
<tr>
<td><em>introloba</em> F. v. M.</td>
<td></td>
<td></td>
<td>AY365387, AY365368, —.</td>
</tr>
<tr>
<td><em>palustris</em> L.</td>
<td></td>
<td></td>
<td>AY365382 AY365376, MF168797.</td>
</tr>
<tr>
<td><em>scaposa</em> Hook. f.</td>
<td></td>
<td></td>
<td>AY365396 AY365379, MF168798.</td>
</tr>
</tbody>
</table>
Appendix C. *Caltha biflora* specimens examined for recircumscription study.

* pollen sampled for SEM, n = 12; † ploidy level estimated via FCM or chromosome count (Wefferling et al., 2017), n = 27.

**Appendix D. Caltha chionophila** specimens examined for recircumscription study.

*pollen sampled for SEM, n = 14; † ploidy level estimated via FCM or chromosome count (Wefferling et al., 2017), n = 8.

Appendix E. *Caltha leptosepala* specimens examined for recircumscription study.

*pollen sampled for SEM; n = 15; † ploidy level estimated via FCM or chromosome count (Wefferling et al., 2017), n = 33.

Appendix F. Fossil calibration details for Ranunculales chronogram.

Fossil details, including calibrated node, taxon name, citation, plant part, type specimen(s) and accession number(s), morphological synapomorphies and considerations of placement, stratum/epoch/age, and locality. Abbreviations: CG = crown group; SG = stem group. All gamma age priors (Alpha, Beta, offset) listed in millions of years before present.

1. CG Proteales: *Nelumbites extenuinervis* Upchurch, Crane and Drinnan (Upchurch, Crane, and Drinnan, 1994). Leaves, perianth parts, and rhizomes. Holotype lacks accession number (simply listed as USNM, Mount Vernon locality; Upchurch et al., 1994: 32). Paratypes include USNM 446029AandB, 446031AandB, FMMH PP43792, PP43793. Grouped with *Nelumbo* in parsimony analysis of Quantico material in Doyle and Endress (2010), though “it is only one step less parsimonious to associate *Nelumbites* with *Brasenia* (Cabombaceae, Nymphaeales), which also has peltate leaves and more than one whorl of carpels” (Doyle and Endress, 2010). For full list of synapomorphies, see Doyle and Endress (2010). Stratum: Quantico Locality (Upper Albian), Lower Cretaceous Potomac Group of Virginia. Late middle to early late Albian (Doyle and Endress, 2010). Gamma age prior (Alpha, Beta, offset): 2, 3.3, 97.5.


3. CG “core Ranunculales” (Circaeasteraceae + Lardizabalaceae + Menispermaceae + Berberidaceae + Ranunculaceae): *Kajanthus lusitanicus* Mendes, G.W.Grimm, Pais et E.M.Friis (Mendes, Grimm, Pais, and Friis, 2014). Single flower. Holotype: Earth Sciences Department, Technology and Sciences College, New University of Lisbon, Portugal, P0093. A cladistic analysis (Mendes et al., 2014) supported this fossil’s affinity with Lardizabalaceae (but see notes on placement, below). “Shared morphological and anatomical traits between *Kajanthus* and all extant Lardizabalaceae include trimerous perianth, trimerous androecium consisting of six stamens in two whorls, anthers with four protruding pollen sacs, anther dehiscence extrorse by longitudinal slits, pollen grains small to medium size, tricolpate, with tectate-perforate pollen wall, and carpels free, plicate, with sessile stigma” (Mendes et al., 2014). However, Mendes et al., (2014) rescored the morphological character matrix of Doyle and Endress (2010), scoring Lardizabalaceae with bisexual flowers (*Kajanthus* flower is bisexual). As Lardizabalaceae usually have unisexual flowers (plants being monoeiceous, dioiceous, or polygamous; Mabberley, 1997, Heywood, Brummitt, Culham, and Seberg, 2007), we placed this fossil at the crown of core Ranunculales based on the following synapomorphies: change [from perianth dimerous] to perianth trimerous (Doyle and Endress, 2010 char. 55 1>0); androecium [dimerous to] trimerous (Doyle and Endress, 2010 char. 64 1>0); stamen positions [double to] single (Doyle and Endress, 2010 char. 66 1>0); carpel [parasyncarpous to] apocarpous (Doyle and Endress, 2010 char. 106 1>0). Bisexual flowers in *Kajanthus*. Stratum: Early Cretaceous (late Aptian–early Albian). Famalicão Member of the Figueira da Foz Formation, Portugal. Gamma age prior (Alpha, Beta, offset): 2, 3.3, 97.5.


5. CG *Anamirta* + *Coscinium* (Menispermaceae): *Anamirta sp.* Colebr. (Jacques and DeFranchesi, 2005). Incomplete endocarp. Specimen 40038 “…kept in the palaeobotanical collections of the Paris Muséum national d’Histoire naturelle (MNHN), collection Dario De Franceschi-Gaël De Ploëg… the spherical outline of the endocarp and a double perforate condyle, suggest affinities to *Anamirta leiocarpa* Manchester from the Clarno Formation” (Jacques and DeFranchesi, 2005). Stratum: Lower Eocene. Le
Quesnoy outcrop (Sparnacian facies, Paris Basin), France. Gamma age prior (Alpha, Beta, offset): 2, 1.8, 47.7.

6. SG *Tinospora* (Menispermaceae): *Tinospora excavata* Reid et Chandler 1933 (Fairon-Demaret and Smith, 2002). Locule cast. Housed at Royal Belgian Institute of Natural Sciences at Brussels collected July 1949. Specimen: IRScNB b3996, IG 20237. “…distinctive boat-shaped locule casts are recorded… conspicuous ventral ridge becoming less sharply defined on the dorsal side but still recognisable even when the cast has been compressed laterally or badly distorted. A well-preserved more or less hemispherical endocarp, with a small rounded ventral aperture, still shows remains of the finely tuberculcate external surface. The tubercles are abraded and partly smoothed away but they nevertheless have retained their elongated shape, radiating from the circular ventral aperture” (Fairon-Demaret and Smith, 2002). Morphologically, very similar to extant *T. hirsuta* (Wefferling, Hoot, and Neves, 2013). Stratum: Paleocene-Eocene transition, Tienen Formation at Dormaal in eastern Belgium (see Coillo et al., 2013 for stratigraphy). Gamma age prior (Alpha, Beta, offset): 2, 0.8, 55.3.

7. SG *Hypserpa* (Menispermaceae): *Eohypserpa parsonii* Reid et Chandler (1933; Jacques and DeFranchesi, 2005). Endocarp. Specimen 40056, “…kept in the palaeobotanical collections of the Paris Muséum national d’Histoire naturelle (MNHN), collection Dario De Franceschi-Gaël De Plôg…” Endocarp horseshoe-shaped, bisymmetric, the lateral faces not excavated [implying presence of hollow lateral chambers, K.M. Wefferling, personal observation], spherical in outline, one limb longer than the other and with a little dorsal median ridge at its end. Locule cast with smooth surface” (Jacques and DeFranchesi, 2005). Clear affinity with extant *Hypserpa* based on unequal limb length, “unexcavated” lateral face, circular lateral outline. Stratum: Lower Eocene. Le Quesnoy outcrop (Sparnacian facies, Paris Basin), France. Gamma age prior (Alpha, Beta, offset): 2, 1.8, 47.7.

8. SG *Stephania* (Menispermaceae): *Stephania palaeosudamericana* Herrera, Manchester, Hoot, Wefferling, Carvalho et Jaramillo sp. nov. (Herrera et al., 2011). Endocarps. Holotype: CJ85-ING-1412. Very similar to extant *S. brevipes* (Wefferling et al., 2013). “Some of the characters that support this affinity include the horseshoe-shaped endocarp, the length of the endocarp considerably less than 10 mm, a thin endocarp wall (<1 mm), only one lateral crest on each side of the endocarp, a straight ventral vascular tube, a long radical limb, conspicuous locule ribs, a straight ventral notch, and the lack of protrusions in the locule chamber” (Herrera et al., 2011). Stratum: Middle to late Paleocene. Guajira Peninsula, Ranchería Basin, Cerrejón coal mine, Cerrejón Formation, Tabaco Extensión localities 0705 and 0712, localities placed below coal bed 175, Colombia. Gamma age prior (Alpha, Beta, offset): 2, 0.8, 55.3.

9. SG *Cocculus* (Menispermaceae): *Cocculus lottii* (Collinson, Manchester, and Wilde, 2012: pp. 43-44, Plate 32 fig. o). Endocarp. Holotype, SM.B Me 8559, “Endocarps lacking dorsal crest but with a groove marking the plane of bisymmetry in an otherwise broad, rounded dorsal surface; with small central depression on each lateral face surrounded by an annular bulged area with radiating rugulae; endocarp with central depression from which coarse rugulae radiate… groove in plane of bisymmetry visible at lower left of specimen” (Collinson et al., 2012). Stratum: Middle Eocene. Messel Flora, SW Germany. Gamma age prior (Alpha, Beta, offset): 2, 1.9, 39.6.

Appendix G. Excluded putative Ranunculales fossil details.

Putative Ranunculales fossils that were considered but not included as calibrations in the present study. To underscore the different divergence time estimates between our study and others, we indicate putative Ranunculalean fossils that have been used to calibrate time trees in other studies with an asterisk (*).

_Archaefructus_ shows “unequivocal angiosperm features” (Friis et al., 2011, p. 95); however, “More detailed structural information is needed before the relationships of _Archaefructus_ can be determined reliably.” (Friis et al., 2011, p. 195).

_Eoaltha zoophila_* (Rodríguez-de la Rosa, Cevallos-Ferriz, and Silva-Pineda, 1998). We discounted this fossil based on an overall lack of morphological similarities to _Caltha_ fruits and seeds: (1) the lack of follicle remains; (2) the suggested position of the seeds in the fruit (see Fig. 9E of Rodríguez-de la Rosa et al., 1998), and (3) the subhemispherical flotation chamber positioned above the embryo sac cavity. In extant taxa that possess such a chamber (_C. palustris_), the flotation chamber is compressed and renders the fruit asymmetrical. *Used by Cheng and Xie (2014).

_Hyrcantha_* (Dilcher, Sun, Ji, and Li, 2007). The authors state: “The systematic affinities are not well placed with any extant angiosperm taxa. _H. decussata_ should be considered an extinct early angiosperm, and it is advisable that it not be used at this time as a reliable node for the origin of the eudicots.” (Dilcher et al., 2007). *Used by Magallón, Gómez-Acevedo, Sánchez-Reyes, and Hernández-Hernández (2015).

_Kenilanthus_ (Friis, Pedersen, and Crane, 2017) is an unresolved eudicot fossil bearing “…a mosaic of plesiomorphic features … _Kenilanthus_ fits this pattern, in showing features that point to a relationship to Lardizabalaceae and other Ranunculales, or perhaps core eudicots.” (Friis et al., 2017, p. 172).


_Myosurus_* (Mai and Walther, 1978). Poor quality of available images of this fossil, along with too few morphological characters for reliable taxonomic placement, preclude use of this fossil as a calibration point. *Used by Wang et al., (2016).

_Paleoactaea_* (Pigg and DeVore, 2005): Although the overall similarities between _Paleoactaea_ and extant species of the _Actaea_ + _Cimicifuga_ clade are striking, the fossil lacks unambiguous morphological synapomorphies for the clade. *Used by Cheng and Xie (2014), Magallón et al., (2015), and Wang et al., (2016).

_Potomacapnos_ (Papaveraceae; Jud and Hickey, 2013). The leaf characters alone seem insufficient for taxonomic placement and use as an age prior.

_Prototinomiscium_* (Knobloch and Mai, 1986). Several authors (Jacques, 2009; Jacques et al., 2011; Herrera et al., 2011; Wefferling et al., 2013) have expressed concern about the placement of this fossil: “… _Prototinomiscium_ is known from a few samples from only two outcrops. Its affinities are with the extant genus _Tinomiscium_, which has the least characteristic endocarp in Menispermaceae, due to its lack of condyle… The ventral concavity with a longitudinal ridge and apical placenta helps support this resemblance but does not confirm it.” (Jacques, 2009, p. 61). *Used by Anderson, Bremer, and Friis (2005), Britton, Anderson, Jacquet, Lundqvist, and Bremer (2007), Wang, Chen, Liu, R.-Q. Li, and J.-H. Li (2007), Jacques et al., (2011), and Magallón et al., (2015). Note that Jacques et al., (2011) analyzed their dataset both with and without _Prototinomiscium_, and found similar age estimates for Menispermaceae with either analysis.

_Ranunculaecarpus quinquecarpellatus_ from East Siberia, Russia, was tentatively placed in Ranunculales by Samylina (Friis et al., 2011), and is currently being re-examined (S. Manchester, Florida Museum of Natural History, personal communication). The fossil bears “…similarities with Ranunculaceae, but mostly in characters that seem to be likely plesiomorphic. We have about five stamens attached at the base of _Ranunculaecarpus_, but the pollen does not look like modern Ranunculaceae.” (S. Manchester, personal communication). We consider the placement of this fossil as too tentative (or taxonomically coarse) to use as a calibration at this point.
*Teixeiraea* (von Balthazar, Pedersen, and Friis, 2005). In the course of a phylogenetic study of Early Cretaceous eudicots (Doyle and Endress, 2010), Doyle and Endress performed a preliminary analysis of *Teixeiraea* but set it aside because its position was too poorly resolved (J.A. Doyle, University of California-Davis, personal communication). Also “…von Balthazar et al., (2005) considered that *Teixeiraea lusitanica* shows most affinities to members of Ranunculales, but it is also similar to some core eudicots, namely to Hamamelidaceae and Daphniphyllaceae (Saxifragales), and to *Berberidopsis* (Berberidopsidaceae, Berberidopsidales)” (Magallón et al., 2015). *Used by Anderson et al., (2005), Britton et al., (2007), and Magallón et al., (2015).
**Appendix H. Extant specimen sampling for Ranunculales chronogram.**

List of Ranunculales and outgroup species used in the order-level phylogeny. Accession information for sequences from current study is listed as follows: species name, GenBank accession number or numbers when multiple accessions were used to compile multiple gene regions. Gene regions are listed in the following order: atpB, rbcl, matK. 26S. — indicates missing sequence data. New accessions (sequences from GenBank) not included in Hoot et al., (2015): Ranunculaceae: Calliantheum taipaicum, Caltha leptosepala, Caltha palustris var. membranacea; Menispermaceae: Cocculus carolinus, Cocculus trilobus, Hyperla nitida, Kolboptalam leonense, Stephania japonica, Stephania longa, Tinospora sinensis, Tinospora smilacina.


**Circaeasteraceae:** *Circaeaster agrestis* Maxim., AF092116, AF093720, KM364776, AF389246. *Kingdonia uniflora* Balf.f. and W.W.Sm., AF092115, AF093719, KM364783, AF3898245.

**Eupteleaceae:** *Euptelea pleiosperma* L., —, AM396510, —. *E. polyandra* Siebold and Succ., U86384, L12645, —, AF389249.


**Papaveraceae s. l.:**


**Ranunculaceae:**

Appendix I. *Caltha* chronogram sampling.

Voucher information for *Caltha* specimens and outgroups used in genus-level phylogeny: species name, GenBank accession number. Gene regions are listed in the following order: internal transcribed spacer region (ITS), *trnL–F*, *rpl32–trnL*. — indicates missing sequence data.


**Outgroups:** *Actaea japonica* Thunb., KT598540, KT709628, —. *Anemonopsis macrophylla* Siebold and Zucc., Z98275, AJ222984, —. *Cimicifuga heracleifolia* Kom., FJ525884, —, —. *Cimicifuga nanchuanensis* P.K.Hsiao, —, KT709620, —. *Eranthis pinnatifida* Maxim, JF505806, JF505932, —. 
Appendix J. Age priors on *Caltha* phylogeny.

MRCA age priors placed on nodes in *Caltha* cpDNA/ITS phylogenies, based on the 95% highest posterior densities in the Ranunculales dataset analysis.

**Clade: all** prior (mean, standard deviation, offset): 43.65, 0.176, 0 [*Caltha chionophila* 1032e cID, *C. biflora* 896w cWA, *Actaea japonica*, *Anemonopsis macrophylla*, *Cimicifuga*, *Eranthis pinnatifida*, *Caltha appendiculata*, *C. dionaeifolia*, *C. introloba*, *C. natans*, *C. novaeezelandiae*, *C. obtusa*, *C. palustris*, *C. sagittata*, *C. scaposa*]

**Clade: all** _but_ *natans* prior (mean, standard deviation, offset): 13.45, 0.426, 0 [*Caltha chionophila* 1032e cID, *C. biflora* 896w cWA, *Caltha appendiculata*, *C. dionaeifolia*, *C. introloba*, *C. novaeezelandiae*, *C. obtusa*, *C. palustris*, *C. sagittata*, *C. scaposa*]

**Clade: og1** prior (mean, standard deviation, offset): 26.75, 0.27, 0 [*Actaea japonica*, *Anemonopsis macrophylla*, *Cimicifuga*, *Eranthis pinnatifida*]

**Clade: og2** prior (mean, standard deviation, offset): 15.1, 0.364, 0 [*Actaea japonica*, *Cimicifuga*, *Eranthis pinnatifida*]
Appendix K. Subalpine marshmarigold sampling for phylogeographic study.

Voucher information for 161 specimens of subalpine marshmarigolds included in this study, organized by species (hexaploid *Caltha biflora*, hexaploid *C. chionophila*, allohexadecaploid *C. leptosepala* with *C. biflora* haplotype, allohexadecaploid *C. leptosepala* with *C. chionophila* haplotype, allonanoploid *C. leptosepala*, hybrids with *biflora* morphology [including a hexaploid, a putative aneuploid, and two with unknown genome size], a hybrid with *chionophila* morphology [with unknown genome size], and a putative aneuploid with *chionophila* morphology) followed by the Hoot lab extraction number. For specimens cited in Wefferling and Hoot (2017): Hoot lab extraction # (in bold, underlined with ploidy level data where inferred by proplidium iodide flow cytometry and/or *chromosome counts), collector and collector #, herbarium and accession number, and GenBank numbers (ITS *biflora* ribotype, ITS *chionophila* ribotype, *rpl32-trnl*, *rps16-trnQ, trnl-trnF*; -- indicates missing data, NA indicates that only a single ribotype is expected (i.e., in *Caltha biflora* or *C. chionophila*). Sequences that are identical to those of another specimen (i.e., redundant sequences) are not given a unique GenBank number and are indicated with = and the Hoot lab extraction number of the identical sequence (with species identification of the identical sequence). Complete voucher information only available for specimens not listed in Wefferling and Hoot (2017) (*n* = 21): Hoot lab DNA extraction #, State/Province, Country, latitude, longitude, elevation, location, collector and collector #, collection date, herbarium and accession #, and GenBank numbers.

=465 (C. chionophila); 1010, 6x, Wefferling K. 59s, UWM65531, =943 (C. leptosepala), NA, =481 (C. leptosepala), –, =481; 1011, Wefferling K. and L. Wefferling 72s, UWM65622, =943 (C. leptosepala), NA, =481 (C. leptosepala), –, =481; 1012, 6x, Wefferling K. and L. Wefferling 94s, UWM65647, =943 (C. leptosepala), NA, =481 (C. leptosepala), –, =481; 1026, 6x, Wefferling K. 47s, UWM65526, MF168929, NA, =466 (C. biflora), –, =1028, 6x*, Wefferling K. CRI, UWM65620, =943 (C. leptosepala), NA, =1028, =481 (C. leptosepala), –, =481; 1029, 6x*, OR, USA, 45.34525, -121.620528, 1570m, Mt. Hood Wilderness, NW end of Elk Meadows, Wefferling K. CR2, 4/27/2015, no voucher, same site as UWM65643, =943 (C. leptosepala), NA, =481 (C. leptosepala), –, =481; 1031, 6x, OR, USA, 45.34525, -121.620528, 1570m, Mt. Hood Wilderness, NW end of Elk Meadows, Wefferling K. and L. Wefferling 149s, 4/27/2015, no voucher, same site as UWM65643, =943 (C. leptosepala), NA, =481 (C. leptosepala), –, =481; 1037, Halse R. 5869, no voucher, same site as UWM63863, =913 (C. biflora), NA, =466 (C. biflora), –, =1039, Wefferling K. and L. Wefferling 74s, UWM65648, =943 (C. leptosepala), NA, =481 (C. leptosepala), –, =481; 1041, 6x, OR, USA, 44.6473, -122.0011, 1244m, Cascade Range, Willamette NF, Bruno Meadows area about 5 air miles SE of Idanha, Halse R. R. 5869, 7/13/2000, OSC199926, =992, NA, =992, =481 (C. leptosepala), –; 1042, Marr K.L. and C. Copley KM6340, V193816, =892 (C. biflora), NA, =892, –, –; 1043, Hebda R. and G. Allen 91-17, V150315, =892 (C. biflora), NA, MF168820, –, –; 1044, Oligivie R. T., W. J. Schofield, and R. J. Hebda 849912, V128306, =892 (C. biflora), NA, =892, –, –; 1049, Jaques 1209, OSC141099, =892 (C. biflora), NA, =892, =950 (C. biflora), –; 1060, 6x, OR, USA, 42.0766, -122.718, 2012m, Klamath NF, Mt Ashland, Pacific Crest Trail, Anderson J. JNSI-1B, 4/27/2015, no voucher, same site as UWM65347, =911 (C. biflora), NA, =933 (C. biflora), –, =481 (C. leptosepala), –; 1062, 6x, OR, USA, 42.0766, -122.718, 2012m, Klamath NF, Mt Ashland Pacific Crest Trail, Bradshaw A. 10451.10 AB2-1, 4/27/2015, no voucher, same site as UWM65347, =911 (C. biflora), NA, =933 (C. biflora), –, =481 (C. leptosepala), –; 1083, 6x, Shrum J. SM5, UWM65606, MF168935, NA, =896 (C. biflora), –, –; 1084, 6x, Hunter G. BL4, UWM65607, =892 (C. biflora), NA, =481 (C. leptosepala), –, =481; 1085, 6x, Hunter G. LR1, UWM65614, =892 (C. biflora), NA, =481 (C. leptosepala), –, –; 1087, Wilson B. L. 6986, SRP013689, =913 (C. biflora), NA, =481 (C. leptosepala), –, =481; 1090, Calder J. A. and K. T. MacKay 29381, US3567701, =892 (C. biflora), NA, –, –.

C. chionophila (6x): 465, Schuettpez E. J. 001, UWM63862, NA, AY365394, MF168802, –, AY365370; 890, Throne A. L. 13776, UWM65377, NA, =893 (C. leptosepala), =905 (C. leptosepala), –, =465 (C. chionophila); 891, Throne A. L. 12538, UWM65376, NA, MF168850, MF168807, –, =465 (C. chionophila); 899, Bursik R. 1240, ID039933, NA, MF168853, =905 (C. leptosepala), MF168829, –, =465 (C. chionophila); 901, Holmgren N. H. and P. K. Holmgren 7330, NYBG s. n., NA, MF168855, =891 (C. chionophila), =889 (aneuploid C. chionophila), =465 (C. chionophila); 907, 6x, Hall C. B. Jacobs and A. Morgan 1565, NYBG s. n., NA, MF168858, =905 (C. leptosepala), =889 (aneuploid C. chionophila), =465 (C. chionophila); 908, Tiehm A. and M. Williams 9675, ID087595, NA, =893 (C. leptosepala), =905 (C. leptosepala), MF168830, =465 (C. chionophila); 909, 6x, Holmgren N. H. and P. K. Holmgren 15424, NYBG01057127, NA, MF168859, MF168812, =899 (C. chionophila), =465 (C. chionophila); 927, 6x, Wefferling K. 19, UWM65341, NA, =465 (C. chionophila), =889 (aneuploid C. chionophila), MF168831, =465; 952, Fertig W. 7481, RM587708, NA, MF168866, =905 (C. leptosepala), =899 (C. chionophila), =465 (C. chionophila); 953, Mantas M. 585, ID105871, NA, MF168867, =905 (C. leptosepala), =899 (C. chionophila), =465 (C. chionophila); 954, Rink G. and L. Stevens s.n., ASC98517, NA, MF168868, MF168818, =899 (C. chionophila), =465 (C. chionophila); 955, 6x, Holmgren N. H. and P. K. Holmgren 16196, NYBG01208759, NA, =889 (aneuploid C. chionophila), =905 (C. leptosepala), MF168833, =465 (C. leptosepala); 982, Refsdal C. H. 6799 with L. Refsdal, RM689262, NA, MF168871, =905 (C. leptosepala), –, MF168845; 983, Evert E. s.n., RM780065, NA, MF168872, =905 (C. leptosepala), –, =465 (C. chionophila); 984, Moseley. B. 1181, ID999679, NA, MF168873, =905 (C. leptosepala), –, MF168846; 985, Brunsfeld S. J. 1737, ID155836, NA, =899 (C. chionophila), =905 (C. leptosepala), –, =465 (C. chionophila); 1021, Wefferling K. 242h, UWM65640, NA, MF168878, =905 (C. leptosepala), –, =465 (C. chionophila); 1022, Wefferling K. 258h, UWM65641, NA, MF168879, =905 (C. leptosepala), –, =465 (C. chionophila); 1023, 6x, ID, USA, 44.033433, -114.624312, 2870m, White
Marr K. L., R. Hebda, and W. MacKenzie 06-0013, V/RBCM_V196348, =892 (C. biflora), =893 (C. leptosepala), =481 (C. leptosepala), =481; 963, 12x, Marr K. L., R. Hebda, and W MacKenzie 07-1648, V/RBCM_V201979, =892 (C. biflora), =949 (C. leptosepala), =481 (C. leptosepala), =481; 980, 12x, Wefferling K. 60, UWM65322, MF168923, =895 (C. leptosepala), =481 (C. leptosepala), =988, Soper J. H. 12754 and M. J. Schepanek, V/RBCM_V056463, =892 (C. biflora), MF168874, =481 (C. leptosepala), =990, Yen A. C. 03-066 with R. Roboehm, S. Bagshaw, L. Van Volkkenburgh, and T. Ohsion, WTU362408, =943 (C. leptosepala), =985 (C. leptosepala), =896 (C. biflora), =991, Wooten G. #GW01566, WU3333192, =892 (C. biflora), =893 (C. leptosepala), =481 (C. leptosepala), =913, 12x, Wefferling K. and L. Wefferling 87s, UWM65628, =943 (C. leptosepala), =895 (C. leptosepala), =481 (C. leptosepala), =481; 1015, 12x, Wefferling K. and L. Wefferling 118th, UWM65631, =980 (C. leptosepala), =895 (C. leptosepala), =481 (C. leptosepala), =481; 1050, Parker C. L. 17964, ALAV170049, =892 (C. biflora), =985 (C. leptosepala), =481 (C. leptosepala), =481; 1051, Taylor S. G. 53, ALA39425, MF169890, =895 (C. leptosepala), =481 (C. leptosepala), =481; 1053, 12x*, Wefferling K. CR3, UWM65618, MF168931, =895 (C. leptosepala), =481 (C. leptosepala), =911, 12x*, collector unknown [seeds obtained from Washington Native Plant Society] WNPS-2, UWM65625, MF168892, =986 (C. biflora), =986, =1080, 12x, Knoke D. 267, WTU354477, =1086 (hybrid, aneuploid?), =947 (C. leptosepala), =481 (C. leptosepala), =481; 1082, 12x, Rocheport R. SP4, UWM65612, MF16934, =947 (C. leptosepala), =896 (C. biflora), =481 (C. leptosepala) with C. chionophila cpDNA, 12x: 900, 12x, Tanaka T. and C. Richardson 82, ID039769, MF168901, MF168854, =905 (C. leptosepala), =899 (C. chionophila), =465 (C. chionophila); 905, 12x, Parks M. L. Stratford and R. McNell 620, ID039909, MF168902, MF16885, MF168811, =899 (C. chionophila), MF168839; 915, 12x, Legler B., S. Gage, W. Gibble, R. Goff, S. Birks and K. Davis 1890, WTU367680, MF168907, MF168860, MF168814, =465 (C. chionophila); 918, Bartolomew B. 4327 and B. Anderson, NYBG00485969, MF168908, MF168861, =915 (C. leptosepala), =908 (C. chionophila), =465 (C. chionophila); 945, 12x, Wefferling K. and L. Woo 62, UWM65375, MF168915, MF168862, MF168817, =908 (C. chionophila), =465 (C. chionophila); 974, 12x, Wefferling K. 67, UWM65642, MF168920, =895 (C. leptosepala), =915 (C. leptosepala), =908 (C. chionophila), =465 (C. chionophila); 977, 12x, Wefferling K. 70, UWM65646, MF168922, =949 (C. leptosepala), =915 (C. leptosepala), =465 (C. chionophila); 1016, 12x, OR, USA, 45.34525, -121.620528, 1570m, Mt. Hood Wilderness, NW end of Elk Meadows, Wefferling K. and L. Wefferling 150s, 7/16/2014, no voucher, same site as UWM65642, =980 (C. leptosepala), =895 (C. leptosepala), =915 (C. leptosepala), =465 (C. chionophila); 1017, 12x, Wefferling K. 1800h, UWM65634, MF168926, =900 (C. leptosepala), =905 (C. leptosepala), =465 (C. chionophila); 1018, Wefferling K. 212lh, UWM65635, MF168927, MF168976, =905 (C. leptosepala), =465 (C. chionophila); 1038, 12x, OR, USA, 45.34525, -121.620528, 1570m, Mt. Hood Wilderness, NW end of Elk Meadows, Wefferling K. and L. Wefferling 151s, 7/16/2014, no voucher, same site as UWM65646, =980 (C. leptosepala), MF168886, =915 (C. leptosepala), =465 (C. chionophila); 1055, 12x*, WA, USA, 46.268447, -121.586248, 1400m, Gifford-Pinchot NF, Takhlahk Meadow, Wefferling K. CR4,
4/27/2015, no voucher, silica material available, =1053 (C. leptosepala), =893 (C. leptosepala), =915 (C. leptosepala), –, –; 1059, 12x, Smith J. F. JS-1, no voucher, same site as SRP40151, MF168933, MF168889, =905 (C. leptosepala), =899 (C. chionophila), =465 (C. chionophila); 1061, 12x*, Anderson J. JNS2-1, UWM65624, =943 (C. leptosepala), =893 (C. leptosepala), =915 (C. leptosepala), =899 (C. chionophila), –; 1072, Moseley B. 1211, ID99807, =911 (C. biflora), MF168890, MF168822, –, –.


C. chionophila (possible aneuploid): 889, undetermined/aneuploid, CO, USA, 39.323, -106.128, 3606m, on County Road 8, 5.1 mi NW of Alma, 0.4 mi S of Kite Lake, Pike NF, Townesmith A. G. Gust and L. Nye 202, 7/15/2005, UWM65105, NA, MF168849, MF168806, MF168826, =465 (C. chionophila).
Appendix L. Permit numbers used for *Caltha* collections.

United States Forest Service National Forest (NF) and United States National Park Service (NPS) permit numbers for *Caltha biflora*, *C. chionophila*, and *C. leptosepala* field collections. All NF permits issued to K. Wefferling.

**National Parks:**
Mount Rainier National Park: **MORA-2015-SCI-0036** (issued to M. Bivin, R. Rochefort, and K. Wefferling)
North Cascades National Park: **NCCO-2015-SCI-0010** (issued to M. Bivin, R. Rochefort, and K. Wefferling)

**National Forests:**
Lassen NF (California): **49702**
Region 1 (Pacific Northwest Region: NF lands in Oregon and Washington): **2012-3, 2012-4 and 2013-7**
Region 4 (Intermountain Region: NF lands in southern Idaho, Nevada, Utah, and western Wyoming):
  - **Wefferling01**
Shasta-Trinity NF (California): **31111**
Sierra NF (California): **2012-003**
CURRICULUM VITAE

Keir Michael Wefferling

Education
B.S., University of Wisconsin–Milwaukee, May 2011
Major: Biological Sciences

Dissertation title
Disentangling the subalpine marshmarigold species complex: the cytogeography, phylogeography, and systematics of Caltha biflora DC., C. chionophila Greene, and C. leptosepala DC. (Ranunculaceae)

Peer-reviewed publications
Wefferling KM, Hoot SB (2017) Species circumscription of the Caltha leptosepala polyploid complex (Ranunculaceae) based on molecular and morphological data. Phytotaxa 316:201–223. doi: http://dx.doi.org/10.11646/phytotaxa.316.3.1

Other publications

Grants funded and Fellowships
Postdoctoral Research Fellowship in Biology, National Science Foundation, September 2018–August 2020
Distinguished Dissertation Fellowship, University of Wisconsin–Milwaukee, September 2014–May 2015
Research Improvement Grant, International Association for Plant Taxonomy, May 2014–May 2015
R. McVaugh Graduate Student Research Grant, American Society of Plant Taxonomists, May 2013–May 2014
Research Improvement Grant, Native Plant Society of Oregon, May 2012–May 2014
Research Improvement Grant, Washington Native Plant Society, May 2012–May 2014
Distinguished Graduate Student Fellowship, University of Wisconsin–Milwaukee, September 2012–May 2013

Recent Awards and Honors
Vernon Ceadle Student Travel Award, Botanical Society of America, July 2017
Clifford H. Mortimer Award, University of Wisconsin-Milwaukee, May 2017
Ruth Walker Grant-in-Aid Award, University of Wisconsin-Milwaukee, May 2016
Lawrence Memorial Award, Hunt Institute for Botanical Documentation, July 2015
James J. Magnino, M.D. Scholarship, University of Wisconsin-Milwaukee, May 2015
Ruth Walker Grant-in-Aid Award, University of Wisconsin-Milwaukee, May 2015
Delzie Demaree Travel Award for Annual Systematics Symposium, September 2014
Travel Grant for Botany 2014, American Society of Plant Taxonomists, July 2014
Graduate Student Research Award, Botanical Society of America, May 2014