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Citation	Kelso, S., Beardsley, P. M., & Weitemier, K. (2009). Phylogeny and biogeography of <i>Primula</i> sect. <i>Parryi</i> (Primulaceae). <i>International Journal of Plant Sciences</i> , 170 (1), 93-106. doi:10.1086/593041
DOI	10.1086/593041
Publisher	The University of Chicago Press
Version	Version of Record
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsfuse

PHYLOGENY AND BIOGEOGRAPHY OF *PRIMULA* SECT. *PARRYI* (PRIMULACEAE)

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Primula sect. *Parryi* comprises five species endemic to western North America: *P. parryi*, *P. angustifolia*, *P. rusbyi*, *P. capillaris*, and *P. cusickiana* with four varieties. This section, derived from a clade with representatives in Asia and Europe, exemplifies a phylogeographic pattern in which a widespread species is accompanied by multiple narrow endemics. We utilized AFLPs and DNA sequences from cpDNA and nuclear internal transcribed spacer/external transcribed spacer regions to examine the hypothesis that sect. *Parryi* represents a split from an ancestral lineage in *Primula* subgenus *Auriculastrum*; within the section, *P. parryi* was the earliest to diverge; other taxa represent allopatric speciation facilitated by Pleistocene climatic and ecological changes. An alternative hypothesis is that all or some of the endemics were derived relatively recently from within the *P. parryi* lineage. Results affirm monophyly of sect. *Parryi* and major species groups, including *P. parryi*. Variable relationships within the *P. cusickiana* complex (*P. cusickiana* s.l. and *P. capillaris*) suggest recent divergence. Phylogenetic perspectives are generally congruent and consistent with a hypothesis of allopatric speciation facilitated by Quaternary landscape changes. Section *Parryi* encompasses a spectrum of variation from well-defined monophyletic species to less well-differentiated taxa. Climatic predictions, limited migration potential, small populations, and reproductive restrictions are reasons for conservation concern.

Keywords: *Primula*, section *Parryi*, subgenus *Auriculastrum*, Primulaceae, endemism, allopatric speciation, niche conservatism.

Online enhancements: appendix tables.

Introduction

The modern flora of the North American Intermountain and Rocky Mountain West inhabits a landscape marked by edaphic, climatic, and topographic diversity. This region, which extends from the Rocky Mountains to the Sierra Nevada–Cascade axis and south of the Mexican border into the Mexican highlands (Brouillet and Whetstone 1993), has been subjected to extreme environmental changes and pervasive diastrophism since the early Tertiary. Its biota is known for indigenous elements with affinities to Eurasia and Mesoamerica (Cronquist 1978; Harper et al. 1978; Tiffney 1985; Qian 1999; Weber 2003) and for endemism (e.g., *Eriogonum*, *Astragalus*, *Penstemon*, *Physaria*, and the major clades in *Mimulus*, *Dodecatheon*, and the Polemoniaceae). Long- and short-term climatic shifts, interactions with new biotic elements from migration over latitudinal and elevational gradients, habitat fragmentation, aridification, and vicariance events such as orogenies of more than 300 mountain ranges have all been suggested as selective forces that shaped distribution patterns and regional diversity over different timescales (Cronquist 1978; Harper

and Reveal 1978; Reveal 1979). The data for vegetation response to environmental change (table 1) is abundant and detailed (Leopold and Macginitie 1972; Tidwell et al. 1972; Cronquist 1978; Reveal 1979; Thompson 1990; Graham 1999; Manchester 1999) and provide a context for biogeographic hypotheses.

A common biogeographic pattern in this region is the occurrence of related allopatric taxa with relatively narrow distributions restricted to specialized habitats (Harper and Reveal 1978; Reveal 1979). These well-defined taxa are likely to have diverged distantly enough in time to demonstrate reciprocal monophyly across lineages. Another common pattern in species-rich groups (*Penstemon*, *Eriogonum*, *Dodecatheon*, and *Mimulus*) is the presence of a widespread species with narrowly distributed endemic relatives apparently derived relatively recently from within the widespread species. In both patterns, endemics are often restricted by elevation or substrate. While evolutionary relationships and patterns of speciation were traditionally interpreted from morphology, molecular genetic data have recently provided nuanced perspectives, in some cases supporting phylogenetic and biogeographic hypotheses based on morphology but in other instances revealing unexpected complexity. This complexity is illustrated by studies from diverse plant groups, such as the Onagraceae (Levin et al. 2004), the Apiaceae (Sun et al. 2004), the Montieae tribe in Portulacaceae (O'Quinn and Hufford 2005), the Physarieae and Boecherae tribes in Brassicaceae (Al-Shehbaz et al. 2006), section *Acrocystis* in *Carex* (Roalson and Friar 2004), *Ribes* (Grossulariaceae; Schulteis

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Manuscript received February 2008; revised manuscript received May 2008.

Table 1
Overview of Cenozoic Environmental Change in Western North America

Era	Climate	Orogenic activity	Vegetation/biogeographic patterns
Early Tertiary (65–35 Myr ago)	Initially warm, moist; some drying by Eocene	Initiation of early Rocky Mountains; Uintahs	Mesothermal, broadleaf to sclerophyll evergreens; Asian affinities
Mid-Tertiary (35–15 Myr ago)	Drying trend, temperature decline	Uplift of the Rocky Mountains	Elements of montane spruce-fir conifer forests established in alpine zones; elevational sorting of vegetation zones; European affinities of some genera
Late Tertiary (29–2 Myr ago)	Continued drying, warming periods, followed by late Pliocene cooling and drying	Elevation of Sierra Nevada, Cascade Mountains, Rocky Mountains; elevation of Great Basin and initiation of fault block mountains	Mixed hardwood conifer forests with Asian affinities in NV, ID; disappearance of subtropical elements, diversification of Cordilleran flora to modern species and possible origins of autochthonous genera; zonal fragmentation of vegetation
Pleistocene (1.6 Myr–10 kyr ago)	Periodic cold-warm and arid-moist episodes of glacial-interglacial conditions	Periodic glaciation in alpine areas; Last Glacial Maximum ends ~18 kyr ago	Vegetation zones and species shift in elevation, latitude and exposure; extensive tundra and montane-subalpine conifer forests
Holocene (10 kyr ago–present)	Increasing warmth and aridification, localized droughts and short term cooling-warming episodes	Rapid deglaciation, drying of interior lake basins	Lowland desert scrub to alpine montane-subalpine conifer forests present; cool forest and tundra increasingly restricted

and Donoghue 2004), *Arnica* (Asteraceae; Ekenas et al. 2007), *Stephanomeria* (Asteraceae; Ford et al. 2006), *Mimulus* (Phrymaceae; Beardsley et al. 2004), *Sidalcea* (Malvaceae; Andreasen and Baldwin 2003a, 2003b), and *Linanthus* (Polemoniaceae; Bell and Patterson 2000). These studies amply demonstrate that reticulate relationships, paraphyly, or polyphyly of taxa traditionally recognized at the generic, subgeneric, or species levels, homoplasies in morphological characters, and/or evidence of recent rapid evolution of segregate taxa may not be exceptional in this region.

One group of endemics conforming to a common regional distribution pattern occurs in section *Parryi* of the genus *Primula* L. (primrose). This section, which contains a broadly distributed species accompanied by relatives with restricted distributions (fig. 1), illuminates regional evolutionary diversification across a spectrum of geographic, genetic, and temporal scales. While this small section in the large *Primula* genus (~500 species worldwide) has close congeners across the North Pacific island arc of the Aleutian-Commander islands into northern Japan as well as in the European mountains, it is the only multispecies section in the genus that is endemic to North America. *Primula* section *Parryi*, described in detail by Smith and Fletcher (1948) and formalized by Wendelbo (1961), consists of eight taxa and is currently recognized as five species: *P. parryi* A. Gray, *P. angustifolia* Torrey, *P. rusbyi* Greene, *P. capillaris* N.H. Holmgren & A.H. Holmgren, and *P. cusickiana* (A. Gray) A. Gray, with varieties *cusickiana*, *maguirei* (L.O. Williams) N. Holmgren & S. Kelso, *nevadensis* (N. Holmgren) N. Holmgren & S. Kelso, and *domensis* (Kass & Welsh) N. Holmgren & S. Kelso.

Primula parryi is common in subalpine/alpine wetlands from northern Montana to Idaho and south into New Mexico, northern Arizona and Nevada. Although usually separated by habitat, *P. parryi* occasionally grows within a kilometer or less of

several of its congeners (S. Kelso, personal observation). Its range encompasses all the other species in the section except one, *P. rusbyi*, which occurs to the south of *P. parryi*. *Primula rusbyi* occurs sporadically in subalpine conifer forests from southern Arizona and New Mexico into the Sierra Madre Occidental of Mexico, a zone known as the “Madrean sky island archipelago” (Warshall 1995) that is recognized for its biotic diversity and biogeographic significance (Felger and Wilson 1995). The remaining taxa, *P. capillaris* (Ruby Mountains, NV), *P. angustifolia* (central Rocky Mountains, Colorado to New Mexico), and the varieties of *P. cusickiana* (Intermountain Region of Oregon, Idaho, Utah, and Nevada), are endemics restricted to certain mountain ranges or anomalously cool, moist habitats such as cliff crevices. Intergrading vegetative characters for this suite of taxa have evoked different taxonomic interpretations (Holmgren and Kelso 2001; Richards 2003; Kelso, forthcoming; table A1 in the online edition of the *International Journal of Plant Sciences*), while the narrow distributions have elicited conservation concerns for many of the taxa.

Previous analyses of sect. *Parryi* were based on morphology (Williams 1936; Smith and Fletcher 1948), and recently, on an analysis of chloroplast DNA sequences from seven regions (*matK* gene, *rpl16* intron, *rps16* intron, *trnL* intron, *trnL/F* spacer, *trnS/G* spacer, and *trnT/L* spacer; Mast et al. 2004). The latter study showed the section to be monophyletic and supported earlier indications (Kallersjö et al. 2000; Mast et al. 2001; Martins et al. 2003) that it is closely related to *Primula suffrutescens* A. Gray (Sierra Nevada of California) and *Dodecatheon* L. (shooting stars; across North America but most abundant and diverse in the western United States), as well as to *Primula* sect. *Cuneifolia*, centered in northern Japan, and sect. *Auricula*, endemic to central European mountain ranges. *Primula suffrutescens*, sects. *Auricula*, *Parryi*, and *Cuneifolia*,

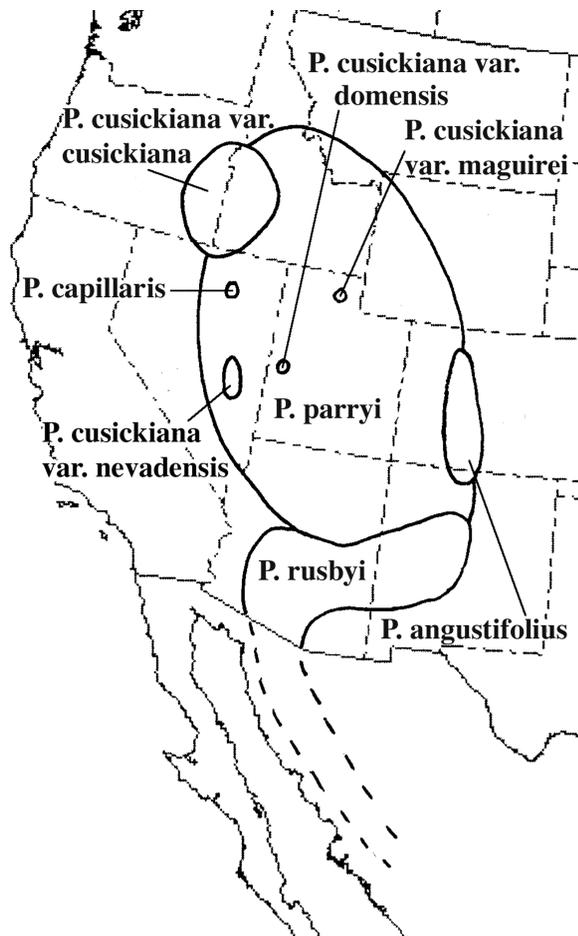


Fig. 1 Distribution of *Primula* sect. *Parryi* in the United States.

plus *Dodecatheon* collectively constitute a monophyletic group of *Primula* subgenus *Auriculastrum* Schott (table 2). Mast and Reveal (2007) transferred *Dodecatheon* to *Primula* based on its paraphyletic position within the subgenus. Mast et al. (2004) confirmed previously hypothesized relationships among these groups with wide intercontinental disjunctions and supported a closer relationship between the morphologically and reproductively dissimilar *Dodecatheon* and *P. suffrutescens* rather than between *Dodecatheon* and *P. parryi*, which share a striking morphological similarity noted by Smith and Fletcher (1948) and Thompson (1953). Within sect. *Parryi*, cpDNA analyses strongly supported a basal split between *P. parryi* and the remaining species in the section. There is also strong support for a sister relationship between *P. rusbyi* and a clade consisting of *P. angustifolia* plus the *P. cusickiana* complex, defined here as the infraspecific varieties of *P. cusickiana* plus *P. capillaris*. Relationships within the *P. cusickiana* complex remained ambiguous or unresolved (fig. 2). However, because the data collected in this study were mostly for one individual per taxon and only one locus was sampled, reticulation among taxa could not be examined. Additionally, because within-taxon sampling was limited to one or two accessions, previous studies did not address the question of whether widely distributed species such as *P. parryi* or *P. rusbyi* are monophyletic.

The cpDNA data suggest a hypothesis that a North American lineage diverged from an Asian common ancestor in subgenus *Auriculastrum* and that *P. parryi* represents an early split within this North American lineage. Later branching within this group led to a southern (“Madrean”) clade represented by *P. rusbyi* and a northern clade consisting of the Rocky Mountain species *P. angustifolia* sister to the Intermountain–Great Basin members of the *P. cusickiana* complex. Intergrading morphology and lack of resolution for members of the *P. cusickiana* complex suggest recent segregation in the context of climatically induced allopatry and ecological vicariance. This pattern is consistent with what is known about late Pleistocene and Holocene climatic and ecological changes within their current ranges, but existing data do not allow for firm conclusions.

The goals of this study were to build upon the hypotheses of relationships provided by chloroplast DNA sequences, and to utilize additional genetic data and wider sampling for developing deeper perspectives on evolution and biogeography of sect. *Parryi* as illustrative of regional plant evolution. Our primary model parallels the cpDNA perspective and hypothesizes that the section *Parryi* is monophyletic, as is the widespread species *P. parryi*. The model predicts strong genetic differentiation for *P. parryi* due to a longer period of separation, with congruent relationships and stable within-taxon clusters from different genetic perspectives for the major species groups. Within the *P. cusickiana* complex, ecological and geographic disjunctions predict some genetic differentiation, but intergrading morphological characters, such as the unresolved cpDNA topology, suggest that relationships and the degree of intertaxon differentiation might be only weakly supported with additional molecular data.

Given the documented portrait of evolutionary complexity and sometimes cryptic relationships for many plant taxa in the region and for other members of the Primulaceae explicitly (Zhang and Kadereit 2004; Dixon et al. 2007; Guggisberg et al. 2008; Reveal, forthcoming), caution might be warranted about assumptions on simple relationships in sect. *Parryi*. An alternative model hypothesizes that *P. parryi* may be more variable than revealed in the limited sampling of chloroplast sequences and that all or some of the endemics within the section were derived relatively recently from within the lineage encompassing *P. parryi*. If so, dissonance between different genetic perspectives, potential paraphyletic positions of endemic taxa nested within *P. parryi*, and genetic affinities between geographically contiguous taxa would be shown with different analyses.

To assess these perspectives, we examined patterns of relationships of closely related taxa, especially in the *P. cusickiana* complex and within *P. parryi* across its geographic range using AFLPs (amplified fragment length polymorphisms), neutral sequences that have been demonstrated to be useful for discriminating between closely related or recently diverged taxa (Althoff et al. 2007). Different taxa that exist in close proximity were preferentially sampled. We also collected DNA sequence data for each taxa from nuclear DNA regions, notably internal transcribed spacer (ITS) and external transcribed spacer (ETS) sequences, to complement data from the chloroplast. We compared phylogenetic hypotheses obtained from individual and combined data sets and analyzed geographic patterns of diversification and speciation in relationship to genetic data and to known environmental history of the geographic region now inhabited by members of sect. *Parryi*.

Table 2
Conspectus of *Auriculastrum*, Based on *Primula* Subg. *Auriculastrum* Schott

Taxonomic group	No. species	Distribution	Ploidy levels (derived from $x=11$)	Reproductive mode
<i>Primula</i> sect. <i>Auricula</i> Duby	~25 (31 taxa)	Mountains of central and southern Europe	6x; aneuploids, 2 high polyploids ($2n=126$; 198)	Heterostylous
<i>Primula</i> sect. <i>Auneifolia</i> Balfour	2 (6 taxa)	Pacific Coastal zone, Japan, Alaska to British Columbia	2x	Heterostylous and homostylous
<i>Primula</i> sect. <i>Suffrutescens</i> A.J. Richards (sine Latin diagnosis)	1	Sierra Nevada, CA	4x	Heterostylous
<i>Primula</i> sect. <i>Parryi</i> W.W. Smith ex Wendelbo	5 (8 taxa)	Western United States; Mexico	4x	Heterostylous
<i>Dodecatheon</i> (traditionally a segregate genus, placed in <i>Primula</i> by Mast and Reveal 2007)	~17 (up to 31 taxa)	North America, primarily western; Russian Far East	4x, 6x, 8x, 12x; aneuploids	Buzz pollination

Note. Taxonomy follows Richards (2003); Mast et al. (2004); Zhang and Kadereit (2004). Taxa share a chromosome number based on multiples of 11, involute vernation of leaves, and morphological characters in seeds, pollen, glands, and fruits (although variation within and among major clades occurs), whereas seedling development patterns, vegetative characteristics, reproductive biology, and floral morphologies vary considerably.

Material and Methods

Taxon Sampling

Leaf material for AFLP analysis and some ITS/ETS analyses were collected in the wild (table A2 in the online edition of the

International Journal of Plant Sciences) from 11 populations of *Primula parryi* (Arizona, Colorado, New Mexico, Nevada, and Utah) six populations of *Primula angustifolia* (Colorado), two populations of *Primula cusickiana* var. *cusickiana* (Idaho), one population of *P. cusickiana* var. *nevadensis* (Nevada), one population of *P. cusickiana* var. *maguirei* (Utah), three populations of

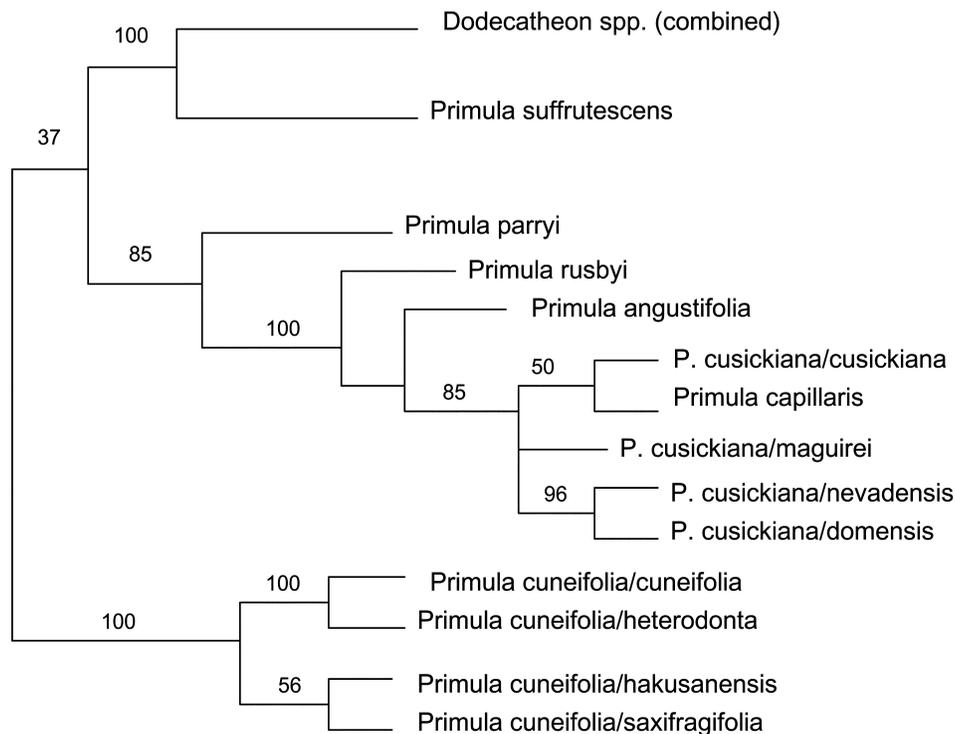


Fig. 2 Combined strict consensus tree of relationships of *Primula* subgenus *Auriculastrum* based on seven chloroplast regions; synthesized from Mast et al. (2004). Original paper provides additional information and discussion. Numbers above branches indicate bootstrap frequencies as a percentage.

Primula rusbyi (New Mexico, Arizona), one population of *Primula capillaris* (Nevada), one population of *Primula suffrutescens* (California), and one population of *Dodecatheon redolens* (Nevada). Three populations of *P. parryi* were sampled in close proximity (within a few hundred m to 3 km) to other taxa: in two locations in Colorado, with *P. angustifolia*, and in Nevada, with *P. cusickiana* var. *nevadensis*. For each population, we sampled a minimum of three individuals, with leaf material preserved in silica gel for later laboratory analysis. Additional material was obtained from garden plants of wild origin (Utah) for *P. cusickiana* var. *domensis* and for *P. rusbyi* (New Mexico). A subset using one individual for each taxon was used for ITS/ETS analysis. We used herbarium specimens as an additional source of material for ITS/ETS analysis for *P. cuneifolia* (vars. *cuneifolia* and *saxifragifolia*; one population of each in Alaska) to serve as more distant outgroups. These samples were not used in the AFLP study. Voucher specimens are deposited at COCO. For GenBank accession numbers for samples used in this study, see table A2 in the online edition of the *International Journal of Plant Sciences*.

AFLPs

Procedures for AFLPs followed protocols described by Vos et al. (1995), with modifications described by Beardsley et al. (2004). Samples were run on an ABI 3130xl automated sequencer (Applied Biosystems, Foster City, CA) and analyzed using Gene-Mapper, version 3.7, with visual inspection of called peaks. The five following primer combinations were all based on the template *EcoRI* 5'-gACTgCgTACCAATTC + XXX, *MseI* 5'-gATgAgTCCTgAgTAA + XXX and involved the following 5' fluorescent dyes: (1) Mse CTC, Eco ACT, 6-Fam; (2) Mse CAg, Eco ACT, 6-Fam; (3) Mse CAT, Eco ACT, 6-Fam; (4) Mse CTC, Eco AAg, NED; and (5) Mse CAg, Eco AAg, NED. Bands were scored as binary characters.

Relationships were estimated using neighbor-joining (NJ) analyses. Bootstrap values were calculated using 10,000 replicate full-branch and bound parsimony searches with the program MULTREES and TBR branch swapping. Distance estimates for the NJ analysis were calculated using the index of Nei and Li (1979). However, recent simulations suggest that standard bifurcating tree-based methods to identify terminal monophyletic groups can be misleading when gene flow between sampled taxa is possible (Reeves and Richards 2007). Therefore, AFLP data were also analyzed using principal coordinate analysis followed by nonparametric modal clustering (PCO-MC). The goal of this analysis was to examine in more detail whether the closely related named taxa in *Primula* section *Parryi* formed distinct groups. Therefore, the *Dodecatheon* and *P. suffrutescens* samples were excluded from the analysis. Support in PCO-MC is measured as a stability value, which reflects the frequency of recovery of the cluster over a scan of R space. R refers to the smoothing parameter used for kernel density estimation at each point and, hence, cluster assignment. Variation in R changes the shape of the density landscape. For each density landscape calculated, a note is made of the assignment of individuals into clusters. Clusters that exist despite changing R receive higher stability values. Stability varies from 1 to 100. Using simulations, P. A. Reeves and C. M. Richards (unpub-

lished manuscript) found that clusters with stability values less than 15 have a high probability of being erroneous. Further details of this analysis procedure are outlined by Reeves and Richards (2007).

Nuclear Sequences

We sequenced two nuclear regions: ITS (primers: ITS2–5; ~700 aligned bases) and a portion of the ETS (Beardsley et al. 2004; ~400 aligned bases). The entire ITS region was amplified using the primers *its4* and *its5* (Baldwin 1992). To amplify a portion of the 3' end of the ETS, we used the 3' 18S-IGS primer of Baldwin and Markos (1998) and the 5' primer ETS-B (Beardsley et al. 2004). Amplification conditions were the same as those described by Beardsley et al. (2004). PCR products were cleaned using Qiaquick spin columns.

DNA sequencing was performed on an ABI 3130xl automated sequencer. The ITS was sequenced using external PCR primers *its4* and *its5* and two internal primers, *its2* and *its3*. The ETS was sequenced using the 18S-E primer of Baldwin and Markos (1998), which is slightly internal to 18S-IGS primer, and the ETS-B primer. We were not able to amplify ETS in *Primula cuneifolia* var. *cuneifolia* or var. *saxifragifolia*. Electropherograms for each region were compiled and compared using the program Sequencher, version 4.2 (Gene Codes, Ann Arbor, MI). Sequences were deposited in GenBank.

Analyses

Consensus sequences for ITS and ETS were aligned manually using the program Se-Al, version 2 (A. Rambaut, University of Oxford, Oxford, UK). Sequences from seven cpDNA regions (*matK* gene, *rp116* intron, *rps16* intron, *trnL* intron, *trnL/F* spacer, *trnS/G* spacer, *trnT/L* spacer) representing 5762 aligned bases were provided by E. Conti and A. Guggisberg and have been published by Mast et al. (2004) with details of data collection and analysis. Alignment of sequences was straightforward, with few manual adjustments.

The chloroplast and nuclear sequences were analyzed individually and in combination, using the program PAUP* 4.0b10 (Swofford 1998). The default PAUP settings were used except as noted. Parsimony searches were conducted using heuristic searches with 10,000 random sequence addition replicates to find multiple islands of trees if present (Maddison 1991). Gaps were scored as missing data. Support for individual branches was estimated using bootstrap values (Felsenstein 1985). Bootstrap values were calculated using 10,000 full heuristic search replicates. Maximum likelihood analyses were performed using a heuristic search with the TIM + I + G model of evolution, which was selected by hLRT using Modeltest 3.7 (Posada and Crandall 1998). Bootstrap values were calculated using 100 full heuristic search replicates.

The incongruence length difference test (Farris et al. 1994; as implemented in PAUP*) was used to assess potential conflicts between the phylogenetic signal from different DNA fragments. These comparisons were made: ITS versus ETS and chloroplast versus combined ITS and ETS. For each test, 100 replicates were analyzed with an heuristic search, each with 10 random sequence addition replicates.

Results

AFLPs

In total, 1716 AFLP fragments were scored for 89 accessions used in the AFLP study. Fragments were scored as binary characters. NJ analyses of these data are presented in figure 3. With the exception of *Primula cusickiana* var. *nevadensis* and

Primula cusickiana var. *domensis*, which show a strongly supported (bootstrap support 89%) but intermixed clade, most of the taxa sampled in the study formed well supported monophyletic groups consistent with the topologies of the nuclear and chloroplast trees. Major clades correspond to *Primula parryi* (100%), *Primula rusbyi* (100%), and the northern clade consisting of the *Primula cusickiana* complex plus *Primula angustifolia* (59%). Within the northern clade, *P. angustifolia* is

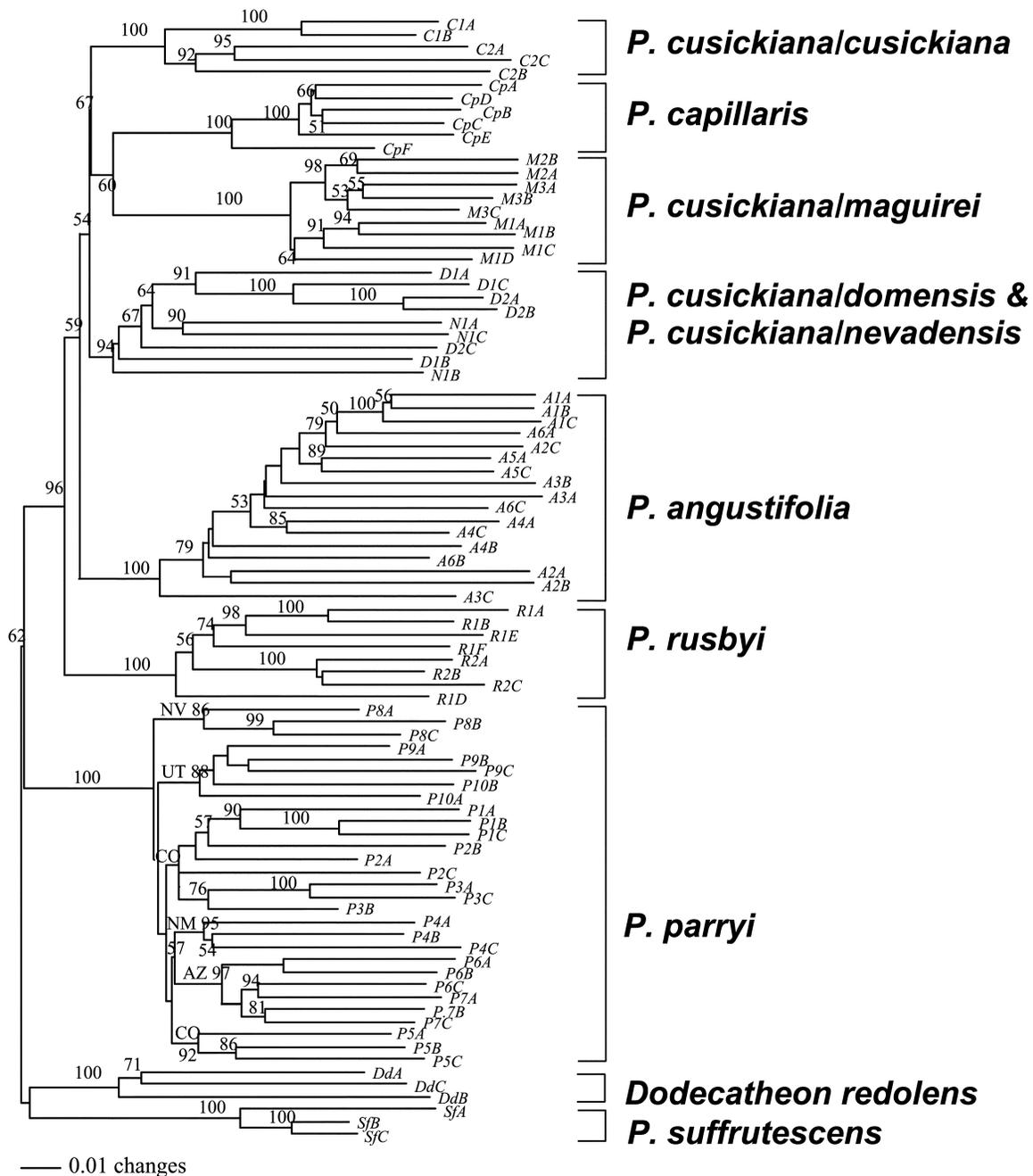


Fig. 3 Inferred neighbor-joining (NJ) tree for all 89 accessions, using 1716 AFLP fragments scored as present or absent. Numbers above the branches indicate NJ bootstrap values. See text and table A2 for sample locations. In the clade representing *Primula parryi*, abbreviations for geographic regions are as follows: NV = Nevada, UT = Utah, NM = New Mexico, AZ = Arizona, CO = Colorado. Numbers after species letter code indicate different populations; the third letter indicates different individuals within a population.

strongly supported as monophyletic (100%), sister to a weakly supported (59%) *P. cusickiana* complex. In the AFLP tree, *Primula capillaris* appears as nested within the *P. cusickiana* complex, sister to *P. cusickiana* var. *maguirei*, rather than sister to var. *cusickiana* as it is in the chloroplast DNA perspective (fig. 2). Within the major clades, most internal nodes are only moderately supported but contain both expected clusters from geographic regions as well as some unexpectedly nested relationships from different geographic regions.

The *Primula parryi* clade contains shallow but recognizable clusters of populations from Nevada (P8; 86%; population identification numbers shown here and throughout are displayed with supporting data in table A2), Utah (P9, P10; 88%), New Mexico (P4; 95%), and Arizona (P6, P7; 97%). Colorado populations (P1–P3, P5) do not demonstrate a unique geographic clade but rather appear as mixed clusters from different geographic regions. Within *P. parryi*, short branch lengths to geographic groupings show limited internal differentiation between samples from different areas that are relatively far apart geographically.

The relationship of a monophyletic *P. rusbyi* as sister to the northern clade of a combined *P. angustifolia* and the *cusickiana* complex is consistent with the chloroplast perspective. Within *P. rusbyi*, samples from the Catalina Mountains of Arizona (R2) nest within south-central New Mexico samples (R1), taken from the Sierra Blanca, 500 km to the southeast of the Catalina Mountains. Although we initially sampled an additional population of *P. rusbyi* (R3 in table A2), these individuals showed weaker banding patterns than were apparent in other samples. Due to ambiguity of interpretation, these are not included in the analysis presented here; however, when included in the NJ analysis (data not shown), they showed a weakly supported (54%) internal cluster, perhaps as an artifact of the weaker amplification. This internal node did not change the recovery of a monophyletic *P. rusbyi*.

Within the *P. angustifolia* clade, internal nodes show embedded relationships of samples from different locations in Colorado. Based on physical proximity, samples from Pikes Peak (A1, A6) and Almagre, an adjoining ridge ~10 km to the south (samples A2, A3), might be expected to show strong affinities, as would those from Hoosier Pass (A5) and Pennsylvania Mountain (A4) in the Mosquito Range ~100 km to the west of Pikes Peak and about 15 km apart. However, the expected geographic clusters are not apparent; instead, the internal nodes are of mixed geographic affinity. Although these nodes show moderate to strong support, they are shallow, indicating little genetic differentiation.

Within the *P. cusickiana* complex, clusters with strong to moderate support correspond to most of the recognized taxa. The only internal cluster (94%) not corresponding to recognized taxa consists of intermixed varieties *domensis* (D1, D2) and *nevadensis* (N2). A weak to moderately supported clade (60%) includes sister *P. capillaris* (Cp: 100%) sister to *P. cusickiana* var. *maguirei* (M1–M3; 100%) with the conjoined cluster sister to *P. cusickiana* var. *cusickiana* (67%).

PCO-MC

Using the AFLP data, distinct clusters with stability values greater than 15 (the level at which apparent monophyly of terminal branches is supported; P. A. Reeves and C. M. Richards, unpublished manuscript) were recovered for *P. parryi* (stability

value = 74), the Sierra Blanca (R1) plus Arizona (R2) populations of *P. rusbyi* (stability value = 22), *P. angustifolia* (stability value = 17), *P. capillaris* (stability value = 31), and *P. cusickiana* var. *maguirei* (stability value = 49) using the PCO-MC method described above. The only other recovered cluster with a stability value greater than 15 was a cluster corresponding to the clade containing all the taxa in the section except for *P. parryi*. *Primula cusickiana* var. *cusickiana* samples did form a cluster, but the stability value was only 5.4. A cluster corresponding only to the *P. cusickiana* complex as a whole was not recovered. Plots of the first three principal coordinates (available from the corresponding author on request) did not further elucidate patterns of relationships among species.

Nuclear DNA Analysis

The ETS and ITS regions are closely linked in the rDNA, therefore, we will present results only for analyses of combined rDNA data. Results from the partition homogeneity test (see below) justify our decision. The ITS data set was 752 bases in aligned length, of which 134 were variable and 64 were parsimony informative. The ETS data set was 494 bases in aligned length, of which 74 were variable and 29 were parsimony informative.

Results of the partition homogeneity test for ITS versus ETS and ITS/ETS versus chloroplast showed that none of the data sets were significantly different from random pairwise partitions of the data ($P = 0.87, 0.10$, respectively). Therefore, we combined the data sets in subsequent analyses.

The maximum likelihood (ML) tree (fig. 4) used the combined ITS and ETS data; it differed from the most parsimonious (MP) tree at the nodes indicated with asterisks. It showed a monophyletic sect. *Parryi* (67%). Both ML and MP trees resolved a clade containing *P. parryi*, *P. suffrutescens*, and *Dodecatheon redolens* with bootstrap support of 100%. A weakly supported (56%/38%) sister relationship between sect. *Parryi* and *Dodecatheon* was also recovered in both the MP and ML trees; this differs from the cpDNA perspective where combined species of *Dodecatheon* are sister to *P. suffrutescens*. Both ML and MP trees show strong monophyly (100%) for a clade composed of the members of sect. *Parryi* excluding *P. parryi* itself, where *P. rusbyi* is sister to a northern clade of the *P. cusickiana* complex plus *P. angustifolia* (99%/100%). In these perspectives, the *P. cusickiana* complex is weakly supported (32%/60%) as monophyletic and *P. capillaris* is sister to *P. angustifolia* but with only minimal support (27%/21%). The estimated phylogeny using nrDNA data is similar to the results obtained using chloroplast DNA (fig. 2) with the following exceptions: (1) All the varieties of *P. cusickiana* formed a monophyletic group in the nrDNA tree. The chloroplast tree indicated that *P. capillaris* and *P. cusickiana* var. *cusickiana* are sister groups, (rather than with *P. angustifolia*) with this group of two taxa nested in a clade with the other subspecies of *P. cusickiana*. (2) *Dodecatheon* and *P. suffrutescens* were strongly resolved as being sister groups in the chloroplast tree, whereas this clade was not supported in the nrDNA tree. However, all of the areas of conflict are only weakly or moderately supported.

Combined Chloroplast and nrDNA Analysis

Analyses of combined chloroplast and nrDNA (fig. 5) resolved a monophyletic *P. cusickiana* that excludes *P. capillaris*,

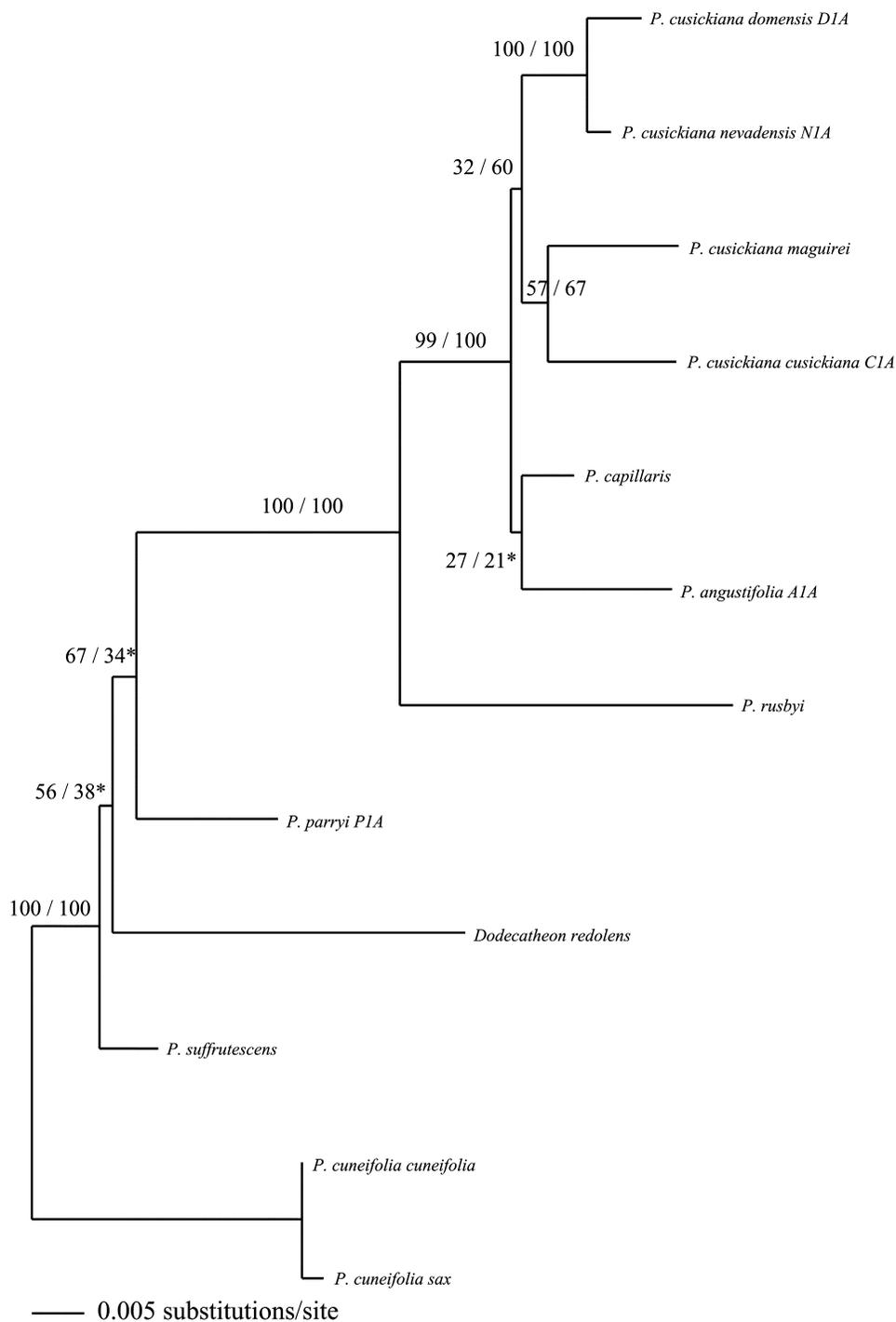


Fig. 4 Maximum likelihood tree inferred using combined ITS and ETS sequences. Numbers above the branches indicate bootstrap values based on maximum likelihood and parsimony analyses, respectively.

with moderate to weak bootstrap support (43% ML bootstrap support, 73% MP bootstrap support). Here *P. capillaris* is shown as sister to *P. cusickiana* s.l. with moderate to weak bootstrap support (43% ML, 71% MP), differing from the cpDNA perspective where it is nested within the complex as a whole (sister to *P. cusickiana* var. *cusickiana*) and the ITS/ETS perspective where it is weakly sister to *P. angustifolia*. Within *P. cusickiana*,

an internal clade consisting of *P. cusickiana* vars. *nevadensis* and *domensis* (100%) is sister to a clade comprised of *P. cusickiana* vars. *cusickiana* and *maguirei*. Other relationships, including a monophyletic sect. *Parryi* and *P. rusbyi* as sister to a northern clade of *P. angustifolia* and the *P. cusickiana* complex, were identical to those resolved using chloroplast, ITS/ETS, and AFLP perspectives.

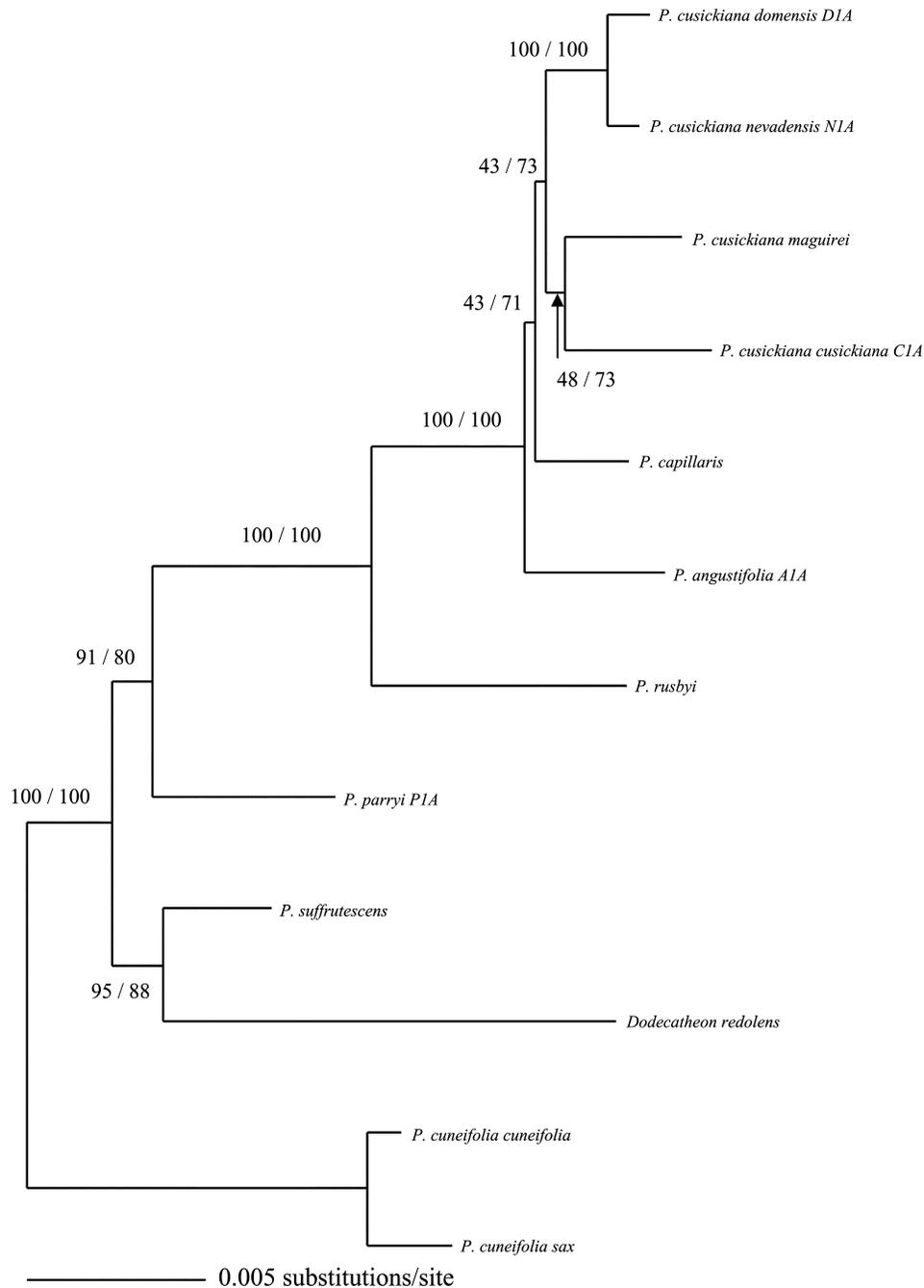


Fig. 5 Maximum likelihood tree inferred using combined chloroplast, ITS, and ETS data. Numbers above the lines indicate bootstrap values based on maximum likelihood and parsimony analyses, respectively.

Discussion

Phylogenetic Perspectives on Evolutionary Models of Primula sect. Parryi

Phylogenies inferred using AFLPs and DNA sequences from both the nucleus are largely congruent. They affirm monophyly of sect. *Parryi*, and for the major species groups within it: *P. parryi*, *P. rusbyi*, *P. angustifolia*, and the *P. cusickiana* complex (including *P. capillaris*). Each species group is discussed individ-

ually below. Nuclear ITS/ETS and AFLP perspectives generally parallel the cpDNA perspective and support our first model for evolutionary diversification of *Primula* sect. *Parryi*; that is, *P. parryi* represents the earliest divergence in this lineage that diverged from a common ancestor in subgenus *Auriculastrum*. The other species are collectively sister to *Primula parryi* and represent later divergence. Our results also affirm *P. rusbyi* as sister to a northern clade composed of *P. angustifolia* (southern Rocky Mountains) and the *P. cusickiana* complex (Great

Basin–Intermountain region). Greater geographic and/or longer isolation appears to have shaped greater genetic and morphological divergence in *P. parryi* and in the southern clade represented by *P. rusbyi* than is apparent between members of the northern clade, *P. angustifolia*, and the *P. cusickiana* complex. We found no dissonance between perspectives provided by different genetic data that would suggest reticulate or paraphyletic relationships among any of the major groups or the possibility that any of the endemic taxa are derived from within *P. parryi*. However, internal relationships within the *P. cusickiana* complex are variable, particularly the position of *P. capillaris*, and suggest that taxa in this clade have not yet achieved reciprocal monophyly.

Primula parryi. We found strong affirmation of significant genetic distinction for this species as a whole, the most widely distributed and morphologically distinctive in the section. *Primula parryi* and *P. angustifolia* can be ecologically parapatric in Colorado (growing at similar elevations in different microsites, sometimes separated by only a few hundred meters), but there is no evidence of hybridization in the wild. Similarly, *P. parryi* can also grow in relatively close proximity (i.e., within a few kilometers) to *P. rusbyi* and to *P. capillaris* (S. Kelso, personal observation), but there is no genetic or field evidence for hybridization between any of these species. In the most detailed analysis of *P. parryi*, AFLP data from diverse geographic areas show recognizable geographic affinity but shallow internal differentiation in spite of the wide range and probable longevity of the species. This parallels the generally consistent morphology, allowing for plasticity with respect to height, leaf shape, and size, all characters recognized as influenced by environmental factors such as water availability, nutrients, or exposure.

Primula parryi differs from other widespread taxa in the Intermountain Region (e.g., *Dodecatheon pulchellum* and *Mimulus guttatus*), where the broad range is accompanied by considerable morphological variation with taxonomic recognition of geographic segregates. This lack of genetic differentiation in *P. parryi* might be due to several factors. The habitat, subalpine bogs and streamsides, is widely distributed and broadly contiguous in the region. Populations are often quite large (sometimes encompassing many thousands of individuals), and gene flow may be facilitated by the integrated nature of hydrological systems; we suspect this species has the capacity to disperse seeds by water and thus can potentially spread seeds over large areas. Its pollinators are known to be diverse and dominated by the genus *Bombus* (Miller et al. 1994), effective pollinators that can travel as much as several kilometers in alpine environments (Schulke and Waser 2001), thus providing pollen dispersal capacity beyond a very localized area. Future studies involving detailed investigation of haplotypes might identify greater resolution of infraspecific differentiation.

Primula rusbyi. As a species, *P. rusbyi* is well marked at the geographic, ecological, and morphological levels and is uniformly segregated genetically in chloroplast, nuclear, and AFLP sequences. This is the only representative of the section in Arizona, southern New Mexico, and the Sierra Madre of western Mexico, and it is consistently shown to be sister to the northern clade of *P. angustifolia* and the *P. cusickiana* complex. Vegetation history of its subalpine conifer forest habitat suggests the likelihood of a more extensive ancestral distribution in the late Pleistocene and that the current distribution represents rem-

nants of this original range. As treated by Williams (1936), *P. rusbyi* encompassed two infraspecific taxa: var. *rusbyi* (found in southwestern New Mexico and southeastern Arizona) and var. *ellisiae* (Pollard & Cockerell) L.O. Williams. Variety *ellisiae*, described from the Sandia Mountains near Albuquerque, New Mexico, and occurring through south-central New Mexico, was theoretically a stouter plant, distinguished by longer calyx lobes, larger flowers, and capsules; later treatments (Kelso, forthcoming) question the integrity and utility of these characters as indicative of a recognizable taxon. Our AFLP results, based on material from Arizona (var. *rusbyi*) and central and south-central New Mexico (material and range inclusive of the putative var. *ellisiae*) show strong support for an integrated single taxon over at least these regions; however, weak segregation of additional material on Sandia Crest (the type locality of var. *ellisiae*) was also indicated. Given the ambiguous sequence data for this population, we cannot draw firm conclusions that would support or reject the appropriateness of a segregate infraspecific taxon within *P. rusbyi*. A future study including samples across the full range of the species with additional genetic markers are needed to clarify this situation and indicate whether substantial and geographically coherent genetic diversification has occurred in any populations; in addition to the Sandia Crest populations, those found in the poorly known and geographically distant southern populations in Mexico are of particular interest.

Primula angustifolia. All the genetic perspectives consistently portray *P. angustifolia* as monophyletic and sister to the *P. cusickiana* complex; these taxa together form a northern clade with respective distributions in the southern Rocky Mountains and in the Great Basin–Intermountain region. The intervening gap in the mountains of western Colorado and eastern Utah represents an area subjected to extensive alpine glaciation during the Pleistocene; these episodes probably constituted vicariance events that facilitated east-west divergence. Although *P. angustifolia* shows a superficial resemblance to *P. capillaris* in Nevada with its diminutive size, few small flowers, and short, spatulate leaves, a close genetic connection between the two species does not appear. These characters are likely to represent convergent responses to similar environmental conditions in their respective alpine habitats. Although populations of *P. angustifolia* are now segregated by topography and ecology in disjunct alpine locations throughout south-central Colorado and northern New Mexico, the shallow internal differentiation and intermixed geographic affinities shown by the AFLP data, well beyond the distance that pollinator-mediated gene flow is likely to occur, suggest that integration of gene pools occurred in the recent past. This is consistent with paleoecological data for this part of Colorado, where it is known that as recently as the mid-Holocene, subalpine forests extended both their lower and upper elevational limits and covered a broader extent range than they do today (Fall 1985).

The *Primula cusickiana* complex. The *P. cusickiana* complex appears to represent a clade derived relatively recently. It includes populations restricted to moist niches and demonstrates more shallow internal divergence than that shown between other major species groups in sect. *Parryi*. Nomenclatural questions have been an issue where intergrading morphologies but disparate ecologies and isolated distributions made it difficult to assess whether the taxa in this group should be recognized at specific or infraspecific levels. Our genetic

perspectives are also somewhat discordant, congruent only with respect to recognizing the complex as monophyletic; relationships within the complex and the position of *P. capillaris* as nested within or as sister to the varieties of *P. cusickiana* are notably dissonant. The lack of morphological distinction and labile patterns of sister relationships indicate limited divergence suggestive of relatively recent allopatry. Our results and conclusions are in accord with those of Bjerregaard and Wolf (2008) with respect to *Primula cusickiana* var. *maguirei*. All of our analyses consistently indicate a very close relationship of *P. cusickiana* vars. *nevadensis* and *domensis* (nested within each other in the AFLP perspective; fig. 3) and suggest that these geographically and morphologically close entities might be considered a single taxon. Additional genetic data and wider sampling of more populations of these taxa are needed to clarify their relationship and assess the potential unity suggested here.

Patterns of Speciation, Biogeography, and Historical Climate Change

Members of sect. *Parryi* currently occupy either upper elevation habitat islands (*P. angustifolia*, *P. rusbyi*, *P. parryi*, *P. capillaris*, and *P. cusickiana* vars. *domensis* and *nevadensis*) or anomalous cool lower elevation microsites that retain moisture (*P. cusickiana* vars. *maguirei* and *cusickiana*). Edaphic affinities include acidic turfs and bogs on granitic or metamorphic bedrock, calcareous ledges or rubble, and alkaline clay-rich swales. These habitats may reflect moisture-retentive substrates rather than a direct pH requirement, since most of the taxa that occur on calcareous substrates in the wild also thrive on neutral to slightly acidic soils under horticultural conditions if sufficient moisture is available. Hard, impermeable limestones or shales like those that support *P. rusbyi* and *P. cusickiana* vars. *domensis*, *nevadensis*, and *maguirei* may play a particularly critical role in providing azonal moist niches by retaining soil water.

In light of what is known about the current ecological profiles and niche conservatism (Wiens 2004) of these *Primula* species (specifically their requirement for moisture and cool growing conditions, at least for some portion of the year) and the regional history of vegetation shifts in response to ongoing climate change, these phylogenetic perspectives on *Primula* sect. *Parryi* consistently support our scenario of ongoing allopatric speciation in which climatic and ecological changes have shaped species barriers and enhanced vicariance in populations isolated over different timescales. For taxa with narrow ecological amplitudes, profound landscape and climatic changes would have fragmented habitats on an ongoing basis; we propose this process has played a decisive role in the evolutionary history of the section.

Primula sect. *Parryi* is likely to be derived from an ancestral Asian lineage in the *Auriculastrum* clade in the late Tertiary (Zhang and Kadereit 2004; Zhang et al. 2004). We do not presume the direct progenitors are extant diploid Asian members of sect. *Cuneifolia* (*P. cuneifolia* s.l. and *P. nipponica*); these species may only represent remnants from an extinct ancestral group. Our analyses support the hypothesis that *Primula parryi* was first to diverge from a common ancestor in North America, with as yet unclear relationships with *P. suffrutescens* and the *Dodecatheon* clade. Further investigations using additional sequences may shed light on those taxa, their relationship to each other, and to sect. *Parryi*. The lack of diploid species in these

North American groups indicates that significant progenitors are no longer extant and critical evolutionary junctions can be only speculative.

Studies by Baker (1983), Spaulding et al. (1983), and Thompson et al. (1993) indicate that conditions ecologically similar to those in which most of the species in sect. *Parryi* grow today were abundant in the Great Basin and Rocky Mountain West during the late Pleistocene. Expanded tundra zones occurred at high elevations, and widespread montane-subalpine conifer forests dominated by spruce and fir lay in lowlands currently dominated by desert scrubland. *Primula*-appropriate (i.e., cool and moist) habitats were thus widespread throughout much of the West and extended into the Mexico during the Late Pleistocene glacial episodes (Straka and Ohngemach 1989). Subalpine cool conifer vegetation became increasingly restricted to higher elevations during postglacial warming as vegetation in the lowlands rapidly changed to the modern pattern of dry lake basins, desert scrub, and grasslands. Contemporary *Primula* habitat fragmentation and its concomitant allopatry may be relatively recent. However, whatever the timescale, as intervening zones became increasingly xeric in the Holocene, they would have inhibited migration for species that require cool, moist conditions. These species would have faced local extirpation and/or increasing fragmentation and isolation in insular alpine habitats and relictual niches. The spectrum of morphological and genetic differentiation seen in *Primula* sect. *Parryi* probably represents separation for different lengths of time, relative strength of local selective forces, and/or aspects of secondary contact during nonglacial episodes. Repeated intervals of Quaternary warming and cooling would be likely to lead to episodic range expansion and contraction or periods of population fragmentation either directly or secondarily via adverse climatic impacts on insect pollinators. Periods of population expansion and greater contiguity might also, however, lead to the potential for gene pool integration and secondary contact (Bjerregaard and Wolf 2008) for populations temporarily isolated in localized Pleistocene glacial refugia; this would consequently promote either only partial genetic differentiation (e.g., between and among members and populations of the *P. cusickiana* complex) or inhibit strong geographic differentiation for a widespread species like *P. parryi*.

Conservation Concerns and Future Climate Change

Wiens and Graham (2005) pointed out that species that demonstrate niche conservatism, evidenced by limited ecological tolerances, are of particular conservation concern. In regions such as the Great Basin and the arid Southwest, populations of moisture-dependent taxa are already small and habitats are restricted; there have been regional climate change predictions of increasing heat and aridity and diminished winter snowpack with earlier melting (Wagner 2003; Field et al. 2007; Kerr 2007), which are likely to pose further stress. Effects may be particularly acute for biota inhabiting higher-elevation sites, where further habitat fragmentation, diminished populations, and species losses are predicted (Murphy and Weiss 1992). These concerns apply to all the taxa in *Primula* sect. *Parryi* but especially to the members of the *Primula cusickiana* complex, where suitable habitats are limited and populations are already small.

In addition to their ecological restrictions, heterostylous species like those in *Primula* sect. *Parryi* face reproductive challenges

because they are highly dependent on insect pollen vectors for successful cross-fertilization of floral morphs (Richards 2003). These vectors are typically bumblebees, halictid bees, or syrphid flies in sect. *Parryi* (Miller et al. 1994; Bjerregaard and Wolf 2008); many of these have relatively limited foraging distances and are not reliable in inclement weather or if plant population numbers (and hence floral display) is low. Thus, when populations become too small, heterostylous primroses have been shown to face significant reproductive limitations (Kery et al. 2000; Brys et al. 2007). Seed dispersal in sect. *Parryi* appears to be limited to short-distance wind or gravity transport or, in the case of *P. parryi*, water. With limited ecological breadth, inhospitable terrain between widely disparate populations, and restrictions on both sexual reproduction and seed dispersal, adaptive and migratory potential for most *Primula* species in sect. *Parryi* would appear to be very low. Seasonal or drought-induced dormancy and some ability for clonal reproduction via sympodial rhizomes are common in the section, however, and suggest that persistence ability at least may be a partially compensatory survival factor.

Our data show that most of the taxa in the *Primula cusickiana* complex represent discrete evolutionary units (excluding *P. cusickiana* vars. *nevadensis* and *domensis*, which are jointly but not individually discrete). Our AFLP results show considerable intrapopulation diversity for all taxa in the section; similar diversity has also been shown within and between populations of *P. cusickiana* var. *maguirei* by Bjerregaard and Wolf (2008). These data indicate that in spite of small population sizes, considerable neutral genetic diversity still exists; if neutral diversity is related to potentially adaptive diversity, then adaptive potential may not yet be critically diminished. However, all members of section *Parryi* could be vulnerable to climatic perturbations that alter pollinator behavior, the amounts and timing of moisture, or temperature range during vulnerable reproductive phases. This western American section of *Primula*, with its clear ancient connections to Asia and Europe via its relatives in subgenus *Auriculastrum* and its spectrum of genetic and morphological differentiation, has much yet to reveal about regional and generic evolutionary responses to short- and long-term environ-

mental change. While extensive adaptive radiation into arid habitats has been well documented in the American West (Stebbins 1952; Bell and Patterson 2000; Sun et al. 2004; Evans et al. 2005), less is known about evolutionary responses in groups like members of *Primula* sect. *Parryi* and their relatives restricted to hydric or mesic environments under conditions of increasing aridity. These species may be exemplary not only of evolutionary potential, in that they have diversified via ecological segregation, but also of the evolutionary constraints imposed by niche conservatism when suitable habitats diminish and intervening terrain expands and becomes increasingly inhospitable. As demonstrated by DeChaine and Martin (2004, 2005), cycles of population expansion and division driven by climatic oscillations between glacial and interglacial conditions during the Pleistocene profoundly impacted both plant and animal species in upper elevation habitats of the American West. These authors suggest that such cycles can act not only as agents of diversification but also of extinction, especially when warm dry phases isolate small populations in shrinking high-elevation refugia. In light of current climatic predictions, ecological uncertainties for the region, and the already apparent picture of stress due to habitat limitation, diminished populations, and reproductive constraints inherent for heterostylous species, all the members of *Primula* sect. *Parryi* warrant conservation concern.

Acknowledgments

We are grateful to the many people who provided help with field work, laboratory analyses, and practical and theoretical techniques, especially J. Andre, M. Bilodeau, L. Bjerregaard, K. Christie, E. Conti, A. Guggisberg, C. Hall, N. Holmgren, P. Holmgren, G. Maentz, D. Mansfield, A. Mast, K. Mauz, C. Parker, P. Reeves, F. Smith, P. Wolfe, and personnel at the Great Basin National Park and Humboldt-Toiyabe National Forest in Nevada. We also thank J. Reveal and an anonymous reviewer for helpful comments on an earlier draft of the manuscript.

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