SYSTEMATIC STUDIES IN THE GENUS *PHLOX* (POLEMONIACEAE): CYTOTYPIC VARIATION IN *PHLOX NANA* NUTT. AND UTILITY OF A LOW COPY NUCLEAR GENE REGION (IDHB) FOR PHYLOGENY DEVELOPMENT

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Abstract

The genus *Phlox* L. presents intriguing opportunities for systematics research, and *P. nana* is of particular interest. *Phlox nana* occurs chiefly in mountains of the Chihuahuan desert to northern New Mexico, and it exhibits much morphological variation across its range. Historically, this taxon has been recognized as a single species (sometimes with infraspecific taxa), or as several species. Perhaps most interesting, variation in ploidy level (cytotypic variation) has been evidenced for *P. nana*. This research employed flow cytometry methods in conjunction with chromosome counts to document patterns of cytotypic variation. Intensive fieldwork in Arizona, New Mexico and Texas enabled excellent sampling, and evaluation of ploidy level for 76 populations was achieved. Diploid and tetraploid chromosome counts were made (four diploid counts; five tetraploid counts), and flow cytometry was conducted on all populations, providing evidence for diploid, tetraploid and hexaploid populations. Polyploids were found to occur in many geographical areas, and in some regions, diploids and polyploids occur in close geographical proximity (e.g., within both the Davis Mountains and the Chisos Mountains of west Texas). Genome size data are presented (with discussion of unusual populations), and geographic patterns of cytotypic variation are presented and discussed. Patterns are also briefly considered with respect to morphology and taxonomy: cytotypic variation does not readily align with historical recognition of taxonomic variation, and this work sets the stage for ongoing, detailed morphometric study.

Research on particular species of *Phlox* benefits from an understanding of a broad phylogenetic context, and low copy nuclear DNA regions are an important resource for phylogeny development. This research further evaluated part of the NADP-dependent isocitrate dehydrogenase gene (*idh*B) for its usefulness in inferring relationships in *Phlox*. Samples were PCR amplified for *idh*B and cloned, and resulting sequences were added to a larger set of *idh*B sequence data previously developed in the lab. A total of 163 samples were included, and Bayesian Inference and Maximum Parsimony analyses were conducted for complete data sets. Phylogenetic findings are discussed in light of previous work based on chloroplast and high copy nuclear DNA regions, and challenges and utility of using *idh*B are discussed.

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Acknowledgements

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Chapter 1 - Polyploidy in *Phlox nana* Nutt. (Polemoniaceae): documentation of cytotypic variation, and relationship to geography

Introduction

Polyploidy (genome doubling) has come to be regarded in recent decades as an important process in plant evolution, and is considered a common and rapid mechanism of speciation in plants (e.g. Levin 1983, Soltis et al. 2007, Beest et al. 2012). The percentage of polyploids present in angiosperms has been long debated. Researchers have varied in recognizing that anywhere from 30% to about 80% of all angiosperms have gone through some form of polyploidization event (e.g. Stebbins 1950, Grant 1981, Masterson 1994). Moreover, ploidy level variation within a species (cytotypic variation) has been increasingly detected in plants (e.g. Husband & Sabara 2003, Ramsey et al. 2008, Balao 2009, Beest et al. 2012), including within the genus *Phlox* L. (Polemoniaceae; e.g. Smith & Levin 1967, Fehlberg & Ferguson 2012a, 2012b, Worcester et al. 2012). While cytotypic variation in some cases is accommodated within a species (e.g. in *Achillea*, Ramsey et al. 2008), cytotypes may be reproductively isolated and represent cryptic species.

In many species, local populations mostly consist of one cytotype and may be geographically separated from differing cytotypes or spatially segregated if cytotypes occur in mixed populations (e.g. *Ranunculus*, Baack 2004; *Dianthus*, Balao et al. 2009; *Turnera*, Elias et al. 2011; *Spartina*, Kim et al. 2012). In other species, local populations may consist of multiple cytotypes (e.g. *Chamerion*, Husband & Schemske 2000; *Senecio*, Schönswetter et al. 2007; *Solidago*, Halverson et al. 2008; *Aster*, Castro et al. 2012). Although the occurrence of multiple cytotypes in a single population may be less common, extensive plant collecting, flow cytometry

and/or chromosome counts are needed in order to better assess patterns of cytotypic variation within a species.

Recent research has been focused on finding ecological or environmental characters that correspond to ploidy levels. While some researchers have found a correlation between the distribution of cytotypes and latitude, longitude or altitude (see Balao et al 2009, Sonnleitner et al 2010, Elias et al 2011, Šafářová et al 2011), other researchers have not found such correlations (e.g. Cires et al 2010). This type of research may be fueled because of the idea that polyploids are better able to adapt to different environments compared to their diploid progenitors (Levin 1983, Ramsey et al 2014). In some cases, polyploids have been found to be more widespread compared to diploids (see Elias et al 2011 and Krejčíkova et al 2013). Polyploids have also been found to be more efficient in water usage (e.g. Manzaneda et al 2011) and certain polyploids may be considered invasive (e.g. Kubátová et al 2008). Based on reproductive and geographic isolation of single cytotype populations, distinct names have also been applied to different cytotypes in some groups (e.g. Koutecký et al 2012). Without regard for environmental or ecological factors, focused research on the documentation of cytotypes and their spatial distributions has been common in recent years (Ramsey et al 2014), but this type of research sets the stage for more advanced studies, such as morphological and ecological correlates of ploidy level.

Phlox nana as a Study System

The genus *Phlox* consists of 60 annual and perennial species that range throughout much of North America, with a concentration of diversity in the western United States (Wherry 1955; Ferguson et al., *Flora of North America*, in prep.). The genus exhibits interesting variation in morphology and geography, and taxonomy has long been complicated. Polyploidy is well known

in *Phlox* (e.g. Flory 1931, 1934, 1948; Smith & Levin 1967; Levin 1966, 1968; Fehlberg & Ferguson 2012b; Worcester et al. 2012), which has a base chromosome number of x=7 (e.g. Flory 1934). Moreover, recent work has found that polyploidy is much more extensive than previously recognized (Fehlberg & Ferguson 2012a, 2012b; Worcester et al. 2012; and Ferguson et al. unpubl.). Because of the diversity of this genus and the extent of polyploidy, it is an excellent system for the investigation of patterns of polyploidy.

Phlox nana Nutt. (Fig. 1.1) is a morphologically variable species (see Figs. 1.2, 1.3) occurring chiefly in the mountains of the Chihuahuan desert (from northern Chihuahua, Mexico, to western Texas, southeastern Arizona, and north throughout much of New Mexico; see Fig. 1.4).

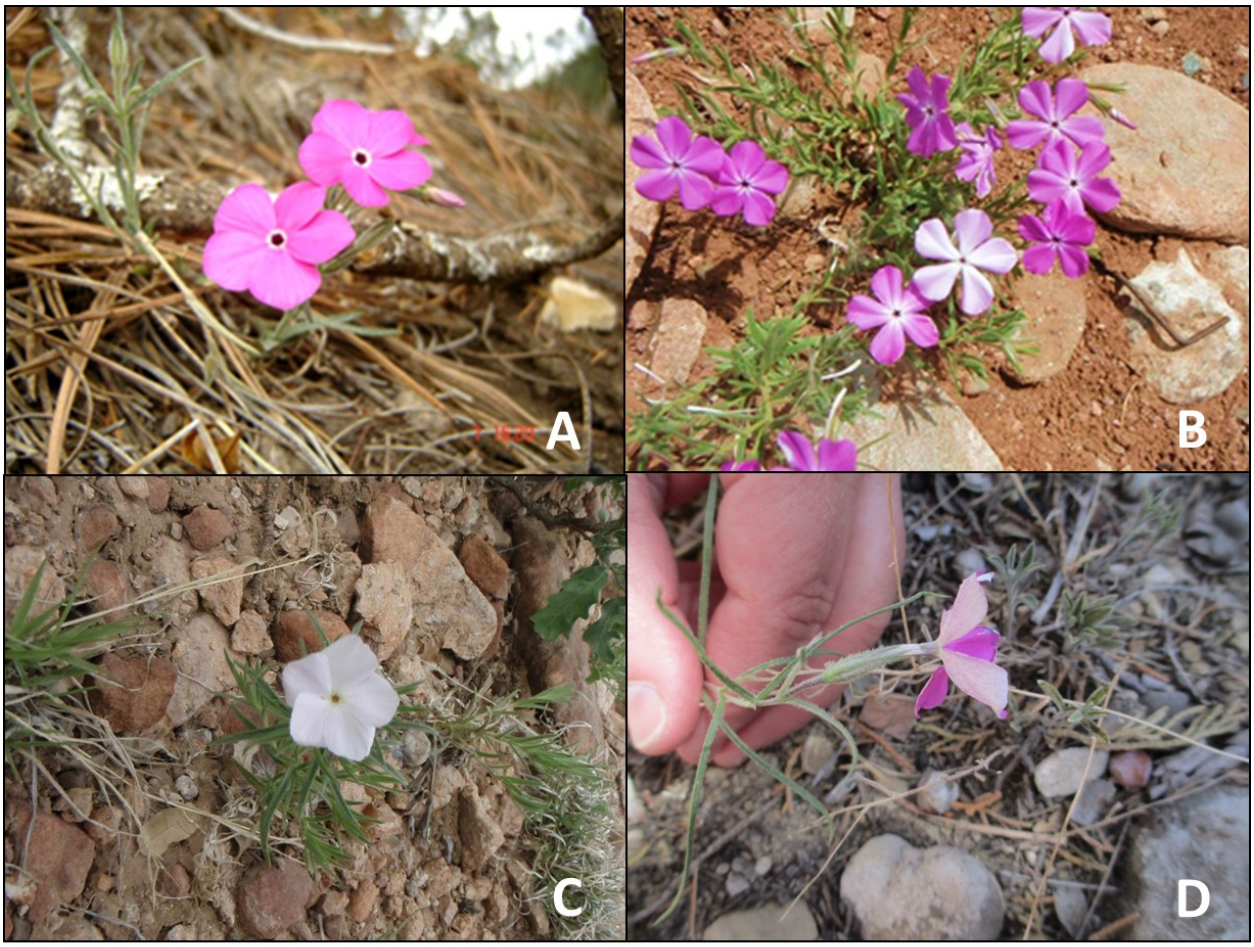

Figure 1.1 Example images of *P. nana*: A. B. Wright l01, Otero Co., NM; B. B. Wright 114, San Miguel Co., NM; C. B. Wright 109, Lincoln Co., NM; D. B. Wright 156, Culberson Co., TX.

Figure 1.2 Some examples of the morphological variation within *P. nana*.
A. dense, eglandular pubescence on calyx (B. Wright 103); B. glandular pubescence on calyx (B. Wright 110); C. dense pubescence on leaves (B. Wright 105); D. sparse glandular pubescence on leaves (M. Mayfield 3984); E. a plant with wide, short leaves (B. Wright 114); F. a plant with long, narrow leaves (C. Ferguson 332).

Figure 1.3 Some examples of morphological similarity between populations growing in the same geographical area in *P. nana*.
A. Plant from the Chisos Mountains (B. Wright 155); B. Plant from the Chisos Mountains (B. Wright 154); C. Plant from the Davis Mountains (B. Wright 145); D. Plant from the Davis Mountains (K. Waselkov 223).

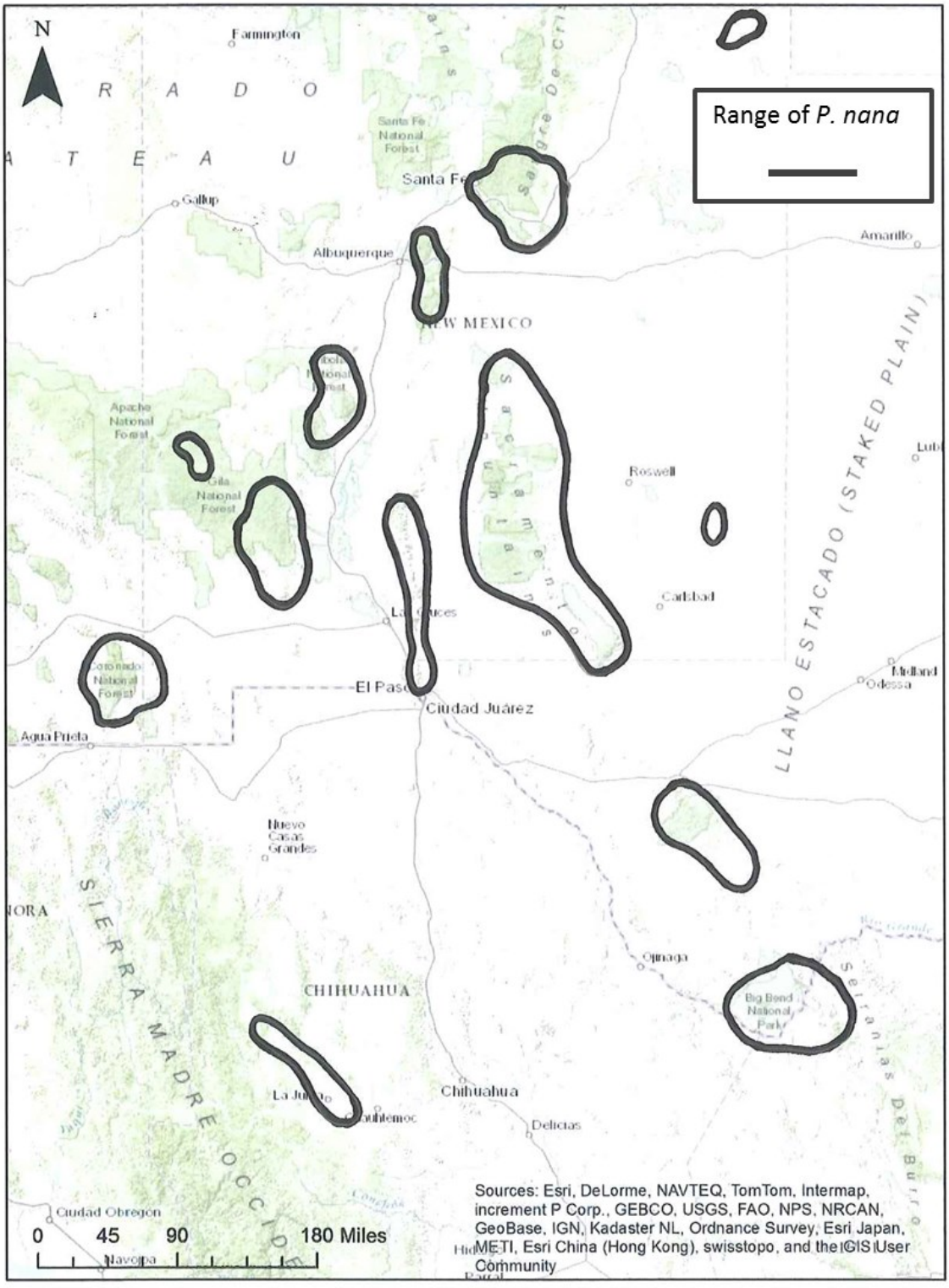

Figure 1.4 Generalized range map of *P. nana* based on herbarium specimen records (data collected in part by L. A. Prather, pers. comm.).

It is intriguing to note that current taxonomy recognizes a broad, variable *P. nana* (e.g. Wilken & Porter, 2005; Heil et al. 2013; Ferguson et al., in prep.), while some authors of floras have recognized multiple species or subspecies following various previous taxonomic treatments (see Taxonomy, below). Table 1.1 summarizes the treatment of *P. nana* sensu lato in pertinent regional floras. Some floras that recognize multiple species additionally note that the species may grade into each other (e.g. *P. nana* and *P. mesoleuca* in Allred & Dewitt Ivey, 2012).

Table 1.1 The recognition of *P. nana*, s.l. in several floras from Texas, New Mexico and Arizona.

Flora	**Authors/Publication Date**	**Taxon or Taxa Recognized**
Arizona Flora	Thomas H. Kearney, Robert H, Peebles (1960, 2nd Edition)	1. *P. nana* Nutt. subsp. *glabella* (Gray) Brand
Manual of the Vascular Plant of Texas	Donovan Stewart Correll, Marshall Conring Johnston (1979)	1. *P. nana* Nutt. 2. *P. triovulata* Thurb. Ex. Torr. 3. *P. mesoleuca* Greene
Atlas of the Vascular Plants of Texas Volume 1	B.L. Turner, Holly Nichols, Geoffrey Denny, Oded Doron (2003)	1. *P. nana* Nutt. 2. *P. triovulata* Thurb. ex. Torr. 3. *P. mesoleuca*
Vascular Plants of Arizona: Polemoniaceae	Dieter H. Wilken, J. Mark Porter (2005)	1. *P. nana* Nutt.
Flora Neomexicana III: An Illustrated Identification Manual	Kelly W. Allred, Robert DeWitt Ivey (2012)	1. *P. nana* Nutt. (*P. nana* Nutt. subsp. *glabella* (Gray) Brand) 2. *P. triovulata* Thurb. ex. Torr. 3. *P. mesoleuca* Greene
Flora of the Four Corners Region:	Kenneth D. Heil, Steve L.	1. *P. nana* Nutt.

Vascular Plants of the San Juan River Drainage	O'Kane, Jr., Linda Mary Reeves, Arnold Clifford (2013)	

Previous studies have suggested that *P. nana* harbors cytotypic variation. A tetraploid chromosome count was reported for *P. nana* material from the Chisos Mountains in Brewster Co., Texas by Flory (1948); and diploid, tetraploid and hexaploid chromosome counts were reported for *P. nana* from various parts of the range (Eater 1967). This context has set the stage for a focused study of patterns of cytotypic variation in *P. nana*.

Taxonomy of Phlox nana

The current circumscription of *P. nana* is broad, with synonymy of some names that have been recognized at the specific or infraspecific levels by some previous workers. The following history of the taxon descriptions and recognition by monographers highlights the challenges of understanding variation in *P. nana*.

Phlox nana was described in 1848 by Thomas Nuttall (Nuttall 1848). *Phlox triovulata* was recognized by George Thurber and was described and published by John Torrey in 1858 (Torrey 1858). In 1899, Elias Nelson, in his taxonomic revision of the western members of *Phlox*, recognized *P. nana* and *P. triovulata* as distinct species. *Phlox triovulata* was discussed as having a more slender habit and narrower leaves than *P. nana* (Nelson 1899). *Phlox mesoleuca* was not described and named until 1905 by Edward Lee Greene (Greene 1905).

In 1907, Brand published a monograph of *Phlox* and believed that much of the variation in *P. nana* warranted taxonomic recognition, so he described subspecies and varieties under the name *P. nana* (See Fig. 1.5 and Table 1.2). He also described a new species, *P. nelsonii* Brand (Brand 1907). He described this taxon as being part of what E. Nelson had included under the

name *P. triovulata* in 1899. In Brand's key to *Phlox*, *P. nelsonii* has calyx lobes almost as long as the corolla tube, while *P. nana* has calyx lobes that are shorter in length compared to the corolla tube. The name *P. mesoleuca* was only included in the addendum, as Brand noted that he had not seen the original specimen. He was not definitive about the position of this species in relation to other *Phlox* species but noted that it probably belonged to the *P. nana* group as it occurred in the same area as those entities described under *P. nana*.

A. Corolla tube ciliate	Subspec. *nana* [1]
a. Leaves lanceolate, 25-35mm long	Var. *albo-rosea*: Flowers white to rose colored
b. Leaves linear	
α. Leaves 3-4 cm long	Var. *lutea*: Flowers yellow
β. Leaves 5-7cm long	Var. *purpurea*: Flowers purple
B. Corolla tube glabrous	
a. Calyx glandular	Subspec. *ensifolia*
b. Calyx eglandular pubescent	Subspec. *glabella*
α. Stem 4-6 cm tall	Var. *depressa*
β. Stem 20-30 cm tall	Var. *triovulata*

Figure 1.5 A key to the subspecies and varieties in the *P. nana* group according to Brand as provided in his monograph (1907).

[1]Brand recognized the nominate subspecies as *P. nana* ssp. *eu-nana* but modern nomenclatural practices dictate proper recognition as *P. nana* ssp. *nana*.

Table 1.2 Morphological characters distinguishing taxa of *P. nana*, as recorded by Brand (1907), based on his key and descriptions.

Subspecies	Variety	Description
nana		Stems 7-20cm, spreading and branched, viscid glandular, leaves glandular and hairy, leaves often curved like a sickle, calyx and pedicel dense glandular, corolla tube ciliate
	albo-rosea	Flowers white to rose colored, leaves lanceolate (25-35mm long)
	lutea	Flowers yellow, leaves linear (3-4cm long)
	purpurea	Flowers purple, leaves linear (5-7cm long)
ensifolia		Stem simple (6-10cm) viscid, glandular, leaves 4-7cm long, linear, glandular calyx, corolla glabrous
glabella		Eglandular, calyx ciliate, leaves 4-5cm long, 2-3mm wide, corolla tube glabrous
	depressa	Low growing, stems 4-6cm tall, white-pubescent, e-glandular, leaves narrow, linear (3-5cm long)
	triovulata	Stem thin, often simple (20-30cm tall), eglandular, lower leaves 5-10cm long, narrow, linear

In his 1955 monograph, Wherry differentiated the variation into three species: *P. nana*, *P. triovulata*, and *P. mesoleuca* (Wherry 1955). Interestingly, he also tried to provide the reader with the choice of recognizing these as subspecies of *P. nana* (and provided the appropriate nomenclature). In so doing, he highlighted their overlapping ranges and potentially minor morphological differences, and seemed to encourage further study on this group. The characteristics that Wherry used to differentiate these species are presented in Table 1.3. Furthermore Table 1.4 presents nomenclatural information for *P. nana* (including locality data for type specimens), based on Wherry's treatment. Wherry described *P. mesoleuca* as "ancestral", putatively having given rise to *P. nana* through a reduction in the height of the plant and to *P. triovulata* through a reduction in the glandularity and pubescence. Wherry also placed a very similar species he described in 1944, *P. mexicana* (based on specimens from the type locality of Durango, Mexico), as closely related to *P. nana* with differences being a longer style (6 to 8mm) and sub-opposite leaves (Wherry 1944, 1955). Only two collections have ever been made in this area, and locality data are very general; more recent attempts to locate populations have been unsuccessful (C. Ferguson, pers. comm.). In his monograph, Wherry provided range maps for the taxa and estimated coordinates for the locations of specimens he considered to be near the borders of their ranges (based on his study of herbarium specimens; Wherry 1955; Fig. 1.6).

Table 1.3 Morphological characters distinguishing taxa of *P. nana* s.l. as shown by Wherry in his 1955 monograph. Some taxon descriptions differed from characters included in Wherry's key and are shown in bold.

Species/Synonym	Leaves/stem	As described in key	Corolla Tube	As described in key	Inflorescence	As described in key	Sepals	As described in key	General Pubescence
P. mesoleuca Synonym: *P. nana* ssp. *ensifolia* Brand Varieties: *P. nana eunana v. lutea* Brand *P. nana eunana v. purpurea* Brand 20 specimens[1]	Few stemmed, leaves linear, lower leaves e-glandular, upper leaves glabrous pubescent or glandular pubescent. Leaves 50 to 85 mm long, 3 to 4 mm wide, longest in the middle. Flowering stems with 5-6 sterile nodes.	Few nodes, somewhat crowded to remote. Leaves linear, 50 to 85 mm long	13 to 18mm long, sparsely pubescent to glabrous. Petal obovate to orbicular (14x11mm), entire or erose.	Sparsely pubescent to glabrous	Flower with white eye, 3 to 6 flowered, glandular pubescent, pedicel 8 to 45 mm	Pedicel length 8 to 45 mm	13 to 18mm long, membranes flat or somewhat plicate, blade linear-subulate.	N/A	Sparsely glandular pubescent
P. nana Synonym: *P. nana α-nana* Wherry Varieties: *P. nana eunana v. albo-rosea* Brand 35 specimens[1]	**Leaves linear** or narrowly elliptic to lanceolate, glandular pubescent, 25 to 45 mm long and 2 to 5 mm wide, short and broad above, short and narrow below, longest in the middle. Flowering shoots with 6 to 12 sterile nodes	Numerous nodes, crowded to sub-remote. **Leaves narrowly elliptic to lanceolate**, 25 to 45 mm long	12 to 18 mm long, pilose with glands or pointed hairs, rarely glabrous. Petal obovate to orbicular (12.5x9mm), entire or erose. (noted that tube pubescence varies)	Glandular pubescent to rarely glabrescent	Densely glandular pubescent, Pedicel 3 to 30mm, 2 to 6 flowered.	Pedicel 3 to 30 mm	10 to 16 mm long, blade linear, cuspidate, membranes flat.	N/A	Glandular pubescent
P. triovulata Synonym: *P. nana* ssp. *glabella* (Gray) Brand Varieties: *P. nana glabella v. depressa* Brand *P. nana glabella v. triovulata* Brand *P. nelsonii* Brand 30 specimens[1]	Leaves linear, long or rarely short acuminate, glabrous to moderately pilose, e-glandular, 40 to 90mm long and 1.5 to 4 mm wide. Flowering shoots with 5-6 sterile nodes. Leaves longest in the middle.	Eglandular, pubescent	13 to 18mm long, glabrous, petal obovate (13x10mm), entire or erose	Glabrous	eglandular pubescent or rarely minutely glandular on pedicel, 3 to 6 flowered, pedicel 7 to 45 mm	N/A	12 to 18mm long, blade linear, sharp cuspidate, **membranes often distinctly plicate**	**Membranes subcarinate**	Mostly eglandular pubescent

[1]Wherry noted the number of specimens upon which his measurements for his descriptions were based. He studied many more specimens; as evidenced by annotations of specimens over many years.

Table 1.4 Nomenclatural information for *P. nana* s. l. as presented by Wherry in his 1955 monograph.
Taxa recognized primarily (green) and secondarily (light yellow) by Wherry are shown. All other taxa listed (white) under those primarily recognized by Wherry are synonyms accepted by Wherry of the corresponding primary taxa. Some additional notes on locality information are included.

Species	Type Status	Collector	Collection Date	Location	Herbarium
Phlox triovulata Thurber ex. Torrey	Lectotype	Thurber 285	May 1851	Mule Springs, NM[1]	NY
Phlox nana ssp. *glabella (*A. Gray) Brand as "acceptable alternative"					
Phlox nelsonii Brand[2]	Isotype	Lemmon J. G. 415	September 1881	SE Arizona, Chiricahua Mts, Rucker Valley	P, US, GH, PH
Phlox nana Nutt.	Holotype	W. Gambel, s.n.	1845	Santa Fe, NM	K
Phlox nana ssp. *nana* as "acceptable alternative"					
Phlox mesoleuca Greene	Holotype	O. B. Metcalfe 1272	June 29, 1904	South end of the Black Range, Kingston, NM	US
Phlox nana ssp. *ensifolia* Brand as "acceptable alternative"					
Phlox mexicana Wherry	Holotype	Damm, F.C., s. n.	September 1934	Durango, Mexico	PH

[1]There is some confusion as to the exact location of the type specimen of *P. triovulata* (Thurber 285). Wherry notes that the type locality of *P. triovulata* is Mule Springs to Santa Rita Copper Mine. The original specimen that is cited only says Mule Springs as the type locality. The closest area that can be located that is named Mule Spring is 23 air miles east-southeast of Santa Rita Copper Mine. Another locality with the name Mule Spring is 57 miles west-northwest of Santa Rita Copper Mine. Further study of historical records, perhaps route information on Wright's exploration, may shed light on the type locality.

[2]Wherry considered *P. nelsonii* as a synonym of *P. triovulata*.

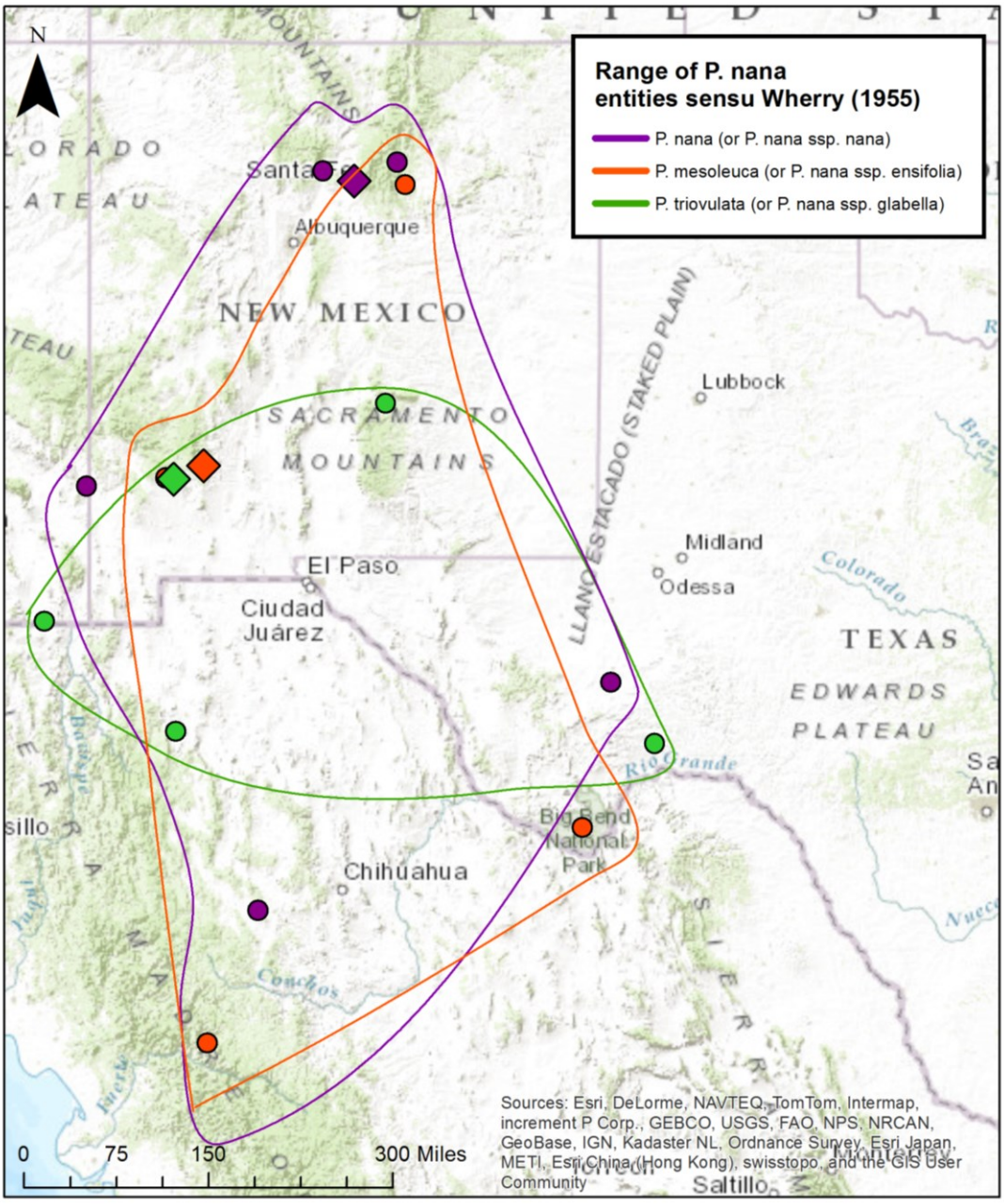

Figure 1.6 Geographic distribution of *P. nana* s. l. as put forth by Wherry (1955). Wherry recognized three species and noted that, alternatively, three subspecies of a single species might appropriately be recognized (see text and Table 1.4; the location of *P. mexicana* to the south near the city of Durango, is not shown). The circled ranges are recreated based on stippling in map figures of Wherry (1955) for each entity. Circle symbols correspond to locations of specimens from points near edge ranges that were cited by Wherry (1955) in support of his distribution maps. Diamond symbols indicate collection localities of type specimens (see Table 1.4). The map was created using ArcGIS® software and Online Map Viewer.

Cytotypic Variation in P. nana

The last in-depth study of *P. nana* s. l. was done by John Eater for his 1967 Master's thesis work, for which he studied numerous populations in the field and also conducted some chromosome counts (Eater 1967). Eater recognized the variation as three species, following the primary recognition in Wherry's 1955 monograph; *P. nana*, *P. mesoleuca*, and *P. triovulata*. He apparently identified plants to species based mostly on glandularity and vestiture of the corolla tube, calyx, leaves, and pedicel along with the length and shape of the leaves (Eater 1967). *Phlox triovulata* was described as having a glabrous corolla tube and an eglandular pubescent calyx, pedicel and leaf, with only a small proportion of the populations having variation in pubescence. *Phlox nana* was described as completely glandular-pubescent. *Phlox mesoleuca* was noted for its variability in the amount and distribution of glandular trichomes but Eater generally described it as having a glabrous corolla tube and a glandular pubescent calyx, pedicel and leaf. A table (Eater 1967, Table 1b) in the introduction of his thesis contains information gathered from Wherry's monograph about the differences between species and was used as a way to identify new populations, although the measurements for leaf length differ slightly from those given by Wherry and it is a very condensed version of Wherry's descriptions. Unfortunately, vouchers for Eater's collections could not be located at Eater's home institution (University of California, Santa Barbara; nor have they been located elsewhere as part of broader taxonomic study; C. Ferguson pers. comm.) and it is likely they may never have been accessioned. Later workers have not been able to study his specimens to gain additional insights into his findings.

Importantly, Eater made diploid and polyploid chromosome counts for material as follows (and following his taxon recognition): *P. nana* (diploid), *P. mesoleuca* (diploid, tetraploid, and hexaploid) and *P. triovulata* (diploid and tetraploid). Most of Eater's chromosome

counts were mentioned in the text of his thesis (rather than in conjunction with figures), including one diploid (2n=14), two tetraploid (2n=28) and one putative hexaploid (2n=42; the figure for the hexaploid is hard for a reader to interpret due to the cell being in metaphase I) count for *P. mesoleuca*, five diploid counts for *P. nana*, and two diploid and nine tetraploid counts for *P. triovulata*. Only those chromosome counts that were included as figures could be linked to specific collection localities based on figure legends (Table 1.5).

Table 1.5 Location of Eater's collections that correspond to chromosome counts for cases in which collection data and chromosome count information can be linked (see text; Eater 1967).

Taxon Identity (sensu Eater)	Location	Collection Date	Eater's Code No.	Ploidy
P. mesoleuca	8 mi NW of Carrizozo, Lincoln Co. NM	5-26-65	6-517	Tetraploid
P. mesoleuca	1 mi S of Kingston, Sierra Co. NM	4-28-66	4341	Hexaploid
P. mesoleuca	2 mi N of Ft. Davis, Limpia Canyon, Ft. Davis Co. TX	4-29-66	4756[1]	Diploid
P. nana	10 mi S of Corona, Lincoln Co. NM	6-22-64	64-71	Diploid
P. nana	3 mi N of Romeroville, San Miguel Co., NM	7-3-64	64-209	Diploid
P. triovulata	3 mi NE of Mescalero, Otero Co. NM	6-23-64	64-80	Tetraploid
P. triovulata	3mi E of Hondo, Lincoln Co. NM	5-2-65	65-16	Diploid

[1]Eater's collection 4756 was not explicitly labeled as diploid in his thesis but there were clearly seven chromosomes presented in his picture of the cell, so herein it is presented as diploid.

Eater observed several separate populations that he described as being intermediate between *P. mesoleuca* and *P. triovulata* or intermediate between *P. mesolecua* and *P. nana*. He proposed hypotheses for this variation including 1) the idea that *P. nana* was the "ancestral" species (and he noted the lack of polyploid chromosome counts) and 2) the idea that *P. mesoleuca* has characteristics intermediate between *P. nana* and *P. triovulata* due to possible

hybridization (which could be taken to contrast with Wherry's idea of *P. mesoleuca* as being "ancestral", although it is not exactly clear what each author means by this as they don't clearly discuss ancestors but only extant taxa). He also suggested that there could be intergradation between species from *P. nana* to *P. mesoleuca* and finally to *P. triovulata* with the suppression of pubescence and glandularity of the corolla tube. Even after Eater was finished with his research, he still seemed to question the relationships between the three. Curiously, however, he did not discuss the possibility of recognition of a single species (even though Wherry clearly posed such a possibility, going so far as to note the appropriate names to be employed for subspecific recognition).

Goals of the Current Study

Current taxonomists recognize this variation as a single, morphologically variable species, *P. nana* s. l., encompassing the past recognition of *P. nana*, *P. triovulata* and *P. mesoleuca*. The current study focuses on cytotypic variation in *P. nana*. Recent advances in flow cytometry allowed the genome size in *P. nana* to be assessed and ploidy level to be inferred (Doležel & Bartoš 2005). Chromosome counts were done in conjunction with flow cytometry in order to carefully document the patterns of ploidy level variation given extensive sampling within *P. nana*. Fieldwork was conducted across the range of *P. nana* in the U.S., cytotypic variation was studied for these populations, and findings were considered relative to morphological characters used by previous taxonomists. This work advances *P. nana* as a study system for work investigating correlates of ploidy, and implications for our understanding of diversity.

Materials and Methods

Field Sampling

Plant material was collected from New Mexico, Texas and Arizona during four seasons: spring 2012, spring 2013 and fall 2013 and spring 2014. Herbarium voucher specimens were collected from each population and accessioned into the KSU Herbarium (KSC; or in a few cases, the herbaria of collaborators). Where available, duplicate vouchers will be sent to herbaria in the region where the plants were collected. In May of 2012, 16 populations were sampled (including, for each population, leaf material from 8 individuals: 5 samples of fresh and silica gel dried leaf material from separate individuals along with extra samples of silica gel dried leaf material from 3 other individuals. In April and May of 2013, 38 populations were sampled (some material collected by collaborators), including fresh and silica gel dried leaf material (from up to 25 individuals) from each population. In August of 2013 silica gel dried material (from up to 25 individuals) of 16 populations (some material collected by collaborators) was collected. In April and May of 2014, 15 populations were sampled (silica dried material from up to 25 individuals) and additional material was also collected for two previously sampled populations.

To enable chromosome counts, during the spring field seasons, buds were collected in 3:1 95% ethanol, glacial acetic acid when they were available. After at least 24 hours, buds were transferred to 70% ethanol for long term preservation. Seeds were additionally collected from populations when available.

Chromosome Counts

Meiotic chromosome counts were conducted on field preserved bud material using B.L. Turner's squash technique (Jones & Luchsinger 1986). Buds were dissected and anthers were

removed, squashed in 1% acetocarmine and observed under a Zeiss (West Germany) photoscope III microscope for metaphase cells. Counts were documented in a lab notebook and by imaging using an Olympus DP71 camera with help from B. Friebe (Gill lab, Kansas State University).

For mitotic chromosome counts, seeds were germinated in a 4°C refrigerator on damp paper towels and after the seeds germinated (3-4 weeks) they were placed on the bench top for several days until the roots were 1-3cm in length. The roots were cut and placed in a vial half full of water. Vials were placed in an ice bucket for 22-28 hours. The water was drained and replaced with 3:1 ethanol, glacial acetic acid at room temperature for 2-4 days. Roots were then transferred to 1% acetocarmine for 3-4 hours and then boiled in 1% acetocarmine immediately prior to squashing. Root tips were then squashed and examined under a light microscope and imaged using an Olympus DP71 camera in the Bikram Gill Lab.

Flow Cytometry and Interpretation of Ploidy

Genome size data for **P. nana**

Flow cytometry was employed in order to estimate the genome size (in picograms) of samples and infer, with comparison to chromosome count data, the ploidy level of each population. Methods that were previously successful in *Phlox* (Fehlberg & Ferguson 2012a, 2012b; Worcester et al 2012) were generally followed. At least one leaf sample (0.05-0.10g; up to 25 samples) per population was chopped with a fresh razor blade in 2ml of a chopping buffer (Davison et al., 2007; modified from Bino et al., 1993). The resulting liquid was then filtered using a 30µm nylon mesh filter (Small Parts Inc.), vortexed and put on ice. Samples were then centrifuged (7 min, 500g) to obtain a pellet. The liquid was decanted leaving the pellet, or in the case no pellet formed, a quarter of the liquid was saved. A staining solution (350-700µl Propidium Iodide; Biosure) was added to fluorescently stain the nuclei and, at this stage, 2µl of

chicken erythrocyte nuclei (CEN; Biosure) was added as a standard. Samples were vortexed and put on ice for 1-3 hours before being transferred to a 5ml round bottom tube with a 40µm cell strainer cap (BD Falcon) and processed. Flow cytometry was run on a Becton Dickinson FACS-Calibur instrument located at the Kansas State University, College of Veterinary Medicine Flow Cytometry Lab. The histograms were analyzed using the program CellQuest (Becton Dickinson). Quality standards included at least 10,000 events (nuclei) per run, and a coefficient of variation (CV) below 5% for each sample (Galbraith et al. 1998; Doležel & Bartoš 2005; discussed by Worcester et al. 2012). Histograms were also checked to ensure that each sample and standard peak were clearly defined. The picograms (pg) of DNA were calculated using the equation from Doležel & Bartoš (2005).

Flow cytometry was run on a single plant in the genus *Phlox* (*P. paniculata*), to test the quality of frozen, silica gel dried, refrigerated and fresh material (all data gathered and analyzed by Jordan McGuinn from the Ferguson Lab). It was found that silica gel dried, refrigerated and frozen material were comparable in quality to fresh material when using flow cytometry to infer ploidy levels. A Kruskal-Wallis test showed that there were no significant differences, with p-values greater than 0.0125, for all comparisons, except in the comparison of frozen (10.62pg) and refrigerated (11.43pg) plant material (p=0.0002; See Appendix A, Tables A.1-A.3). This ancillary study indicates that ploidy levels in *Phlox* can be confidently inferred using material preserved through various methods. Fresh material was used for flow cytometry when possible during this study.

Interpretation of Ploidy Levels

Ploidy levels were determined by qualitatively looking for natural cut off points or gaps in the picogram values from flow cytometry. Chromosome counts for material from populations

also used for flow cytometry enabled linking of the two lines of evidence. The inference of the ploidy levels for the remaining populations were determined by observing picogram values near the average of those populations with corresponding chromosome counts.

Comparison of Different Internal Standards

The use of chicken nuclei as a standard has been seen by some researchers as a potentially problematic reference because of disagreement on the size of the chicken genome (see Doležel & Bartoš 2005). Published 2C-values range from 2.33 to 2.5pg (see Galbraith et al. 1983; Tiersch et al. 1989; the equation from Doležel & Bartoš 2005, uses 2.5pg as a size for CEN). To explore the use of a plant reference standard, we additionally used *Sorghum bicolor* (BTx 623) for the internal standard for measurements of several populations. *Sorghum bicolor* is estimated to have a genome size of 818MB (Price et al. 2005). Using the equation from Dolezel et al. (2007; DNA content [pg]=DNA content [bp]/[0.978x109]) we converted this into picograms in order to estimate the picogram value of our sample. The *S. bicolor* picogram value was estimated to be 1.67pg. Samples of *S. bicolor* were also run with CEN for comparison. Six samples from one population of each ploidy level were run with CEN and then run again on the same day with *S. bicolor*. This gives a way to compare the DNA content of the same sample using both CEN and *S. bicolor* as standards. It is important to note that the commercial CEN standard used can be expected to be consistent in values; but the estimation of the chicken genome size per se is not straight forward.

Geographical Patterns of Cytotypic Variation

Resulting ploidy data for populations were mapped using ArcGIS® desktop software and Online Map Viewer. Field work in later seasons targeted some areas of particular interest based on previous data.

Results

Field Sampling

Samples were obtained from a total of 76 populations (22 populations from Texas, 48 populations from New Mexico, 4 populations from Arizona, and 2 populations from Mexico; see Table 1.6). Flow cytometry was conducted on all 76 populations with three populations being chosen for intensive flow cytometry. Some preserved material, from previously sampled populations prior to this study, were also used in flow cytometry. Bud material from 4 populations was useful for obtaining meiotic chromosome counts and seeds from 5 populations were useful for mitotic chromosome counts.

Table 1.6 Samples of *P. nana* included in the study of cytotypic variation.
Resulting data for populations (flow cytometry, chromosome counts, and inferred ploidy level), are discussed in the text. Populations are ordered by state (TX, NM, AZ and Mexico) and then alphabetically by county.

Pop. code	Collection Number and Preservation Method[1]	Date of collection	General Locality	State	Locality	Altitude (ft.)	Latitude	Longitude	Associated collectors[2]	Chromo-some Count	# of samples[3]	Picogram values for all samples	Average picogram value for population[4]	Inferred ploidy level[5]	Calyx vestiture (Glandular or Eglandular)
1	SDF 42309-1 Fresh	23-Apr-09	Chiricahua Mountains	AZ	COCHISE Co.: Along Forest Road 74 at mile marker 20, south side of the road.	5574	31.75547	-109.37611	SDF, KF		1	17.57	15.57	4x	
	SDF 42413-1A Fresh	24-Apr-13			COCHISE Co.: A) Along Forest Road 74 between mile marker 20/21, south side of the road; B) Along FR74E, from Rucker Lake Dam to parking lot, along SE slope; C) Along road to Rucker Lake (FR74E)	5574	31.75547	-109.37611	SDF, KF		1	13.58			
2	SDF 42309-2 Fresh	24-Apr-09	Chiricahua Mountains	AZ	COCHISE Co.: Near Cypress Park Campground; along Forest Road 74E, about 3.6 road miles from junction with Forest Road 74.	5927	31.76847	109.31855	SDF, KF		2	17.18, 16.83	15.9	4x	
	SDF 42413-1B Fresh	24-Apr-13			COCHISE Co.: A) Along Forest Road 74 between mile marker 20/21, south side of the road; B) Along FR74E, from Rucker Lake Dam to parking lot, along SE slope; C) Along road to Rucker Lake (FR74E)	5574	31.75547	-109.37611	SDF, KF		1	13.7			
3	SDF 42413-1C Fresh	24-Apr-13	Chiricahua Mountains	AZ	COCHISE Co.: A) Along Forest Road 74 between mile marker 20/21, south side of the road; B) Along FR74E, from Rucker Lake Dam to parking lot, along SE slope; C) Along road to Rucker Lake (FR74E)	5574	31.75547	-109.37611	SDF, KF		3	15.61, 14.69, 14.05	14.78	4x	

4	SDF 42413-3 Fresh	24-Apr-13	Chiricahua Mountains	AZ	COCHISE Co.: Along Forest Road 74, at the junction with Forest Road 4818, primarily along north side of the road	5763	31.7213	-109.35413	SDF, KF		6	15.64, 14.49, 15.38, 15.27, 15.07, 14.62	15.078	4x	
5	BW 170 Fresh	15-May-13	Cibola National Forest, Manzano Mountains	NM	BERNALILLO Co.: NE facing slope off FR 515, N of Deadman Campground, approx. 0.1 rd miles S of HWY 337. Cibola National Forest.	7430	35.00464	-106.33585	MHM		5	6.93, 6.85, 7.14, 7.24, 7.14	7.061	2x	G
	BW 203	9-Sep-13			BERNALILLO Co.: NE facing slope off FR 515, N of Deadman Campground, approx. 0.1 rd miles S of HWY 337. Cibola National Forest.	7417	35.00499, 35.00935	106.3359, 106.33469	KW, AP, KF		0				G
6	MHM 3978 Silica	29-Apr-12	Cibola National Forest, Manzano Mountains	NM	BERNALILLO Co.: At the southern boundary of the Cibola NF on HWY 337, roadside bank on northeast corner of junction with Oak Flat Road.		34.98986	-106.33011			3	7.05, 7.57, 7.30	7.306	2x	G
7	SDF 50514-2 Silica	5-May-14	Gila National Forest	NM	CATRON Co.: Wilson Canyon, State Route 233, approx. 9.5 road miles east of its junction with highway 12, east-northeast of Reserve, Gila National Forest		33.732138	-108.60929	KF		5	7.89, 7.83, 8.20, 7.86, 8.06	7.969	2x	
8	BW 158 Fresh/Silica	10-May-13	Roadside	NM	CHAVES Co.: S of HWY 82, 0.3 rd miles E of its jct with Sagebrush Valley Rd., 11.9 rd miles WNW of Hope (jct with CR 12), 5.4 rd miles W of Eddy County line.	4770	32.85568	-104.93	CJF	2n=28	5	14.29, 14.02, 13.66, 15.84, 15.91	14.552	4xC	No flowers
	BW 194 Silica	4-Sep-13			CHAVES Co.: S of HWY 82, 0.3 rd miles E of its jct with Sagebrush Valley Rd., 11.9 rd miles WNW of Hope (jct with CR 12), 5.4 rd miles W of Eddy County line.	4770	32.85568	-104.93	KW, AP		2	14.09, 14.06			E

9	BW 159 Fresh	10-May-13	Southern Lincoln National Forest, Northern Guadalupe Mountains	NM	CHAVES Co.: Near FR 67/Rim Road, 0.2 rd miles SW of cattle guard, 2.4 rd miles SW of forest boundary, 3.5 rd miles SSW of jct of Rim Rd and FR 44/Russell Gap Road. Southern Lincoln National Forest, Guadalupe Mountains.	6055	32.58086	-105.09237	CJF		3	6.38, 8.42, 6.88	7.2	2x	No flowers
	BW 195 Silica	4-Sep-13			CHAVES Co.: Near FR 67/Rim Road, 0.2 rd miles SW of cattle guard, 2.4 rd miles SW of forest boundary, 3.5 rd miles SSW of jct of Rim Rd and FR 44/Russell Gap Road. Southern Lincoln National Forest, Guadalupe Mountains.	6055	32.58086	-105.09237	KW, AP		2	7.36, 6.97			E
10	SDF 50814-1 Silica	8-May-14		NM	CHAVES Co.: Roadside of Tower Road/NM Route 172, 0.4 miles north of its junction with Aberdeen Road (Route 249) and ca. 11 road miles north-northwest of its junction with US Route 82, north-northeast of Artesia.		33.023579	-103.80096	KF		6	16.09, 15.48, 15.76, 15.69, 23.60, 23.43	15.76 (18.34)	Unknown Polyploid	
11	BW 198 Silica	6-Sep-13	Organ Mountains	NM	DONA ANA Co.: Near Aguirre Spring Rd, approx. 3.9 air miles SE of Organ.	4669	32.3715	-106.5606	KW, AP, KF		5	6.09, 6.38, 6.11, 6.31, 6.69	6.316	2x	E
12	BW 163 Fresh	11-May-13	Gila National Forest, West of Mimbres Mountains	NM	GRANT Co.: Slopes and wash of Santa Rita Creek, S of FR 73 (Georgetown Road), from a point starting SW of its jct. with FR 4084Z, approx. 2.4 air miles WSW of Mimbres. Gila National Forest.	6772	32.82727	-108.04366	CJF		2	6.72, 7.06	6.89	2x	No flowers

13	BW 164 Fresh/Silica	11-May-13	Gila National Forest, West of Mimbres Mountains	NM	GRANT Co.: N facing slope near FR 73 (Georgetown Road), 0.3 rd miles W of FR 4205E, approx. 2.6 air miles W of Mimbres.	6540	32.85207	-108.02277	CJF		3	6.68, 6.62, 7.07	7.704	2xA	E
	BW 199 Silica	7-Sep-13			GRANT Co.: SE facing slopes near FR 73 (Georgetown Road) starting at its jct with FR 4085L. Approx. 4.4 air miles WSW of Mimbres along Santa Rita Creek.	6540	32.82347, 32.82538	108.04750, 108.04606	KW, AP, KF		18	6.81, 7.08, 6.58, 6.73, 6.82, 7.24, 6.98, 7.53, 11.18, 7.59, 7.48, 7.39, 7.76, 7.65, 7.56, 11.10, 11.35, 7.37			E
14	AP 3839 Silica		Gila National Forest	NM	GRANT Co.: Along Windmill Pasture Rd., and extending east, 1.1 mi N of the junction with Georgetown Road, Gila National Forest		32.83328	-108.06049			5	8.21, 8.54, 8.40, 8.62, 8.37	8.427	2x	
15	AP 3840 Silica		Roadside	NM	GRANT Co.: Roadside along NM Hwy 35, 4.6 mi N of the intersection with NM Hwy 152, on W side of road		32.84828	-107.97495			5	8.58, 8.15, 8.87, 8.27, 8.57	8.486	2x	
16	BW106 Fresh/Silica	10-May-12	Roadside	NM	LINCOLN Co.: Along HWY 37, 0.1 miles S of junction with County Road CO17, 5 miles S of Nogal, NM (mile markers 6 and 7).	7000	N 33.51896	-105.67727	DW		3	13.82, 14.73, 15.04	14.529	4x	G
	BW 138	12-Apr-13			LINCOLN Co.: Along HWY 37, 0.1 rd miles S of junction with CO17/Cora Dutton Road/FR 488, 5 rd miles S of Nogal, NM (mile markers 6 and 7).	7044	33.51882	-105.67726	CJF		0				No flowers

17	BW107 Fresh/Silica	10-May-12	Roadside	NM	LINCOLN Co.: E of County Road AO22, 1.5 miles N of junction with County Road AO23. Cibola National Forest.	7000	34.19478	-105.69905	DW		3	7.54, 7.45, 7.62	7.536	2x	G
18	BW108 Fresh/Silica	10-May-12	Cibola National Forest, Red Cloud Canyon	NM	LINCOLN Co.: Near County Road AO23 about 2 miles W of junction with County Road AO22. Cibola National Forest.	7150	34.18235	-105.71146	DW		3	6.72, 6.63, 7.12	6.821	2x	G
19	BW109 Fresh/Silica	10-May-12	Cibola National Forest, Red Cloud Canyon	NM	LINCOLN Co.: Near County Road AO23 about 2.25 miles W of junction with County Road AO22, 0.1 miles E of cattle guard and Cibola National Forest sign. Cibola National Forest.	7200	34.18385	-105.71478	DW		3	6.79, 6.80, 7.06	6.883	2x	G
20	BW113 Fresh/Silica	11-May-12	Santa Fe National Forest, East of Sangre de Cristo Mountains	NM	LINCOLN Co.: Near Forest Road 86 (County Road B64), 0.2 mi. E of junction with Forest 83 Road (County Road B44A). Santa Fe National Forest.	7400	35.58556	-105.63608	DW		3	6.64, 6.67, 7.64	6.983	2x	G
21	BW 168 Fresh/Silica	14-May-13	Roadside	NM	LINCOLN Co.: Along Jicarilla Road (CR A044), 0.5 rd miles ESE of jct with rd A036 at Ancho, 0.4 rd miles SE of railroad crossing and 0.1 rd miles E of jct with 1946/A036.	6196	33.93514	-105.73193	MHM		5	7.46, 7.46, 6.97, 7.08, 7.45	7.284	2x	G
22	BW 169 Fresh	14-May-13	Cibola National Forest, Red Cloud Canyon	NM	LINCOLN Co.: Red Cloud Canyon near CR A023, E facing slope and along top of bank, 2.2 rd miles NW of Cibola National Forest sign, 3.75 rd miles NW of junction with CR A022. Cibola National Forest.	7465	34.20114	-105.74148	MHM		5	7.01, 6.87, 7.14, 6.99, 6.90	6.982	2x	G
23	MHM 3979 Fresh/Silica	30-Apr-12	Cibola National Forest, Red Cloud Canyon	NM	LINCOLN Co.: Red Cloud Canyon Campground (new relocated) in Cibola NF, between main road and creek bed to south.	7250	34.19044	-105.72682			2	6.44, 6.88	6.658	2x	G
24	KW 228 Silica	15-Apr-14		NM	LINCOLN Co.: SW-facing slope on the east side of highway 380 at the junction with highway 70, northwest of Hondo.		33.39171	-105.27372	CJF		5	8.30, 8.16, 8.39, 8.36, 8.13	8.269	2x	

25	KW 229 Silica	15-Apr-14		NM	LINCOLN Co.: Along highway 380, 1.8 road miles NW of its junction with highway 70, west of Hondo		33.41191	-105.29005	CJF		5	8.40, 7.71, 8.25, 8.44, 7.63, 8.51	8.247	2x	
26	KW 230 Silica	15-Apr-14	Lincoln National Forest	NM	LINCOLN Co.: Salazar Canyon off FR 57, approx. 0.5 road miles north of its junction with highway 380, north of Double Crossing, Lincoln National Forest.		33.53296	-105.43607	CJF		5	16.87, 15.97, 15.69, 16.26, 16.42	16.241	4x	
27	KW 231 Silica	15-Apr-14		NM	LINCOLN Co.: Roadside of FR A040, west of highway 246, 6.2 miles north of Capitan.		33.67841	-105.57083	CJF		6	15.21, 15.06, 15.19, 15.18, 15.00, 15.07	15.12	4x	
28	KW 232 Silica	15-Apr-14	Lincoln National Forest	NM	LINCOLN Co.: Above forest road 84/Indian Divide Road, approx. 2 road miles west of its junction with highway 380, west of Capitan, Lincoln National Forest.		33.61592	-105.66472	CJF		6	14.90, 15.06, 15.24, 15.21, 15.29, 14.71	15.069	4x	
29	AP 3843 Silica		Roadside	NM	LUNA Co.: About 0.5 air mi. along an old mining road toward Cookes Peak, after it splits from the road to Riley Spring, Cookes Range		32.54610, 32.54364	-107.72019, to -107.72356			5	16.61, 17.58, 17.07, 16.31, 17.20	16.95	Unknown Polyploid	
30	BW100 Fresh/Silica	9-May-12	Sacramento Mountains, Roadside	NM	OTERO Co.: Near Russia Canyon Road (Forest Road 247), N of junction with Cox Canyon Highway (HWY 130), N of bridge. West of Cloudcroft. Lincoln National Forest.	7900	32.89438	-105.67844	DW	n=14	2	13.07, 15.26	14.535	4xC	E
	BW 162 Fresh/Silica	10-May-13			OTERO Co.: Near Russia Canyon Road (FR 247), 0.05 rd miles S of junction with Cox Canyon Highway (HWY 130), N of bridge, SE of Cloudcroft.	7900	32.89438	-105.67844	CJF		5	15.21, 13.36, 16.42, 14.28, 14.14			E

31	BW101 Fresh/Silica	9-May-12	Sacramento Mountains, Roadside	NM	OTERO Co.: Near Russia Canyon Road (Forest Road 247), N of junction with Cox Canyon Highway (HWY 130), S of bridge. West of Cloudcroft. Lincoln National Forest.	7900	32.89423	-105.6785	DW		10	11.94, 12.73, 14.77, 12.68, 12.97, 13.26, 13.01, 13.15, 13.73, 14.30	13.253	4x	E
32	BW102 Fresh/Silica	9-May-12	Sacramento Mountains, Roadside	NM	OTERO Co.: One mile W of Russia Canyon Road (Forest Road 247), N of Cox Canyon Highway (HWY 130). West of Cloudcroft. Lincoln National Forest.	7800	32.88768	-105.66287	DW		3	12.16, 13.78, 15.10	13.682	4x	E
33	BW 161 Fresh	10-May-13	Sacramento Mountains, Roadside	NM	OTERO Co.: Eroded roadside bank along Cox Canyon Highway (130), 4.6 rd miles SE of its jct with Russia Canyon Road, approx. 6.8 air miles NW of Weed.	7300	32.86494	-105.61067	CJF		2	12.53, 14.09	13.025	4x	E
	BW103 Fresh	9-May-12			OTERO Co.: N of Cox Canyon Highway (HWY 130). About 0.2 miles E of Sivells Baptist Retreat and Conference Center. West of Cloudcroft.	7300	32.86494	-105.61067	DW		2	13.44, 12.04			E
34	BW104 Fresh	9-May-12	Sacramento Mountains, Roadside	NM	OTERO Co.: N of HWY 82, 0.2 miles E of junction with Lariat Road, across from Robin Hood Ranch. West of Cloudcroft. Lincoln National Forest.	7600	32.94503	-105.62359	DW		3	11.85, 11.67, 12.74	12.088	4x	E
35	BW105 Fresh/Silica	9-May-12	Sacramento Mountains, Roadside	NM	OTERO Co.: Near Cox Canyon Highway (HWY 130), 1 mile E of junction with Valley Road (mile markers 4 and 5). West of Cloudcroft. Lincoln National Forest.	8500	32.92143	-105.72217	DW		2	16.28, 16.39	16.339	4xA	E

36	BW160 Fresh/Silica	10-May-13	Lincoln National Forest, Sacramento Mountains	NM	OTERO Co.: Off Bear Canyon Road (FR 621), approx. 0.35 rd miles SW of its jct. with HWY 24, N of Weems Spring, Lincoln National Forest, NW of Weed.	6963	32.84744, 32.84794	105.54379, 105.54334	CJF	2n=28	5	15.03, 13.42, 14.63, 13.60, 15.13	14.876	4xC	E
	BW 196 Silica	5-Sep-13			OTERO Co.: South of Bear Canyon Road (FR 621), approx. 1.3 rd miles SW of its jct. with HWY 24, SW of Weems Spring, Lincoln National Forest, NW of Weed.	7128	32.84334	-105.55226	KW, AP		20	13.49, 13.29, 13.62, 13.55, 13.26, 14.71, 15.48, 14.95, 14.50, 15.51, 15.99, 17.35, 14.53, 14.99, 15.57, 16.75, 15.38, 15.26, 15.78, 16.13			E
37	BW 197 Silica	5-Sep-13	Lincoln National Forest, Sacramento Mountains	NM	OTERO Co.: East of Mountain View Rd , just SE of Calico Peak, 2.1 rd miles N of jct with Mountain Park Rd. Approx. 1.6 miles NNE of High Rolls.	6583	32.97316	-105.82592	KW, AP, KF		5	13.59, 14.20, 14.52, 14.94, 13.47	14.15	4x	E
38	BW110 Fresh/Silica	11-May-12	Sangre De Cristo Mountains, Roadside	NM	SAN MIGUEL Co.: Near HWY 50, 0.1 miles W of junction with County HWY 63 in Pecos, NM (mile markers 1 and 2). Santa Fe National Forest.	7200	35.56736	-105.7463	DW		3	7.28, 7.57, 7.21	7.352	2x	G
39	BW111 Fresh/Silica	11-May-12	Roadside	NM	SAN MIGUEL Co.: S of Old Colonias Road (County Road B60), near junction with Vista Grande Road in Pecos, NM and near Santa Fe National Forest.	7000	35.57121	-105.65681	DW		3	6.55, 7.34, 7.70	7.196	2x	G

40	BW112 Fresh/Silica	11-May-12	Roadside	NM	SAN MIGUEL Co.: Dead end of Old Colonias Road (County Road B60). About 0.2 mi. E of junction with Los Llanitos Road. Near Santa Fe National Forest.	7200	35.57343	-105.64341	DW	n=7	5	6.37, 6.51, 6.56, 6.56, 6.50	6.5	2xC	G
41	BW114 Fresh/Silica	11-May-12	Roadside	NM	SAN MIGUEL Co.: Along HWY 50 in Glorieta, NM, 3.25 miles W of its junction with HWY 63, and 0.2 miles E of gas station, N of HWY 50. Near Santa Fe National Forest.	7000	35.57069	-105.70658	DW		3	6.69, 7.40, 6.99	7.027	2x	G
42	BW115 Fresh	11-May-12	Roadside	NM	SAN MIGUEL Co.: Pecos, NM, along HWY 50, 3 miles W of HWY 63, near gas station on N side of HWY 50. Near Santa Fe National Forest.	7100	35.57351	-105.70121	DW	n=7	1	6.34	6.726	2xC	G
	BW 171 Fresh	15-May-13			SAN MIGUEL Co.: N of HWY 50 near Pecos, 3 miles W of jct with HWY 63 (N Main St), approx. 4 air miles E of Glorieta.	7100	35.57351	-105.70121	MHM		5	6.92, 6.88, 6.62, 6.91, 6.70			G
43	BW 172 Fresh/Silica	15-May-13	Santa Fe National Forest, Sangre De Cristo Mountains	NM	SAN MIGUEL Co.: Top of S facing slope off Dalton Canyon Rd/FR 123 and W of jct with rt 63, N of Camp La Salle (seen also along the N side of road), approx. 5.9 air miles N of Pecos. Santa Fe National Forest.	7351	35.65888	-105.69112	MHM	2n=14	5	7.22, 6.88, 7.38, 8.21, 7.20	7.377	2xC	G
	BW 204	9-Sep-13			SAN MIGUEL Co.: Top of S facing slope off Dalton Canyon Rd/FR 123 and W of jct with rt 63, N of Camp La Salle (seen also along the N side of road), approx. 5.9 air miles N of Pecos. Santa Fe National Forest.	7351	35.65888	-105.69112	KW		0				G
44	BW 173 Fresh	15-May-13	Santa Fe National Forest, East of Sangre De Cristo Mountains	NM	SAN MIGUEL Co.: Near FR 203A, from a point just N of its jct with 83MBC and approx. 1.3 rd miles S of its jct with Rt 83, SE of Pecos. Santa Fe National Forest.	8014	35.54384	-105.54627	MHM		1	7.51	7.51	2x	No flowers

45	BW 174 Fresh/Silica	15-May-13	Santa Fe National Forest, East of Sangre De Cristo Mountains	NM	SAN MIGUEL Co.: E of FR 203A, approx. 0.2 rd miles N of jct with FR 83MBA and 0.1 rd miles S of jct with Rd 83, E of Pecos. Santa Fe National Forest.	8182	35.55884	-105.54432	MHM		4	6.99, 6.62, 6.80, 7.08	6.873	2x	No flowers
46	BW 141	13-Apr-13	Roadside, Mimbres Mountains, Black Range	NM	SIERRA Co.: Near rt 152, 0.2 air miles E of Kingston.	6190	32.91812	-107.70084	CJF		0		21.29	6x	G
	MHM 3985 Silica	1-May-12			SIERRA Co.: Gila NF, vicinity of Kingston Campground, east of Kingston.	6190	32.91812	-107.70084			3	21.14, 22.99, 21.83			G
	BW 200 Silica	7-Sep-13			SIERRA Co.: Near rt 152, approx. 0.2 air miles E of Kingston and 7.5 air miles W of Hillsboro.	6190	32.91812	-107.70084	KW, AP, KF		3	21.04, 21.15, 19.5			G
47	BW 142 Fresh	13-Apr-13	Gila National Forest, Mimbres Mountains, Black Range	NM	SIERRA Co.: N facing slope S of FR 4145, E of rt 152 where it makes a hairpin turn over Southwest Canyon, approx. 2.5 air miles above (SW of) Kingston.	7000	32.90165	-107.72853	CJF		4	21.21, 21.79, 19.86, 21.57	21.168	6x	No flowers
	MHM 3987 Fresh/Silica	1-May-12			SIERRA Co.: Gila NF, at sharp curve in HWY where it crosses Southwest Canyon, above Kingston. Above and South of FR 4145M.	6880	32.90146	-107.72867			1	21.42			G
48	AP 3822 Silica	6-Sep-13	Roadside, Mimbres Mountains, Black Range	NM	SIERRA Co.: Along Highway 152 as it follows Percha Creek, 4.4 miles east of Kingston.	5620	32.91827	-107.6357	KW, AP, KF	2n=28	6	13.40, 13.81, 13.22, 13.23, 13.30, 15.93	13.82	4xC	G

49	BW 202 Silica	8-Sep-13	Gila National Forest	NM	SIERRA Co.: NW of rd Co03, approx. 0.5 rd miles W of jct with county rd Co01, 5.4 air miles SSW of Winston, near Monument Spring, Gila National Forest.	6298	33.25763, 33.25034	-107.68322, to -107.70459	KW, AP, KF		23	19.66, 21.13, 21.23, 19.14, 20.75, 20.33, 20.30, 19.82, 20.18, 20.40, 21.10, 21.83, 22.14, 20.85, 20.86, 21.05, 22.15, 21.33, 20.94, 20.93, 21.00, 22.91, 21.86, 21.30	20.97	6x	G
50	Alexander 1436 Herbarium specimen[6]		White Sands Missile Range	NM	SIERRA Co.: Northern San Andres Mountains near the top of Sweetwater Canyon, 1.8 miles WNW of Salinas Peak, 2.0 mi NE of Ladybug Peak. White Sands Missile Range.		33.3065	-106.5618			10	12.54, 12.49, 13.18, 12.29, 12.14, 12.33, 12.73, 13.10, 12.73, 13.60	12.71	4x	
51	BW 165 Fresh/Silica	12-May-13	Cibola National Forest, Magdalena Mountains	NM	SOCORRO Co.: Off Water Canyon Rd (FR 235), approx. 0.7 rd miles SW of its jct with FR 406 (near Water Canyon Campground), 0.2 rd miles SW of Mesa trailhead. Cibola National Forest.	6965	34.01616	-107.13534	CJF		5	13.74, 12.59, 14.72, 14.46, 15.09	14.12	4x	G
	BW 201	8-Sep-13			SOCORRO Co.: Off Water Canyon Rd (FR 235), approx. 0.7 rd miles SW of its jct with FR 406 (near Water Canyon Campground), 0.2 rd miles SW of Mesa trailhead. Cibola National Forest.	6965	34.01616	-107.13534	KW, AP, KF		0				G

52	SDF 90913-1 Silica	9-Sep-13	San Mateo Mountains	NM	SOCORRO Co.: Along Forest Road 330; 4.1 road miles from junction with 107; 2 road miles past forest boundary.	7506	33.80555	-107.41951	KF		5	14.62, 16.82, 13.40, 13.86, 14.08	14.56	4x	
53	BW 152 Fresh/Silica	7-May-13	Roadside	TX	BREWSTER Co.: E side of HWY 118, 5.3 rd miles SE of jct with US 90 in Alpine.	5072	30.30324	-103.59919	CJF		6	17.39, 17.28, 17.75, 16.62, 17.29, 17.17	17.195	Unknown Polyploid	G
	BW 191 Silica	2-Sep-13			BREWSTER Co.: E side of HWY 118, 5.3 rd miles SE of jct with US 90 in Alpine.	5072	30.30324	-103.59919	KW, AP		2	16.74, 17.32			G
54	BW 153 Fresh/Silica	7-May-13	Roadside	TX	BREWSTER Co.: E side of HWY 118, 6.2 rd miles SE of jct with US 90 in Alpine. Growing along S end of rocky road cut	5173	30.28901	-103.59455	CJF		2	16.87, 16.71	16.387	4xA	G
	CF 337 Frozen	9-Aug-96			BREWSTER Co.: Along E side of route 118, 6.2 miles S of junction with US 90 in Alpine.		No GPS listed	No GPS listed			3	14.83, 17.56, 15.96			G
55	CF 335 Frozen	8-Sep-96	Roadside	TX	BREWSTER Co.: Along E side of route 118, 5.3 mi S of junction with US 90 in Alpine.		30.30324	-103.59919			3	8.05, 8.12, 8.07	8.082	2x	E
56	BW 154 Fresh/Silica	8-May-13	Big Bend National Park, Chisos Mountains	TX	BREWSTER Co.: N facing slope above (S of) Lost Mine Trail, approx. 0.3 air miles SE of point where trail meets Chisos Basin Rd (trailhead), S of Panther Pass into Chisos Basin, Big Bend National Park.	5894	29.27144	-103.28173	CJF		5	15.34, 15.29, 14.73, 14.38, 15.42	15.032	4x	No flowers
57	CF 332 Frozen	6-Sep-96	Big Bend National Park, Chisos Mountains	TX	BREWSTER Co.: Big Bend National Park, near top of pass into Chisos Basin, by beginning of Lost Mine Peak Trail.		29.2744	-103.2864			2	14.13, 14.21	14.168	4x	G

58	KW 226 Silica	13-Apr-14	Big Bend National Park, Chisos Mountains	TX	BREWSTER Co.: NNW-facing slope of Casa Grande Peak above trailhead for Lost Mine Peak trail, Chisos Mountains, Big Bend National Park.		29.27403	-103.28728	CJF		11	17.80, 16.13, 16.86, 17.20, 18.04, 22.67, 17.83, 18.23, 18.26, 17.95, 18.08	18.1	Unknown Polyploid	
59	BW 155 Fresh	8-May-13	Big Bend National Park, Chisos Mountains	TX	BREWSTER Co.: Scattered on slope above Laguna Meadows Trail, approx. 1.2 air miles N of Emory Peak, approx. 0.4 air miles SSW of trailhead. Chisos Mountains Basin, Big Bend National Park.	5550	29.2626 4	-103.3057	CJF		5	6.87, 7.55, 7.76, 7.39, 7.46	7.407	2x	G
60	CF 333 Frozen	7-Sep-96	Big Bend National Park, Chisos Mountains	TX	BREWSTER Co.: In Big Bend National Park, on slope above trail to Laguna Meadows, near top of trail close to short trail to lookout.		29.26903	-103.30362	Denise Louie		2	7.27, 7.01	7.139	2x	G
61	KW 227 Silica	14-Apr-14	Big Bend National Park, Chisos Mountains	TX	BREWSTER Co.: Slope above dry wash off Laguna Meadows trail, Chisos Mountains, Big Bend National Park.		29.26241	-103.30501	CJF		5	8.61, 8.51, 8.60, 8.78, 12.62	9.42	2x	
62	BW 156 Fresh	9-May-13	Guadalupe Mountains National Park	TX	CULBERSON Co.: Pine Spring Canyon, above (S of) Devil's Hall Trail in NE facing slope/wash, approx. 0.3 air miles W of trailhead, approx. 1.7 air miles ENE of Guadalupe Peak, NW of Pine Springs. Guadalupe Mountains National Park.	6000	31.8969	-104.83177	CJF		3	14.07, 14.43, 13.13	14.53	4x	
	BW 192 Silica	3-Sep-13	Guadalupe Mountains National Park	TX	CULBERSON Co.: Pine Spring Canyon, above (S of) Devil's Hall Trail in NE facing slope/wash, approx. 0.3 air miles W of trailhead, approx. 1.6 air miles ENE of Guadalupe Peak, WNW of Pine Springs. Guadalupe Mountains National Park.	6020	31.89811	-104.8336	KW, AP		7	15.28, 13.47, 13.17, 15.43, 14.04, 14.90, 17.34			E

63	BW 157 Fresh/Silica	9-May-13	Guadalupe Mountains National Park	TX	CULBERSON Co.: McKittrick Canyon, S of McKittrick Canyon Trail, approx. 0.6 air miles NW of trailhead, approx. 4.4 air miles N of Nickel Creek Station. Guadalupe Mountains National Park.	5059	31.98427	-104.75983	CJF		5	14.49, 13.40, 12.65, 13.90, 14.89	13.866	4x	
	BW 193	4-Sep-13			CULBERSON Co.: McKittrick Canyon, S of McKittrick Canyon Trail, approx. 0.6 air miles NW of trailhead, approx. 4.4 air miles N of Nickel Creek Station. Guadalupe Mountains National Park.	5065	31.98421	-104.75977	KW, AP		0				E
64	BW 140 Fresh	13-Apr-13	Franklin Mountains	TX	EL PASO Co.: S of Mundy's Gap West/West Cottonwood Trail, 0.25 miles ESE of trail head/parking lot. Franklin Mountains State Park.	5157	31.91571	-106.50613	CJF		5	14.06, 15.12, 14.03, 13.75, 13.92	14.178	4x	G
	SDF 82613-1	26-Aug-13			El Paso Co.: North facing slope south of Agave Loop Trail, where trail descends into the canyon.	5349	31.91368	-106.50412	KF		0				
65	BW 143 Fresh/Silica	14-Apr-13	Davis Mountains	TX	JEFF DAVIS Co.: Along North 78 Spur, 0.7 rd miles SE of jct of N 78 spur with HWY 118, below McDonald Observatory.	6288	30.67963	-104.01871	CJF		5	21.44, 21.28, 19.89, 21.85, (16.89)	21.113 (20.27)	Unknown Polyploid	G
66	CF 237 Frozen	16-Aug-95	Davis Mountains	TX	JEFF DAVIS Co.: McDonald Observatory, by water faucet near 87" telescope.	6791	30.67166	-104.02722	Phyllis Boyd		3	15.65, 15.81, 15.50	15.652	4x	G
67	BW 144 Fresh/Silica	15-Apr-13	Davis Mountains	TX	JEFF DAVIS Co.: Davis Mountains State Park: near trail S of amphitheater in canyon, S and W of road to Lookout Tower, approx. 2.2 air miles W of Fort Davis.	5073-5102	30.59113	-103.93013	CJF	n=14	13	19.60, 16.99, 18.17, 19.69, 24.17, 17.36, 17.38, 17.99, 18.39, 19.32, 19.13, 18.13, 17.28	18.74	4xAC	G

68	BW 151 Fresh/Silica	7-May-13	Davis Mountains	TX	JEFF DAVIS Co.: S side of HWY 118, 2.4 rd miles E of Davis Mountains State Park entrance, just W of small picnic area, approx. 1.3 air miles N of Ft. Davis, Davis Mountains.	4925	30.60677	-103.89125	CJF		6	19.75, 23.74, 21.60, 20.87, (17.07, 16.41)	23.02 (22.33)	Unknown Polyploid	G
	KW 225 Silica	12-Apr-14			JEFF DAVIS Co.: Near roadside picnic area on south side of highway 118, 2.4 road miles east of Davis Mountains State Park, Davis Mountains		30.60677	-103.89125	CJF		12	30.48, 23.78, 22.17, 24.70, 24.37, 25.19, 22.74, 23.64, 22.08, 20.91, 21.53, 20.82			
69	BW 145 Fresh	15-Apr-13	Davis Mountains	TX	JEFF DAVIS Co.: Limpia Canyon, S of Wild Rose Pass along HWY 17, 0.7 rd miles S of roadside park, 2.7 rd miles S of top of Wild Rose Pass, NE of Ft. Davis. Along E side of road starting at guard rail S of crossing and continuing south.	4541	30.68096	-103.79282	CJF		5	8.04, 9.60, 9.69, 8.09, 8.27	8.737	2x	G
70	BW 148 Fresh	7-May-13	Davis Mountains	TX	JEFF DAVIS Co.: E side of HWY 17, along Wild Rose Pass, approx. 11 air miles NE of Ft. Davis on W facing slope near fence, Davis Mountains.	4575	30.71499	-103.78239	CJF	2n=14	5	11.76, 9.00, 11.17, 11.61, 12.08	11.124	2xAC	G
71	BW 149 Fresh	7-May-13	Davis Mountains	TX	JEFF DAVIS Co.: Limpia Canyon, W side of HWY 17, 3 rd miles S of Wild Rose Pass, approx. 8.6 air miles NE of Ft. Davis. Growing near roadside picnic area, Davis Mountains.	4500	30.67703	-103.79438	CJF		5	9.57, 9.78, 9.79, 8.93, 9.05	9.424	2x	G
72	BW 150 Fresh	7-May-13	Davis Mountains	TX	JEFF DAVIS Co.: Rocky slope of Limpia Canyon, E side of HWY 17, 7.1 rd miles NE of jct with HWY 118 in Ft. Davis, approx. 12.9 air miles E of Mt. Locke, Davis Mountains.	4640	30.65743	-103.80524	CJF		5	11.21, 10.58, 8.87, 9.50, 8.82	9.796	2x	G

73	KW 223 Silica	12-Apr-14	Davis Mountains	TX	JEFF DAVIS Co.: Limpia Canyon south of Wild Rose Pass along highway 17, Davis Mountains.		30.689722	-103.78917	CJF		5	14.34, 13.59, 14.49, 13.19, 14.85	14.092	4x	G
74	KW 224 Silica	12-Apr-14	Davis Mountains	TX	JEFF DAVIS Co.: Along County Road 1832, east of Scout Camp, Davis Mountains.		30.81458	-103.93585	CJF		5	24.51, 22.93, 20.03, 22.63, 19.68	21.955	6x	
75	CF 244 Frozen	18-Aug-95	Roadside	Mexico	Municipio de Guerrero: Along Chihuahua highway 16 W of Cuauhtemoc, approx 12.8 mi (20.6 km) by road E of the cemetery at La Junta, near "Pizzas y mas--Los Rosales" sign on north side of road.	7382	28.42194	-107.1275			2	19.43, 19.38	19.407	6xA	G
76	CF 245 Frozen	18-Aug-95	Roadside	Mexico	Municipio de Temosachic: Along Chihuahua highway 16, 1.3 mi (2.1km) by road southeast of cruce with road to San Jose de Bavicora, 0.5 mi (0.8km) by road southeast of 100km marker.	6361	29.110833	-107.89889	Phyllis Boyd		2	16.95, 19.07, 16.73, 18.58	18.826	Unknown Polyploid	G

[1]The preservation method listed represents the type of preserved material used when running flow cytometry samples.

[2]CJF: Carolyn J. Ferguson, KW: Kate Waselkov, AP: Alan Prather, KF: Kevin Fehlberg, MHM: Mark H. Mayfield, DW: Deborah Wright, SDF: Shannon D. Fehlberg.

[3]In most cases, many samples from each populations were collected and only a portion of them were used to do flow cytometry. The number of flow cytometry runs that passed all the quality checks is listed.

[4]Values in parentheses are separate averages that include picogram values that were outside the normal for that population.

[5] "Unknown Polyploid" samples are discussed explicitly in the text. All populations marked with a "C" have a recorded chromosome count. Inference of ploidy for samples marked with an "A" are considered atypical and are addressed in the Discussion.

[6]The Alexander 1436 material used for flow cytometry was removed from pressed herbarium specimens within just a few weeks of collection, and then placed in silica gel and sent to the Ferguson Lab for study.

Chromosome Counts

Chromosome counts (see Fig. 1.7-1.17) were obtained for nine populations of *P. nana* (four diploid and five tetraploid; see Table 1.6). Chromosome counts from buds were obtained from 0.8-1.0mm long anthers for diploids and about1.5mm long anthers for tetraploids. Slides of squashed cells from meiotic chromosome counts were preserved and will be archived in the Kansas State University Herbarium (KSC). In material for all but one population, chromosomes were observed as expected. In population BW148 a small fragment of a chromosome was found in several cells (see Fig. 1.7 (E), 1.11 (left))

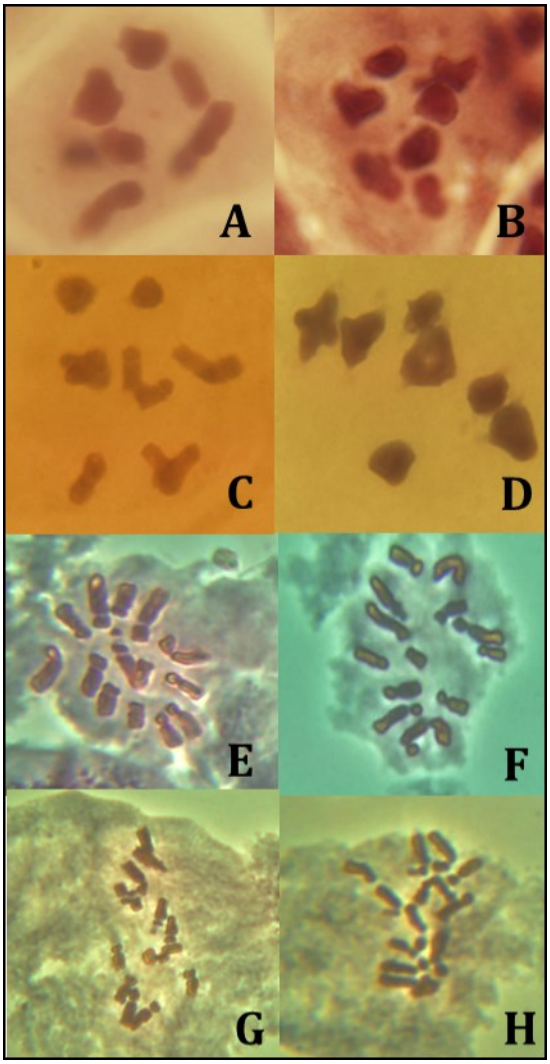

Figure 1.7 Diploid chromosome counts: meiotic counts of BW 112 (A, B), and BW 115/171 (C, D) and mitotic counts of BW 148 (E, F; containing small fragment of chromatin), and BW 172/204 (G, H).

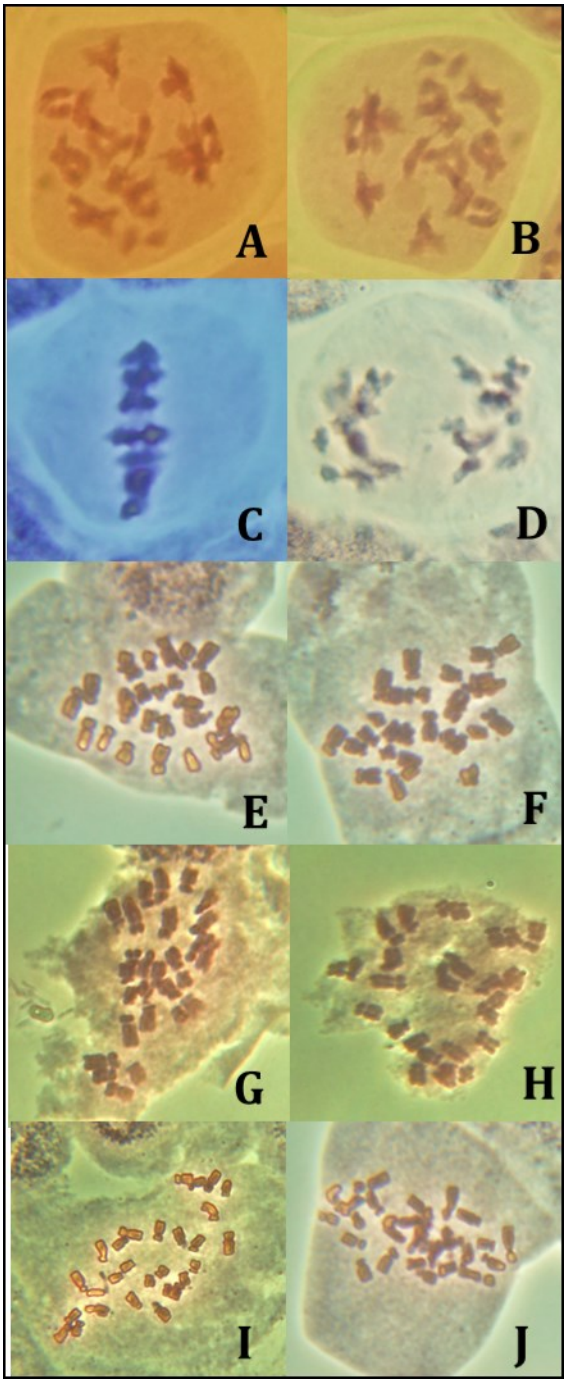

Figure 1.8 Tetraploid chromosome counts: meiotic counts of BW 144 (A, B) and BW 100/162 (C, D) and mitotic counts of AP 3822 (E, F), BW 160/196 (G, H) and BW 158/194 (I, J).

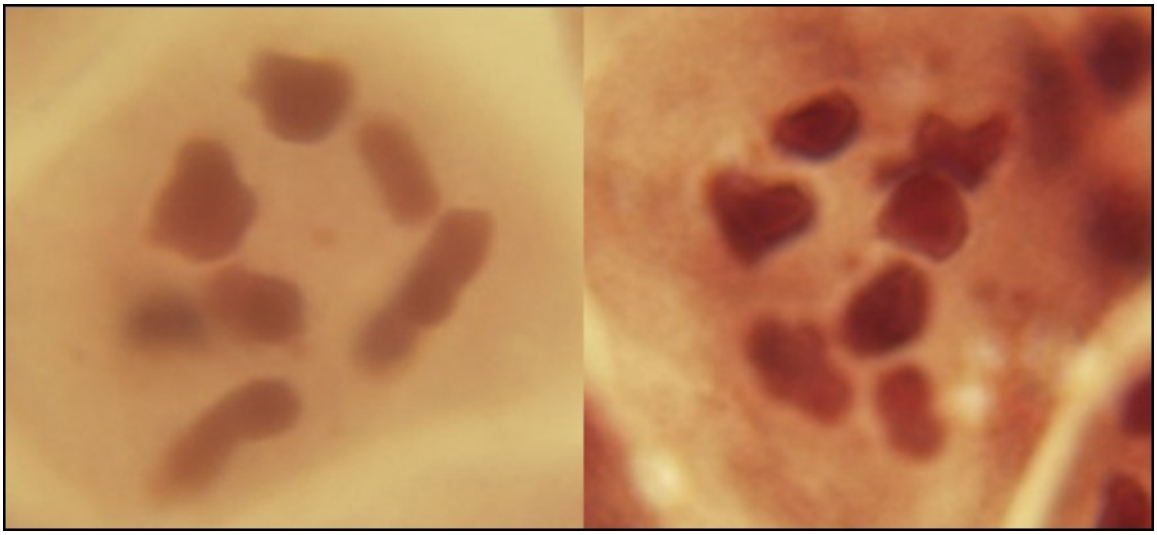

Figure 1.9 Meiotic chromosome counts of BW 112 showing seven chromosomes in metaphase I cells of meiosis (diploid; corresponds to Fig. 1.7 A and B).

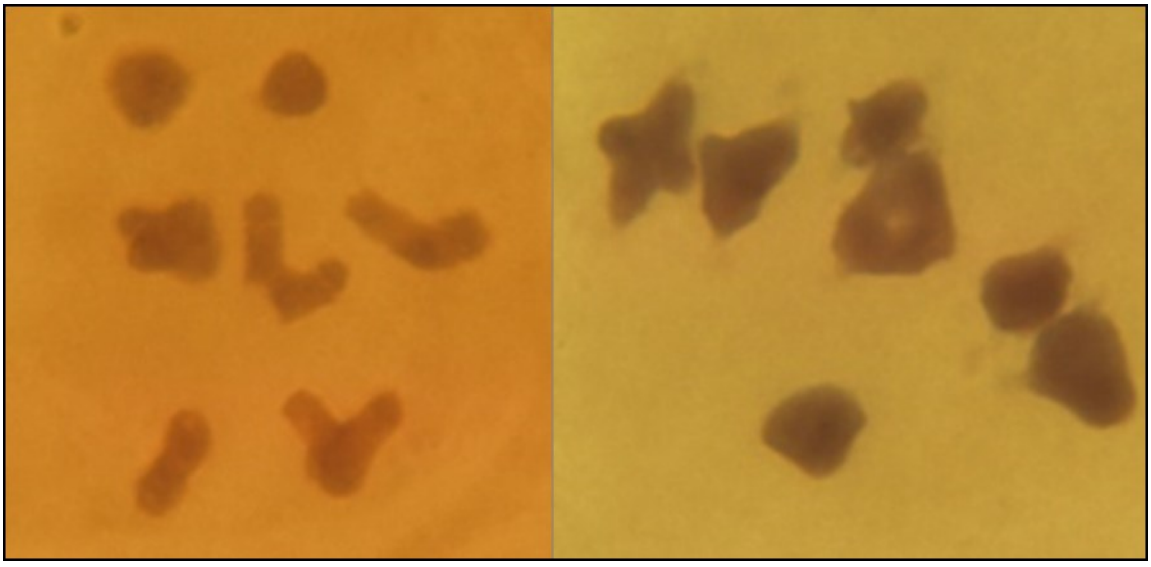

Figure 1.10 Meiotic chromosome counts of BW 115/171 showing seven chromosomes in metaphase I cells of meiosis (diploid; corresponds to Fig. 1.7 C and D).

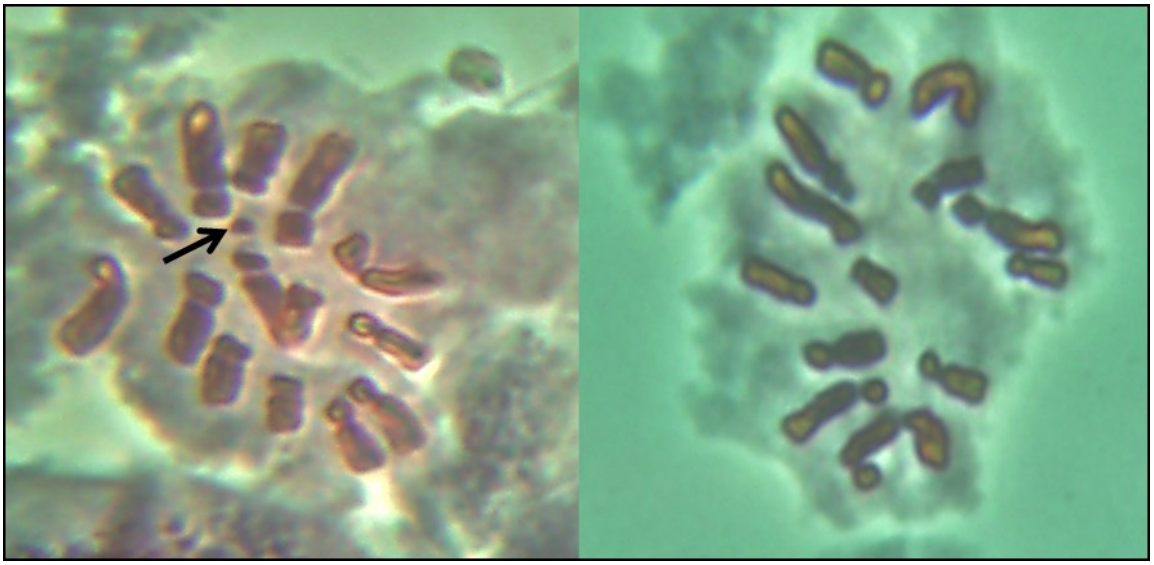

Figure 1.11 Mitotic chromosome counts of BW 148 showing 14 chromosomes in early metaphase cells (diploid; corresponds to Fig. 1.7 E and F).
A small fragment can be seen in the picture on the left (arrow). BW 148 was the only population with a fragment of a chromosome.

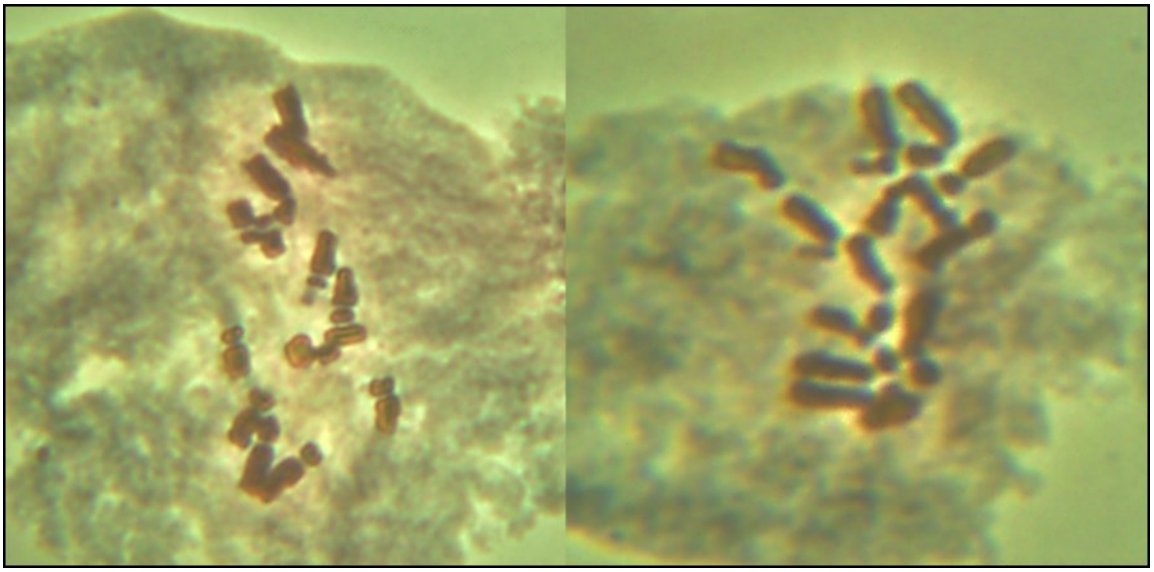

Figure 1.12 Mitotic chromosome counts of BW 172/204 showing 14 chromosomes in early metaphase cells (diploid; corresponds to Fig. 1.7 G and H).

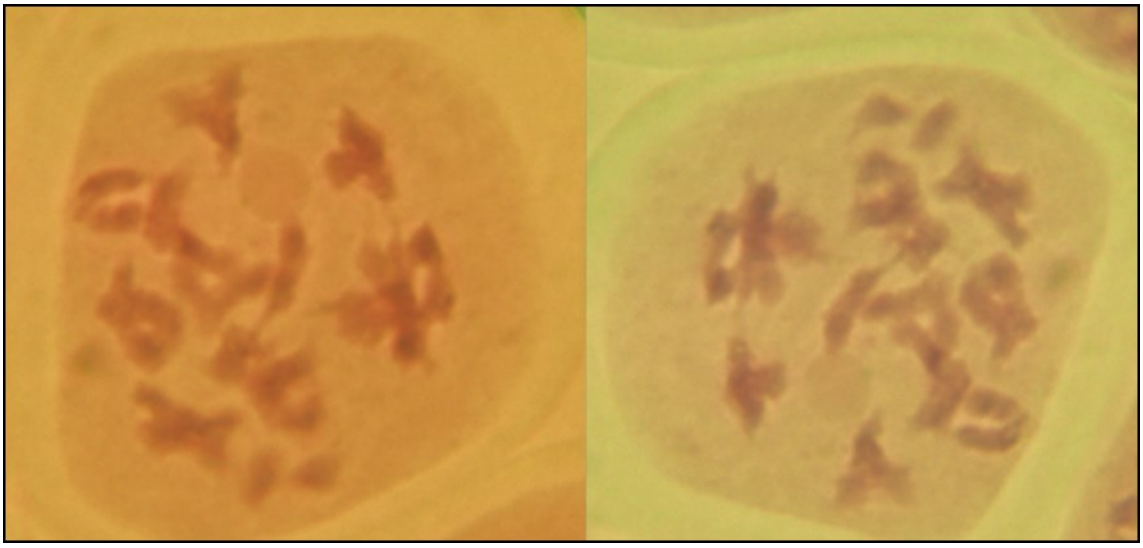

Figure 1.13 Meiotic chromosome counts of BW 144 showing 14 chromosomes with cells in metaphase I of meiosis (tetraploid; corresponds to Fig. 1.8 A and B).

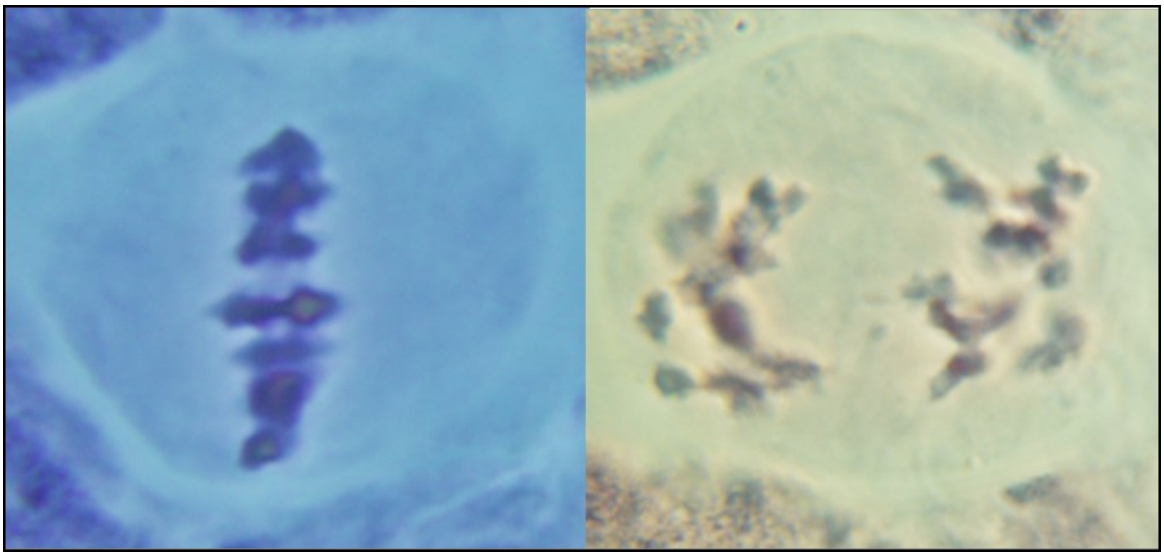

Figure 1.14 Meiotic chromosome counts of BW 100/162 with cells in anaphase and telophase of meiosis (tetraploid; corresponds to Fig. 1.8 C and D).
Better cells were not found in order to get an accurate count for this population although we infer that there are more than seven chromosomes present.

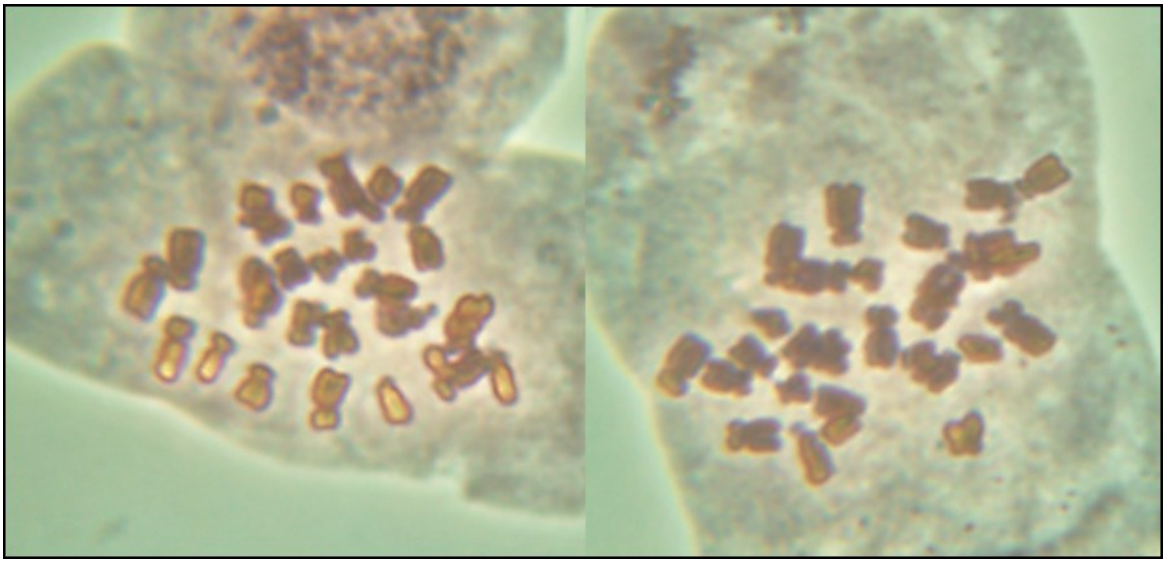

Figure 1.15 Mitotic chromosome counts of AP 3822 showing 28 chromosomes in early metaphase cells (tetraploid; corresponds to Fig. 1.8 E and F).

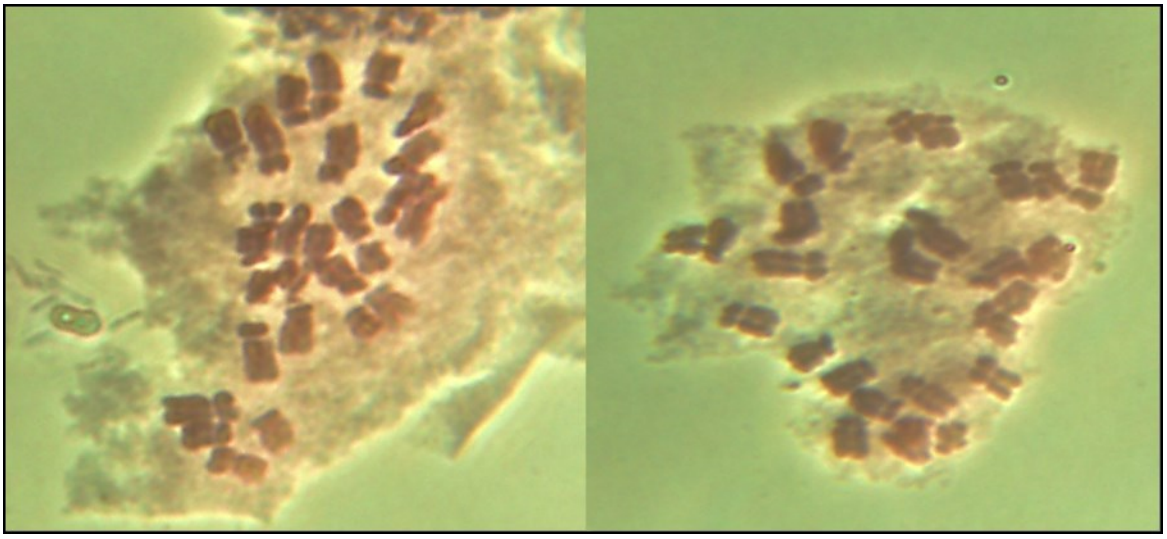

Figure 1.16 Mitotic chromosome counts of BW 160/196 showing 28 chromosomes in early metaphase cells (tetraploid; corresponds to Fig. 1.8 G and H).

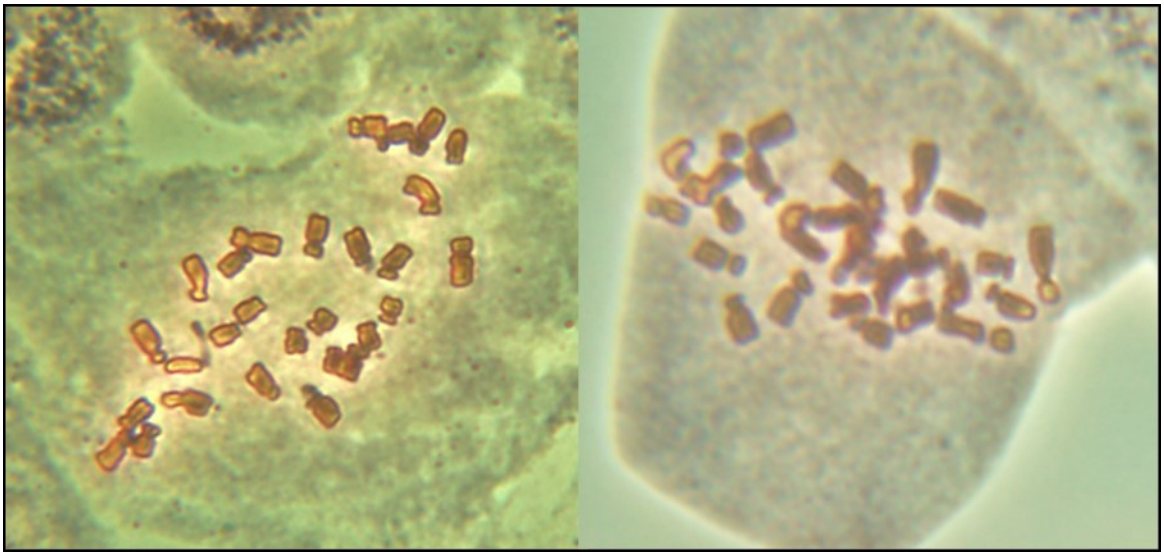

Figure 1.17 Mitotic chromosome counts of BW 158/194 showing 28 chromosomes in early metaphase cells (tetraploid; corresponds to Fig. 1.8 I and J).

Flow Cytometry and Interpretation of Ploidy

Genome size data for P. nana

Flow cytometry was conducted on fresh, frozen or silica gel dried samples from all 76 populations with a range of 1 to 25 samples run per population (usually 5 runs per population). The picogram values from each population were averaged and data are presented in Table 1.6. Samples had a coefficient of variation that ranged from 1.35-4.99% and 10,020-18,268 nuclei were scored with most samples having around 10,100 scored nuclei.

Interpretation of Ploidy Levels

Chromosome counts were used to link to picogram values gathered from flow cytometry in order to estimate picogram ranges for each ploidy level. Diploid counts were made for populations with average picogram values of 6.5, 6.73, 11.12 and 7.38 (pop.40: BW112, pop. 42: BW115/171, pop. 70: BW148 and pop. 43: BW172/204 respectively; see Fig. 1.7 (A-D), 1.9-

1.12). Tetraploid counts were made for populations with average picogram values of 18.74, 14.54, 14.55, 14.88 and 13.82 (pop. 67: BW 144, pop. 30: BW100/162, pop. 8: BW158/194, pop. 36: BW160/196, and pop. 48: AP3822 respectively; see Fig. 1.8 (C-F), 1.13-1.17). Chromosome counts for hexaploid populations were not obtained during this study although a value of 21.29pg can be connected to a hexaploid population due to a chromosome count from Eater's Masters Thesis (1967) that was near the population 46 (MHM3985/BW141/200) collection site.

The average picogram values for populations determined to be diploid, tetraploid or hexaploid as well as the ranges are shown in Table 1.7 (including 34 diploid, 30 tetraploid, 5 hexaploid and 7 unknown populations). Several populations are excluded from the first "average" due to atypical picogram values and neither of the averages for each ploidy level includes the "unknown polyploid" populations.

Table 1.7 Average picogram values and ranges corresponding to each inferred ploidy level. Chromosome counts were made for population 70 (BW148; diploid) and population 67 (BW144; tetraploid) but picogram values were elevated for these populations (as shown in parentheses for the range and are not included in the first average). All other populations labeled with an "A" in the "Inferred ploidy level" column in Table 1.6 are excluded and are addressed in the Discussion, and all populations listed as "unknown polyploid" are excluded from both averages.

Ploidy Level	Number of populations	Average picogram value	Average including all values[1]	Range
Diploid	34	7.559	7.669	6.31-9.79 (11.12)
Tetraploid	30	14.482	14.688	12.08-16.33 (18.73)
Hexaploid	5	21.346	20.958	19.40-21.95

[1]This average includes all values that are considered atypical ("A"; see Table 1.6) but excludes populations labeled as "Unknown Polyploid".

Diploids were found to have the widest range in picogram values with the highest value being 1.55 times larger than the lowest. The range in picogram values decreased with increasing ploidy level. The highest tetraploid value was 1.35 times larger than the lowest and the highest hexaploid value was 1.13 times larger than the lowest value.

Comparison of different Internal Standards

The picogram values of samples run with both CEN and *S. bicolor* were compared for six samples per ploidy level using population 13 (BW199; diploid), population 36 (BW196; tetraploid) and population 49 (BW202; hexaploid). The average difference between all samples was 0.7pg, with diploids having the lowest average difference at 0.5pg, tetraploids with an average difference of 0.74pg, and hexaploids with the highest average difference at 0.87pg (see Tables 1.8-1.9). To compare the samples run with CEN versus the same samples run with *S. bicolor*, a paired t-test was used. Only small changes in the picogram value of the sample was observed, which in no case changed our ploidy level estimation (see Table 1.8) and the paired t-test showed that the differences were not significant (p=0.787). We can confidently say that CEN is giving us an accurate value for our samples in order for us to estimate ploidy level for the remaining populations.

Table 1.8 Picogram values for individuals run with CEN and *S. bicolor* as standards.

Sample	Ploidy Level	DNA content (pg)	Difference in picogram value
BW199C CEN	2x	7.73194	-0.185405
BW199C SOR		7.54653	
BW199D CEN		8.23323	-0.627661
BW199D SOR		7.60557	
BW199H CEN		7.37309	0.465011
BW199H SOR		7.8381	
BW199K CEN		7.64027	1.120718
BW199K SOR		8.76099	
BW199J CEN		7.56564	0.364002
BW199J SOR		7.92964	
BW199Y CEN		8.02737	0.212097
BW199Y SOR		8.23946	
BW196O CEN	4x	16.0976	-1.018697

BW196O SOR		15.0789	
BW196P CEN		15.563	-0.716594
BW196P SOR		14.8464	
BW196A CEN		15.7711	-0.698712
BW196A SOR		15.0724	
BW196E CEN		14.9041	-1.884139
BW196E SOR		13.02	
BW196L CEN		15.427	-0.012845
BW196L SOR		15.4141	
BW196Q CEN		14.6426	-0.122408
BW196Q SOR		14.5202	
BW202A CEN	6x	21.7021	0.246018
BW202A SOR		21.9481	
BW202AA CEN		22.3876	1.65253
BW202AA SOR		24.0402	
BW202G CEN		21.8792	0.784138
BW202G SOR		22.6633	
BW202N CEN		21.8554	1.212606
BW202N SOR		23.068	
BW202J CEN		21.4196	0.7985
BW202J SOR		22.2181	
BW202M CEN		21.9426	0.513957
BW202M SOR		22.4565	

Table 1.9 The average difference in picogram values between samples run with CEN and samples run with *S. bicolor* as standards for each ploidy level.

Population	Ploidy Level	Average Difference in picogram value
BW199	2x	0.22
BW196	4x	0.74
BW202	6x	0.87

Geographical Patterns of Cytotypic Variation

Phlox nana exhibits cytotypic variation across its range (see Fig 1.18). In New Mexico diploid populations are widespread, tetraploid populations are concentrated in the southern half of the state, and there is a small congregation of hexaploid populations in the southwest but we do not see a clear pattern of distribution of ploidy levels. Populations of differing ploidy levels in New Mexico seem to be well isolated from each other with the exception of a small area near the Black Range.

Populations in west Texas do not show a defined pattern of distribution (see Fig 1.18-1.20) as populations of diploids, tetraploids and hexaploids are widespread and occur within short ranges of each other (some within a mile of each other; see Fig 1.19-1.20). Some populations show a higher picogram value compared to what we would expect for that population (given a chromosome count) or have a picogram value that falls between the averages of two ploidy levels.

There was only one ploidy level detected in most of the populations sampled, although there are two populations for which mixed ploidy levels are inferred based on current sampling and are discussed below.

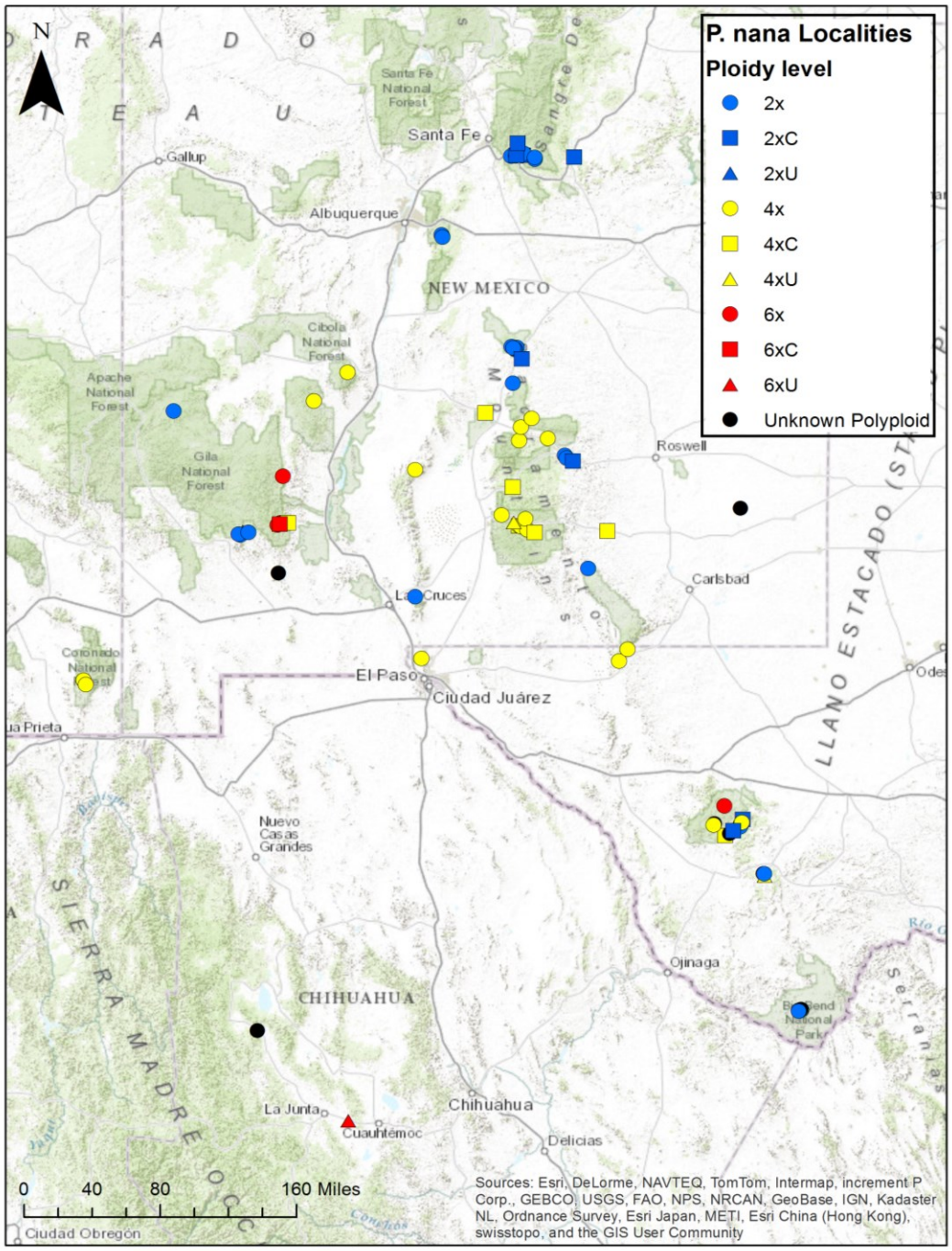

Figure 1.18 The distribution of 83 *P. nana* populations (including 7 populations from Eater [1967], in addition to the 76 populations in the present study) in Arizona, New Mexico, Texas, and Chihuahua, Mexico.
Blue markers are diploid populations, yellow markers are tetraploid populations, red markers are hexaploid populations and black markers are uncertain. Squares represent chromosome count of the corresponding ploidy level and triangles represent unusual populations (see Table 1.6).

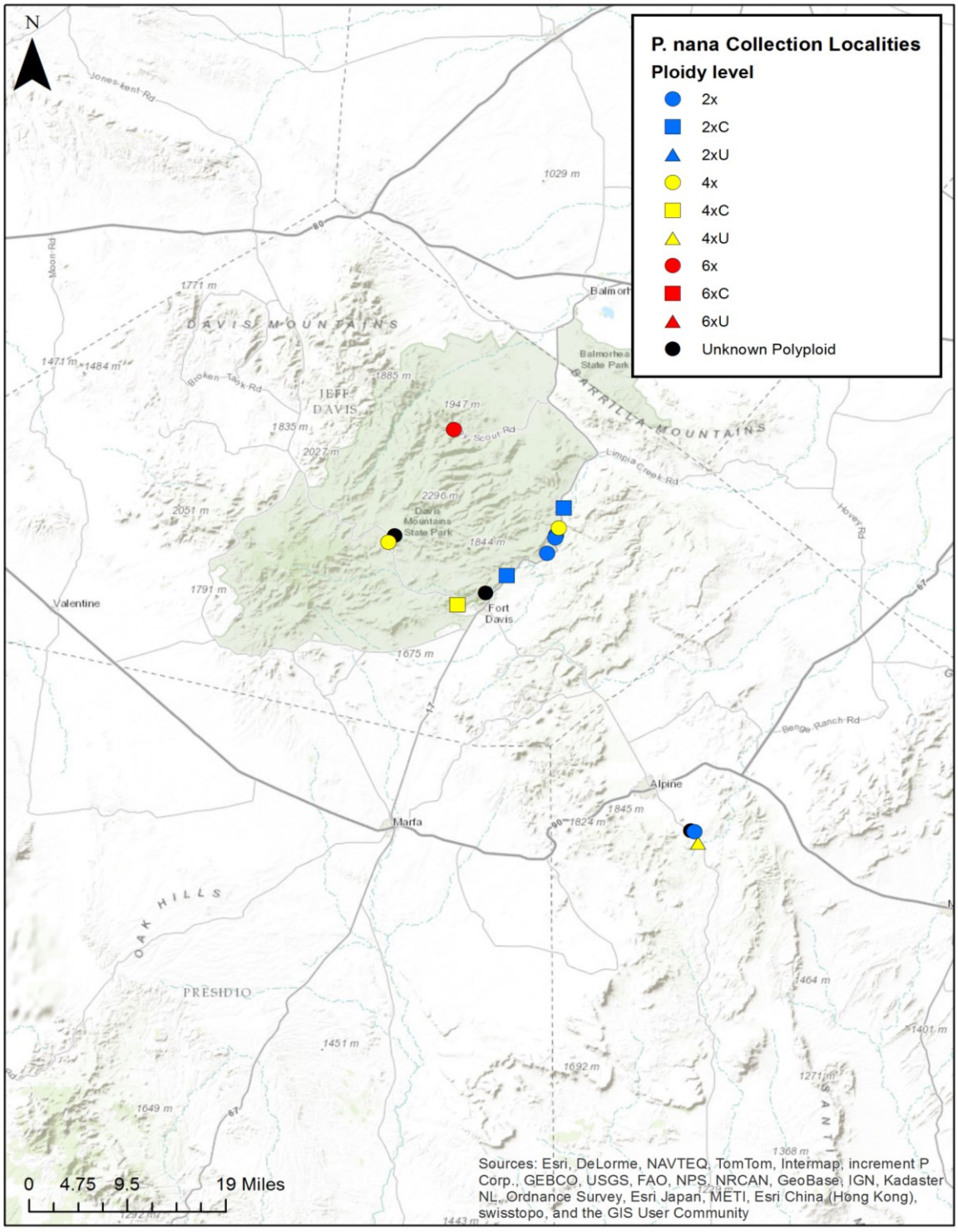

Figure 1.19 Distribution of *P. nana* populations in the Davis Mountains and Alpine, Texas. Blue markers are diploid populations, yellow markers are tetraploid populations, red markers are hexaploid, and black markers are uncertain. Squares represent chromosome count of the corresponding ploidy level and triangles represent unusual populations.

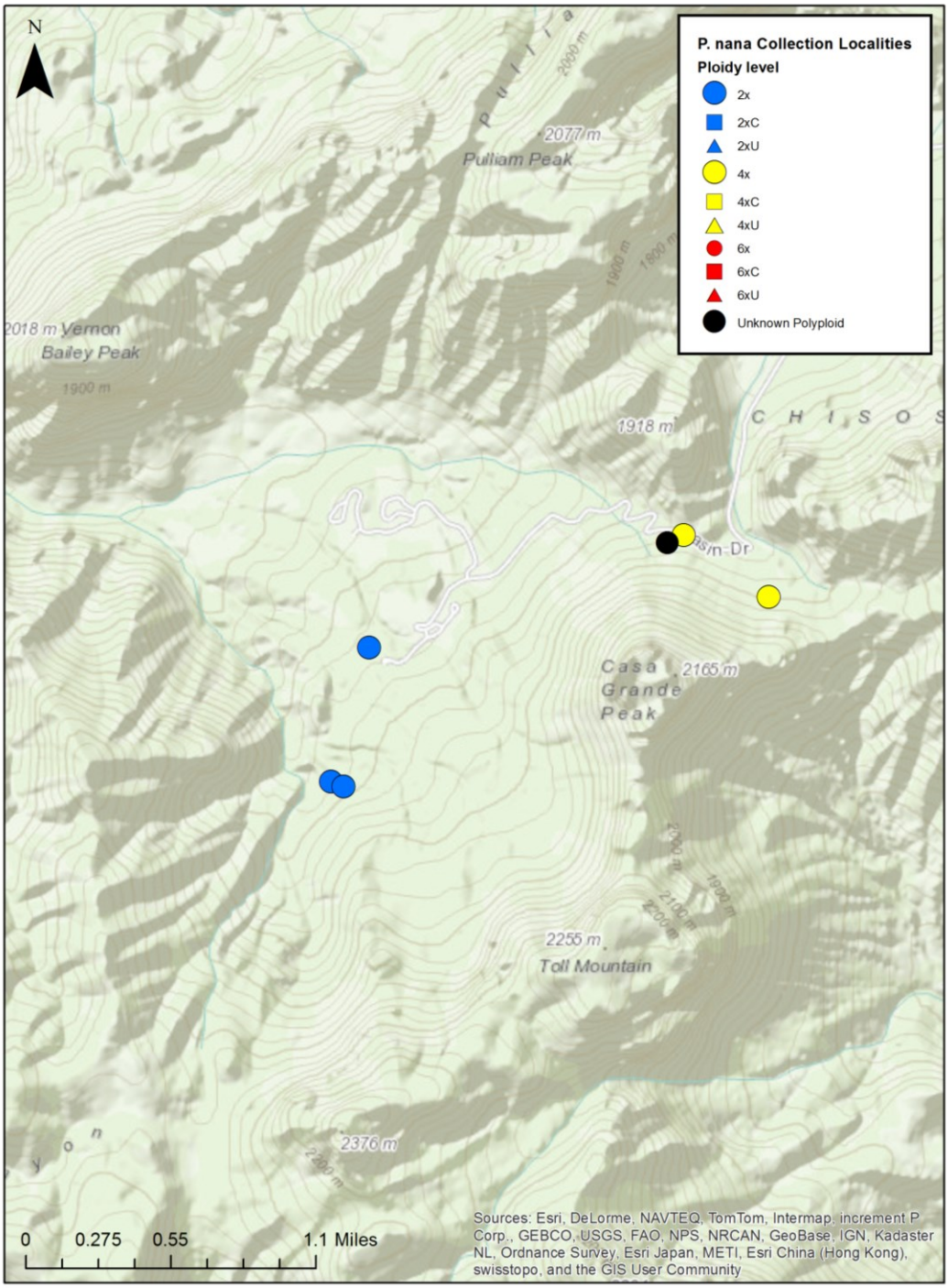

Figure 1.20 Distribution of sampled populations in the Chisos Mountains, Big Bend National Park, showing the close proximity of diploid and tetraploid populations. Blue markers are diploid populations, yellow markers are tetraploid populations, and black markers are uncertain.

The Davis Mountains, Texas

In west Texas, in the Davis Mountains, population 67 (BW 144) had an average picogram value of 18.74pg which may be considered a value between a tetraploid (range: 12.08-16.33pg) and a hexaploid (range: 19.40-21.95pg). This population is thought to be tetraploid as evidenced by a chromosome count (see Figs. 1.8 A and B, 1.13, and 1.19) but we are unable to directly link this chromosome count to a specific flow cytometry run done on the same plant. This population also exhibits a large range of picogram values (16.88-24.17pg) so this hints to the possibility of being a mixed ploidy population, although, we cannot be definitive without more chromosome counts. We can confidently say that there is at least one tetraploid plant in this population but we are unable to confidently assign a ploidy level to the entire population. For simplicity this population is marked as tetraploid on the map due to the tetraploid chromosome count.

Both population 65 (BW 143) and population 68 (BW 151/KW 225) also had elevated average picogram values (20.27pg and 23.02pg [average of 22.33pg including two low values of 16.41 and 17.07pg] respectively) but chromosome counts for these populations were not obtained. Both of these populations also had one (pop. 65) or two (pop. 68) picogram values just above the range for a tetraploid (16.89pg for pop. 65 and 16.41pg and 17.07pg for pop. 68) The majority of these picogram values are in the range of normal hexaploid populations but combined with the unusual low values and without a chromosome count we are unable to confidently assign a ploidy level to these populations.

In the same area, population 70 (BW 148) also had an elevated average picogram value of 11.12pg. A chromosome count for this population lets us infer that the population is diploid which aligns with flow cytometry data showing that the other populations along Wild Rose Pass are also diploid. A chromosome count for population 70 was obtained showing 14 clear

chromosomes (Figs. 1.7 E and F, 1.11) but in several of the cells observed there was a fragment of a chromosome. The fragment of the chromosome was not found in all cells and does not seem to be large enough to increase the average picogram value from 7.49pg (overall average of diploid populations) to 11.12pg (average for pop. 70).

We can be confident about the diploid populations occurring along Wild Rose Pass in the Davis Mountains in Texas, but for most polyploid populations we are unable to confidently assign them as tetraploid or hexaploid due to atypical picogram values. We can be confident about two tetraploid populations (Pops. 66 and 73) and a single hexaploid population (pop. 74) due to the picogram values being consistent and their average values being within the range for tetraploids or hexaploids.

Alpine, Texas

Population 53 (BW152/191) south of Alpine, Texas had an average picogram value (17.195pg) between what would be considered a tetraploid and a hexaploid and this population is considered an unknown polyploid. Population 54 (BW 153/CF 337) had a picogram value (16.39pg) around the range for normal tetraploid populations (12.08-16.33pg) and may be considered tetraploid but is currently marked as atypical (see Fig. 1.19). Unfortunately chromosome counts for these populations were not obtained in order to observe the exact ploidy level or any unusual factors that might influence the picogram value. We are certain that population 53 is polyploid but due to the unusual picogram values that tend to surround Texas polyploid populations we are not assigning a specific ploidy level to this population.

The Chisos Mountains, Texas

Diploid and tetraploid populations occur within a mile of each other in Big Bend National Park in the Chisos Mountains. An unknown polyploid population (pop. 58; KW 226) with an

average picogram value of 18.10 and a wide range (16.13-22.67pg; see Table 1.6 and Fig. 1.20) was found in close proximity to tetraploid populations. We cannot be certain of the ploidy level of this population. Populations in the Chisos Mountains warrant further exploration.

The Black Range and surrounding area, New Mexico

Population 13 (BW 164/199) near Georgetown, NM in the Gila National Forest (see Fig. 1.21) was a population selected for intensive flow cytometry. A total of 23 samples were run for this population and the average picogram value was 7.74pg of DNA (diploid). Three samples from this population had an elevated picogram value (BW199Q, BW199N, BW199O; 11.18pg, 11.10pg, and 11.35pg respectively; see Table 1.6) which is out of the normal range for a diploid (see Table 1.7), while all other samples were consistently in the range of a normal diploid. These elevated values may be triploids in this population. The average picogram value for this population excluding these atypical values is 7.16pg ±0.39. Further exploration is necessary in order to discover the source of these elevated values.

South of the Black Range, near Cookes Range, population 29 (AP 3843) had picogram values above the normal for tetraploid populations (average of 16.95pg; see Table 1.6). This is currently the only sampled population from the area and is marked as an "unknown polyploid".

The Sacramento Mountains, Guadalupe Mountains and surrounding area, New Mexico

In the Sacramento Mountains, population 35 (BW 105) had an average picogram value (16.33pg; see Table 1.6) at the highest range for tetraploids. This population is still considered tetraploid due to its proximity to other tetraploid populations and because there have not been other ploidy levels discovered in this part of the Lincoln National Forest. The population warrants further exploration in order to discover the cause of the elevated picogram value compared to the surrounding populations. The remaining populations in and around the

Sacramento Mountains and the Guadalupe Mountains seem to be within the normal range for diploid and tetraploid populations.

Southeastern New Mexico

East of the Lincoln National Forest, population 10 (SDF 50814-1) consists of picogram values ranging from 15.76 - 23.60 picograms (see Table 1.6). This population may be a mixed ploidy population with tetraploid and hexaploid plants. This population warrants further sampling to be sure of its mixed ploidy status. This population is marked as an "unknown polyploid" on the map (see Fig 1.18).

Chihuahua, Mexico

Population 76 (CF 245) has an average picogram value of 18.83pg which is between the average for a tetraploid and a hexaploid. We consider this population to be an unknown polyploid. Population 75 (CF 244) had an average picogram value of 19.41pg which we would normally call hexaploid. We have only run two flow cytometry samples for this population and infer it to be hexaploid but further samples may make us more confident in this inference.

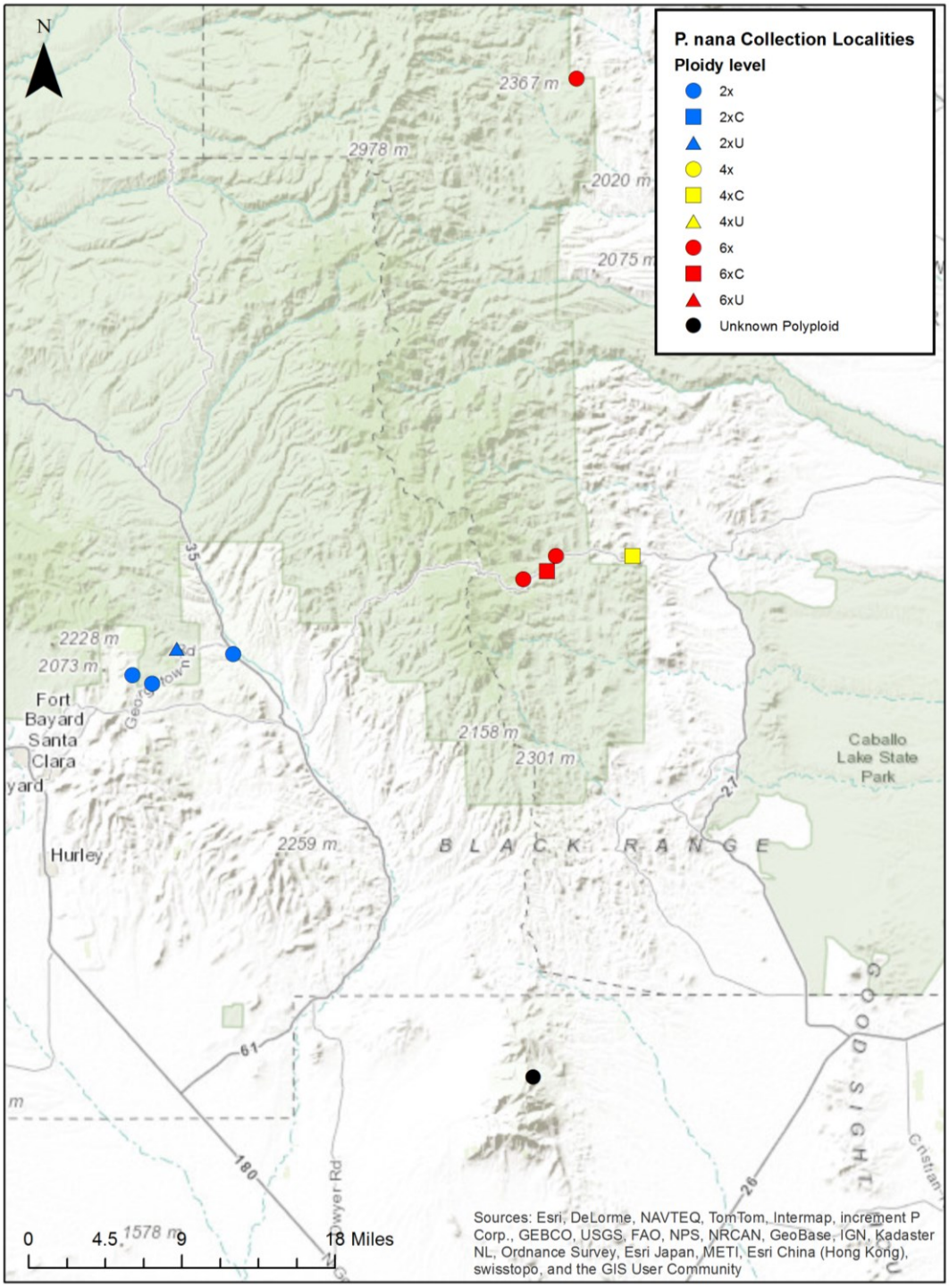

Figure 1.21 Distribution of sampled populations in the Gila National Forest showing the close proximity of tetraploid and hexaploid populations.
Blue markers are diploid populations, yellow markers are tetraploid populations, red markers are hexaploid populations and black markers are uncertain. Squares represent chromosome count of the corresponding ploidy level and triangles represent unusual populations.

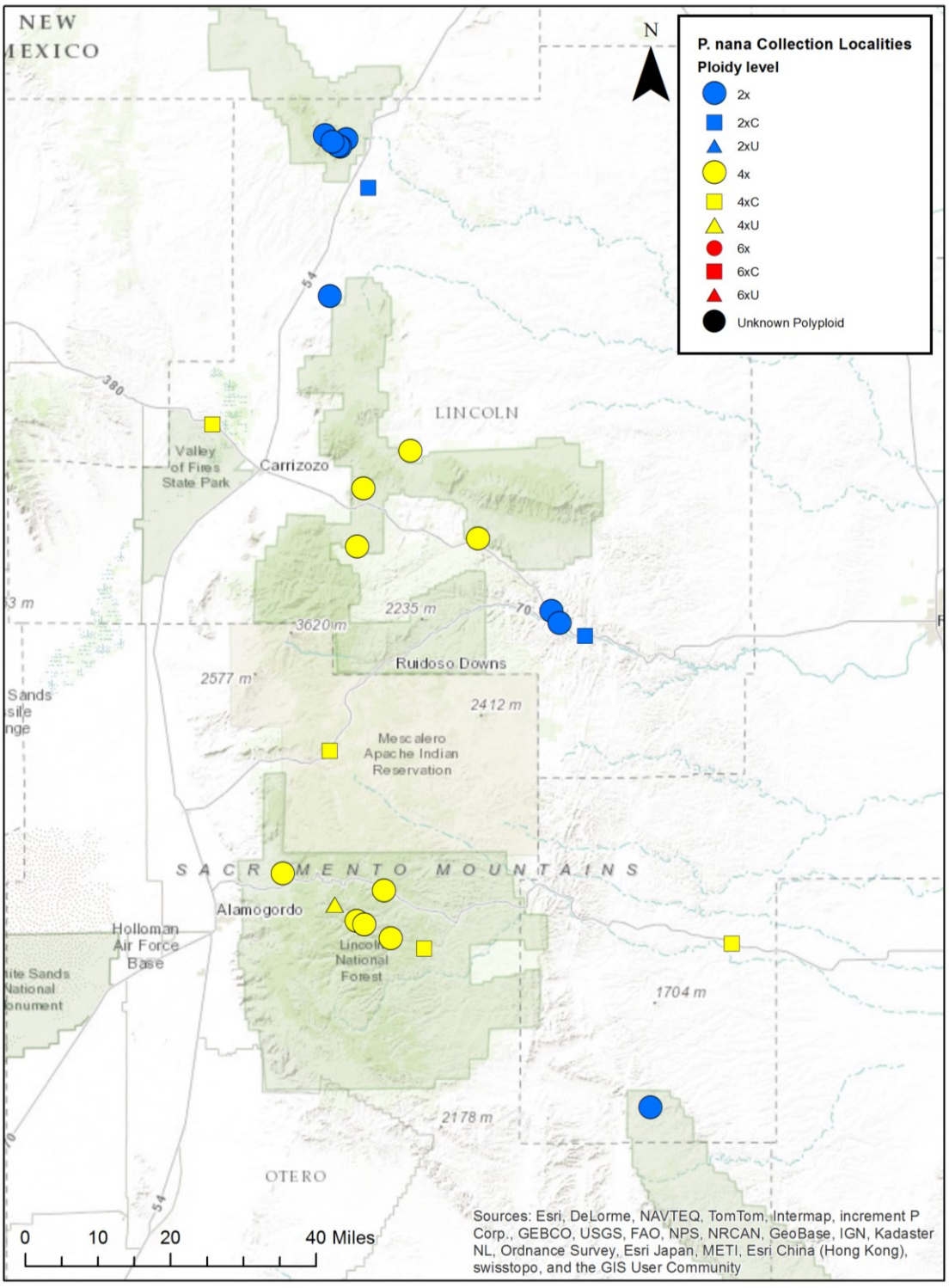

Figure 1.22 Distribution of sampled populations throughout the Lincoln National Forest. Blue markers are diploid populations, yellow markers are tetraploid populations, and black markers are uncertain. Squares represent chromosome count of the corresponding ploidy level and triangles represent unusual populations.

Discussion

This study highlights the presence of cytotypic variation in *P. nana* (diploid, tetraploid, and hexaploid populations) with most populations being diploid or tetraploid. There are only a few inferred hexaploid populations concentrated near the Gila National Forest in New Mexico where the only previously known population was located (Eater 1967). Other hexaploid populations are indicated in the Davis Mountains and in Chihuahua, Mexico.

The average genome sizes for diploids, tetraploids and hexaploids in *P. nana* (see Table 1.7) were found to be several picograms smaller than those reported for other species in the genus (including *P. amabilis*, *P. woodhousei* and *P. pilosa*; see Fehlberg & Ferguson 2012a; Worcester et al. 2012). The range of each ploidy level in *P. nana* (about a 3 pg range for each ploidy level) was found to be larger than those reported for *P. amabilis* and *P. woodhousei* (1-3pg; Fehlberg & Ferguson 2012a), but smaller than ranges reported for *P. pilosa* (4-6pg; Worcester et al. 2012), although these differences may be due to differences in sampling size.

There is no clear correlation between the morphological characters that Wherry described (as dividing *P. nana* into three separate species) and the cytotypic variation in the species. When we look at calyx vestiture, for example, trichomes in diploid and tetraploid populations are found to be either glandular or eglandular (see Table 1.6). Diploid populations tend to be glandular and hexaploid populations (those that have recorded calyx vestiture in Table 1.6) have exclusively glandular trichomes on the calyx. Most populations have glandular trichomes on the calyx and most populations with eglandular trichomes on the calyx are concentrated in the Guadalupe Mountains and around the Sacramento Mountains in southern New Mexico. It is possible that morphological characters such as the calyx vestiture are linked to specific geographic localities

more so than they are to ploidy level but ongoing research on morphological correlates of polyploidy may be able to shed some light on the morphological variation in this species.

Geographical Patterns of Cytotypic Variation

The Davis Mountains, Texas

We are only able to say that there are diploid and polyploid populations occurring in the Davis Mountains area. We are unable to confidently assign specific ploidy levels to most polyploid populations in this region (with the exception of 3 populations) due to picogram values that seem to be out of the range for normal polyploid populations when linking to chromosome counts. It is possible that some populations may be of mixed ploidy levels or that all the populations in the Davis Mountains and surrounding areas have unusually high picogram values compared to Big Bend National Park and populations occurring in New Mexico and Arizona. This area is very unique and warrants more exploration.

A tetraploid chromosome count for population 67 (BW144) was obtained for a single plant but we are unable to link this chromosome count to a flow cytometry run on the same plant due to the process of bud collection. We can confidently say that there is at least one tetraploid plant in this population. All of the flow cytometry runs for this population, as well as the average, are consistent, showing an increased picogram value (see Table 1.6) from that of the overall average of tetraploids (see Table 1.7). We believe this population may be all tetraploid due to the consistency in the flow cytometry but further investigation is needed to be confident. All other polyploid populations in the Davis Mountains area, with the exception of population 66 (CF 237), population 73 (KW 223) and population 74 (KW 224), have also been regarded as "unknown polyploid" at this time.

A chromosome count for population 70 (BW148; diploid) was obtained showing a fragment of a chromosome. This was present in several cells but may not explain this populations increased picogram value due to the fragments small size and its random occurrence in cells. Chromosomal fragments have been recorded in *Phlox* (Smith & Levin 1967; Worcester et al. 2012) so this discovery is not surprising. Without evidence from chromosome counts from other populations in the area we cannot be sure if other populations contain fragments. Other diploid populations in the Davis Mountains area, including population 69 (BW145), population 71 (BW149), and population 72 (BW150), also have higher picogram values although they are not as high as population 70 (see Table 1.6). We are confident that these are all diploid populations.

Alpine, Texas

Material from south of Alpine, TX (population 53; BW152/191 and population 54; BW153/CF337) show unusual picogram values as well. Both populations will need further exploration in order to pinpoint the reason for their increased picogram values but we suspect that these populations are tetraploid because of their consistency in picogram values. Picogram values from population 55 (CF335) are also slightly elevated in regards to being diploid. We are unable to directly link these populations to chromosome counts and polyploids are considered unknown.

The Chisos Mountains, Texas

Populations occurring in Big Bend National Park occur within a short distance from each other. Population 58 (KW 226), near to a previously sampled tetraploid population, was found to have elevated picogram values. The cause of the elevated picogram values is unknown.

The Black Range and surrounding area, New Mexico

The Kingston area near the Gila National Forest is the only area in New Mexico where populations of different ploidy levels occur within several miles of each other. This area is also the only area of a known hexaploid chromosome count (see Eater 1967). Future efforts in the area may consist of collecting buds or bagging plants to collect seed in order to get an accurate hexaploid chromosome count for the species.

The Sacramento Mountains, Guadalupe Mountains, and surrounding area, New Mexico

The Sacramento Mountains consist mainly of tetraploid populations. A few diploid populations were recorded east of the forest in and around low lying areas along roads, as well as in the northern portion of the Guadalupe Mountains. There were few issues in the inference of ploidy levels for these populations due to their consistent diploid and tetraploid values. A chromosome count from the area also confirms tetraploid populations from the Sacramento Mountains area (see Fig. 1.6 C and D, 1.12).

Southeastern New Mexico

A population from southeastern New Mexico (east of the Lincoln National Forest; see Fig. 1.18), is the only population sampled (collected by collaborators in 2014) that far east in New Mexico. Currently it is one of the only populations that may be of mixed ploidy level (tetraploids and hexaploids) inferred from four tetraploid values from separate plants and a single hexaploid value. Further, intensive sampling and chromosome counts from this population may be needed in order to be sure if there are truly tetraploid and hexaploid plants present.

Chihuahua, Mexico

Phlox nana material from Mexico is scarce and in the case of population 76 (CF245) picogram values for two probable hexaploids and two values that are slightly elevated from a normal tetraploid are recorded. It is possible that these are all tetraploid with a large amount of

DNA (similar to areas in Texas) but due to the limited amount of material we cannot be certain. There is a large, seemingly unsampled, area between specimens collected in Texas and New Mexico and those collected in Mexico although the majority of this terrain is low-land desert where conditions are not favorable for the growth of *P. nana*. Only two populations are sampled from Mexico and there are only a few recorded from the area. Future research could entail exploring the mountains west of Chihuahua, Mexico.

Other Areas of Cytotypic Variation

Out of 76 populations, population 13 (BW 164/199) and population 10 (SDF 50814-1) were the only populations that we believe may harbor two cytotypes. Population 13 had several samples that were recorded at 3.5 picograms (on average) above the normal for that population and population 10 (SDF 50814-1) had a range from 15.48-23-60pg. There is a possibility that several of the Texas populations may also harbor within population cytotypic variation but limited current sampling, and elevated picogram values for all polyploid populations near the Davis Mountains and Alpine makes it impossible to say this confidently.

All other populations sampled, including those samples from the Santa Fe National Forest and the Cibola National Forest areas, had average picogram values within the range of normal diploids or tetraploids.

Figure 1.3 shows plants from the Chisos Mountains (A and B) and the Davis Mountains (B and C) where populations grow within only a few miles from each other. Pictures A and C represent diploid populations from those areas and pictures B and D represent tetraploid populations growing in the same areas (corresponding to A and C respectively). In this species, morphology seems to vary by geographic area (or possibly ecology) and not by ploidy level, and ongoing investigations (including some study with collaborators) may shed some light on this.

The morphological similarity of populations in close geographical proximity (see Fig. 1.3) suggests independent origins of polyploidy in different geographical areas, and this should be further investigated. A common garden or reciprocal transplant experiment may be useful in the future.

Populations with atypical picogram values have mostly been inferred to be the nearest ploidy level below their picogram value. Several chromosome counts found in the Davis Mountains point to picogram values being elevated from normal diploids and tetraploids in relation to the number of chromosomes found. In a study done on the *Claytonia perfoliata* complex (McIntyre 2012), it was found that some hexaploid populations had a 30% larger genome and the colder temperatures (or warmer in the case of tetraploid *C. parviflora*) during the winter could increase the genome size (McIntyre 2012). It is possible that this increase in the genome size for some populations of *P. nana* is related to ecological factors (such a temperature). It is alternatively possible for transposable elements to be responsible for increases in genome size as polyploidy can be accompanied by the transposition of transposable elements which could ultimately lead to changes in genome size (Oliver et al 2013). Ultimately, populations in west Texas seem to have gone down distinct evolutionary pathways compared to populations in New Mexico.

Overall, we now have a thorough understand of ploidy levels and their ranges in *P. nana*. Previously, only one population was recorded to be hexaploid, from an obscure chromosome count by Eater (1967). Currently, several other possible hexaploid populations have been located, including areas near to the original documented area, which has been corroborated here with flow cytometry data, as well as localities in west Texas and Mexico that had previously not

been documented. Odd areas needing further study have been located due to their atypical picogram values and the variation within populations had never previously been studied.

A number of recent studies have documented contact zones consisting of populations of mixed ploidy levels (e.g. Mazaneda et al 2011, Keeler et al 1999, Halverson et al 2008, Marhold et al 2010, Mráz et al 2012). In *P. nana*, we have only recorded the possibility of two populations (out of 76) as consisting of mixed ploidy levels. These populations are separated by many miles and several mountain ranges. Further sampling within each currently sampled population as well as the identification of new populations may help locate more mixed populations or may help solidify the idea of *P. nana* populations consisting mostly of a single cytotype.

Conclusions and Ongoing Research

Polyploidy within *P. nana* presents the opportunity to explore cytotypic diversity relative to geography, and to investigate morphological and ecological correlates. This research provides a valuable, focused study of ploidy level variation while extensively exploring cytotypic diversity in *Phlox nana*. Several other species in *Phlox* exhibit cytotypic diversity, including *P. woodhousei* and *P. amabilis* which occur in Arizona. *Phlox nana*, *P. woodhousei* and *P. amabilis* are part of an ongoing study exploring polyploidy and aspects of diversity, including phylogenetics, population genetics, morphological and ecological correlates, with collaborators S. Fehlberg, at the Desert Botanical Garden in Arizona and L. Alan Prather, at Michigan State University in Lansing, MI. This research will help us explore the links between ploidy level and morphology in this species and may also help explore the possibility of multiple origins of polyploidy.

Populations in Texas and several populations from New Mexico will need further study to explore the elevated picogram values occurring in a few areas. Few populations from Mexico

were included in this study due to the inability to travel to this area. Exploring the cytotypic variation within *P. nana* in Mexico may raise some interesting questions.

This extensive study of the cytotypic variation in *P. nana* was a vital contribution for research to continue on *Phlox* polyploid complexes. This study as well as ongoing investigations could hold implications for conservation efforts, as well as a taxonomic revision of *P. nana* and other polyploid complexes in light of morphological and ploidy level data.

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Chapter 2 - The utility of a low copy nuclear gene region (*idh*B) in the investigation of relationships in the genus *Phlox*

Introduction

The discovery of new gene regions for use in phylogenetics has become very important to plant systematists over the years. Phylogeny development relies on the use of multiple gene regions in cases where current regions don't resolve relationships or when more regions are needed because there are disagreements between gene trees. Finding informative, variable gene regions becomes valuable to determining species relationships as some approaches enable researchers to infer species trees from multiple gene trees.

The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA is a commonly used gene region in plants for phylogeny development (Baldwin et al. 1995, Alvarez & Wendel 2003). The ITS region is a repetitive, high copy region that is thought to undergo concerted evolution. It is an easy region to amplify (compared to low copy nuclear regions) with universal primers because it is found in thousands of copies (Alvarez & Wendel 2003) and can be directly sequenced from DNA samples of individuals (Baldwin et al. 1995). Variation within individuals due to incomplete homogenization of genes (Alvarez & Wendel 2003) may be ignored through direct sequencing so it is important to consider ITS data in conjunction with data from other regions in order to fully understand relationships.

Chloroplast DNA (cpDNA) regions are also widely used in phylogeny development, in part because they are easy to work with. Chloroplast regions are variable and can be directly sequenced although the chloroplast is typically maternally inherited in plants and some regions do not have a lot of variation for the examination of low taxonomic levels. Chloroplast regions

can also be short sequences so should be used in conjunction with other regions to produce a more resolved phylogeny (Judd et al. 2008).

Low copy DNA regions can be an important resource for the development of phylogenies in plants. These regions can be more variable than high copy nuclear genes and chloroplast genes and do not undergo concerted evolution. Although these regions are very useful, they have their challenges. At times it may be hard to find regions with enough variation and primers usually have to be designed or optimized for their use in each genus of interest. Low copy regions can be hard to amplify and PCR products must be cloned in order to capture all the alleles present in each individual. Despite the challenges, these regions can be highly variable and informative and can play an important role in determining species relationships.

The genus *Phlox* L. (Polemoniaceae) consists of ca. 60 annual and perennial species (Wherry 1955). The phylogeny of *Phlox* deserves further investigation because of the role that polyploidy and hybridization play within the genus which may cause issues in determining relationships (see Ferguson & Jansen 2002). The evolutionary relationships within the genus have been previously analyzed with the inclusion of phylogenies from nuclear (ITS) and chloroplast DNA (Ferguson et al. 1999, Ferguson & Jansen 2002, Ferguson et al. 2008; and unpublished). Although previous phylogenies have shown complicated relationships due to the role of hybridization, polyploidy, and possible incomplete lineage sorting (Ferguson & Jansen 2002), the discovery and inclusion of more variable and informative regions may shed more light on these relationships.

The low-copy nuclear region *idh*B (in the NADP-dependent isocitrate dehydrogenase gene family), is a highly variable region that might result in a clearer understanding of the relationships in *Phlox*. The region was developed by the L. Johnson Lab (Weese & Johnson

2005, Johnson & Johnson 2006) and was found useful for determining relationships between other members in the Polemoniaceae. The *idh*B region (between exons C and J) includes 8 exons and 7 introns. In the discovery of the usefulness of the region Weese and Johnson (2005) found that the exons were highly conserved and were useful for understanding deeper relationships and the introns were highly variable in as much as they provided insights into relationships among closely related species. Exons and introns used in conjunction may provide a more resolved phylogeny for the genus *Phlox*.

Members of the Ferguson Lab have gathered evidence that *idh*B is a promising region for phylogeny development in *Phlox*. A preliminary *idh*B dataset was developed in the Ferguson Lab (unpublished ; through the work of researcher S. Fehlberg and others) and is used as a basis for the work presented. The goal for this project was to advance the work on *idh*B in the genus *Phlox* by increasing sequencing for *idh*B, rigorously analyzing the data and comparing findings from phylogenies to previous work on ITS and cpDNA. In the future we aim to use this region along with additional regions to provide phylogenetic context for research on *P. nana* (see Chapter 1) and other southwestern polyploid complexes in the genus.

Methods

Extraction and Sampling

Genomic DNA was extracted from fresh, frozen or silica dried material using the small-scale CTAB extraction protocol of Loockerman and Jansen (1996), modified from Doyle and Doyle (1987). The genus *Microsteris* was also sampled, which is closely related to *Phlox* and is thought to be sister to *Phlox* (Porter & Johnson 2000, Ferguson et al. 1999, Ferguson & Jansen 2002). *Leptosiphon* was chosen as an outgroup (Porter & Johnson 2000) as it is closely related to

the genus *Phlox* (Bell et al. 1999, Bell & Patterson 2000). A total of 53 samples were included in the sampling and analyses (see Table 2.1).

Table 2.1 Samples included in the *idh*B analyses with corresponding voucher information and the number of clones included in the complete dataset.

Sample Name	Voucher	DNA[1]	Locality	Number of clones	Sequence data development
P. adsurgens	M. Sidells 263	1276	Jackson Co., OR	3	S. Fehlberg/lab
P. amabilis	SDF 51707-1	1921	Mohave Co., AZ	3	S. Fehlberg/lab
P. amabilis	SDF 51507-1	1885		3	S. Fehlberg/lab
P. amoena GA2	CJF 286	413	Charlton Co., GA	3	S. Fehlberg/lab
P. austromontana ssp. *austromontana* UT2	CJF 623	1024	Washington Co., UT	3	BW
P. austromontana ssp. *austromontana* UT3	SCS 10	1110	Washington Co., UT	4	S. Fehlberg/lab
P. bifida	G. Mayfield 9 April 1995	2591	Rutherford Co., TN	2	BW
P. caryophylla	CJF 653	1108	Archuleta Co., CO	3	S. Fehlberg/lab
P. cespitosa OR2	M. Sidells 170	1239	Harney Co., OR	1	S. Fehlberg/lab
P. cluteana	CJF 651	1106	Apache Co., AZ	3	S. Fehlberg/lab
P. cluteana AZ2 [2]	SDF 51008-2	2090	Apache Co., AZ	3	S. Fehlberg/lab
P. cuspidata	CJF 75	2583	Refugio Co., TX	2	BW
P. diffusa ssp. *longistylis* OR1	M. Sidells 156	1176	Deschutes Co., OR	1	S. Fehlberg/lab
P. divaricata NY	D. Goldman 912	2575	Genesee Co., NY	4	BW
P. dolichantha	CJF 641	1026	San Bernardino Co., CA	4	S. Fehlberg/lab
P. drummondii ssp. *mcallisteri*	CJF 86	2565	Lampasas Co., TX	4	S. Fehlberg/lab
P. drummondii ssp. *johnstonii*	CJF 348	2555	Kent Co., TX	1	BW
P. glaberrima ssp. *triflora*	R. Mayfield 18 July 1995	2577	Avery Co., TN	4	BW
P. hoodii ssp. *canescens* ID1	CJF 587	2542	Nez Perce Co., ID	5	BW
P. hoodii ssp. *canescens* ID2	CJF 588	1008	Custer Co., ID	3	S. Fehlberg/lab
P. hoodii ssp. *hoodii* WY1	SCS 1	1113	Fremont Co., WY	3	S. Fehlberg/lab
P. hoodii ssp. *hoodii* WY2	D. Ferguson 2	400	Natrona Co., WY	3	S. Fehlberg/lab
P. idahonis	CJF 985	985, 2541	Clearwater Co., ID	3, 4	S. Fehlberg/lab
P. longifolia ssp. *longifolia* AZ1	SDF 51008-1	2115	Apache Co., AZ	3	S. Fehlberg/lab
P. longifolia ssp. *longifolia* AZ2	SDF 50208-2	2166	Yavapai Co., AZ	3	S. Fehlberg/lab
P. longifolia ssp. *longifolia* AZ3	SDF 50808-1	2323	Mojave Co., AZ	3	S. Fehlberg/lab
P. longifolia ssp. *longifolia* CA2	CJF 630	1033	Mono Co., CA	3	S. Fehlberg/lab

P. longifolia ssp. *longifolia* ID1	CJF 603	1022	Clark Co., ID	4	S. Fehlberg/lab
P. longifolia ssp. *longifolia* NV	SCS 18	1120	Lander Co., NV	2	S. Fehlberg/lab
P. longifolia ssp. *longifolia* UT2	CJF 611	1032	Summit Co., UT	3	S. Fehlberg/lab
P. longifolia ssp. *longifolia* WY2	CJF 215	2552	Sublette Co., WY	2	BW
P. longifolia ssp. *superba*	CJF 634	994	Inyo Co., CA	3	S. Fehlberg/lab
P. maculata	CJF 206	691	Worth Co., IA	3	S. Fehlberg/lab
P. missoulensis	M. Sidells 149	1209	Missoula Co., MT	1	S. Fehlberg/lab
P. multiflora CO	CJF 645	1050	Routt Co., CO	3	S. Fehlberg/lab
P. multiflora WY1	S. W. Harper 18	2573	Fremont Co., WY	5	BW
P. multiflora WY2	CJF 573	1011	Sheridan Co., WY	3	S. Fehlberg/lab
P. nana Chi.	CJF 245	369	Mpio. Temosachic, Chihuahua, Mexico	3	S. Fehlberg/lab
P. nana TX	CJF 332	431	Brewster Co., TX	3	S. Fehlberg/lab
P. oklahomensis	CJF 269	418	Woods Co., OK	3	S. Fehlberg/lab
P. opalensis	CJF 610	989	Lincoln Co., WY	3	S. Fehlberg/lab
P. paniculata	CJF 233	693	Madison Co., AR	3	S. Fehlberg/lab
P. pattersonii	CJF 256	672	Mpio. Bustamante, Nuevo Leon, Mexico	3	S. Fehlberg/lab
P. pilosa ssp. *pilosa* TX2	CJF 121	1258	Montgomery Co., TX	3	S. Fehlberg/lab
P. pulchra	CJF 169	695	Bibb Co., AL	3	S. Fehlberg/lab
P. pulvinata ssp. *condensata* CA	CJF 629	993	Mono Co., CA	3	S. Fehlberg/lab
P. pulvinata ssp. *pulvinata* UT	SCS 8	2570	Iron Co., UT	3	BW
P. pulvinata ssp. *pulvinata* MT	CJF 582	984	Carbon Co., MT	3	S. Fehlberg/lab
P. pungens WY2	CJF 609	988	Lincoln Co., WY	4	S. Fehlberg/lab
P. stolonifera	G. Mayfield 26 April 1995	425	Yancey Co., NC	4	S. Fehlberg/lab
P. tenuifolia	M. Mayfield 3606	1348	Pima Co., AZ	1	S. Fehlberg/lab
P. woodhousei AZ1[3]	SDF 51207-3	1837	Coconino Co., AZ	3	S. Fehlberg/lab
Microsteris gracilis CA	A. David 274	2580	San Mateo Co., CA	3	BW
Leptosiphon floribundus	L. A. Prather 1583	388	San Bernardino Co., CA	4	S. Fehlberg/lab

[1]DNA number is an internal tracking number in the Ferguson Lab, and is included here to facilitate ongoing work.
[2]This is the same locality as CJF 651
[3]This is the same locality as CJF 642

Amplification and Cloning

The *idh*B region of the nuclear DNA was amplified using 4µl of DNA, 5µl of each primer (*idh*BJ1R and *idh*BC1F; Johnson & Johnson 2006), 2µl of dNTPs, 1.3µl dH2O, 0.2µl GoTaq (Promega), and 2.5µl $MgCl_2$. The program was run for five minutes at 94°C, one minute at 72°C, (30 sec at 94°C, 30 sec at 52°C, 1 min at 72°C; repeat 29x) eight minutes at 72°C, then the product was refrigerated.

The PCR product was run on a 1.2% agarose gel in order to visualize the bands. Some PCR product was cleaned via spin columns (QIAquick PCR Purification Kit, Qiagen) for future cloning, while other PCR product was used directly in the ligation process without being cleaned. The ligation was done overnight using a pGEM®-T Easy Vector System I kit with 2.5µl Buffer, 0.5µl Vector, 1.5µl PCR product and 0.5µl Ligase.

DH5α competent cells were made following standard protocols (Sambrook & Russell 2001) and stored at -80° until used for cloning. Competent cells were placed on ice for several minutes until thawed and 2µl of the ligation reaction was added. The tubes were incubated on ice for 20 minutes, heat shocked for 45 seconds at 42°C and returned to ice for two minutes. SOC (950µl) was added to each tube and incubated at 37°C for 1.5 hours, 50-100µl was plated on LB plates containing Ampicillin, IPTG, and X-GAL. Plates were incubated for about 12 hours at 37°C then stored at 4°C. About five colonies from each plate were grown separately in 3ml of LB broth containing 3µl of ampicillin overnight.

Once the clones were grown in LB, autoclaved, distilled water (38µl) was combined with 2µl of cells grown in broth and 5µl of the mixture was added to a PCR tube and run at 95°C for ten minutes. The bacteria were then added to a tube with 8.35µl H2O, 5µl 5x Buffer, 2.5µl MgCl2, 2µl dNTPs, 1µl of each primer (M13U, M13R), and 0.15µl GoTaq. The clones were run

for two minutes at 94°C, (45sec at 94°C, 1min at 51°C, 2min at 72°C; repeat 30 times) five minutes at 72C° and then refrigerated before running on a 1.2% agarose gel in order to check for the *idh*B region before plasmid prepping. The remaining bacterial growth in the 3ml tubes was spun down for five minutes at 10,000rpm and the LB was poured out leaving only the bacterial cells to be stored at -20°C for future use and/or used in the plasmid prep process via spin columns (Wizard Plus SV Minipreps DNA Purification System, Promega).

Sequencing

The plasmid prepped product was used in the sequencing reaction with 3.5µl plasmid prepped DNA, 2µl 5x big dye buffer, 1.7µl autoclaved ddH_2O, 0.8µl primer (M13U, M13R), and 2µl BigDye® terminator v3.1 (Applied Biosystems). Tubes were run at 96°C for 15 seconds, 50°C for five seconds, 60°C for four minutes (cycle repeated 24 times), 30°C for one minute and then stored at 4°C until filtered through Sephadex filled spin columns. Samples were then vacuum dried for 30 minutes before sending to Iowa State DNA Facility and run on an Applied Biosystems 3730xl DNA Analyzer. Sequences were edited using Sequencher (Gene Codes Corp.) and sequences were exported in a FASTA format to manually align in Se-Al (Rambaut 1996). Se-Al files were exported as nexus (.nex) files to be used in analysis.

Analyses

A parsimony analysis was run using PAUP* (version 4.0d64; Swofford 2003) for the whole dataset (163 samples; 159 ingroup and 4 outgroup clones) using the heuristic methods with the tree bisection and reconnection (TBR) branch swapping. Maxtrees was set at 20,000 and gaps were coded as missing data. A bootstrap analysis was run with 10,000 replicates using the fast-stepwise addition.

A Bayesian analysis was run on the whole dataset with only one outgroup clone (160 clones total) using MrBayes 3.2.2 through CIPRES Science Gateway (V. 3.1; http://www.phylo.org/sub_sections/portal/) for the entire dataset. The GTR + G model (nst=6, rates=gamma) was used with the number of generations set at 2,000,000.

In most cases, multiple clones of the same sample grouped together on trees constructed from the entire dataset. A subset of the data was determined by only using one clone from each sample when they grouped together in order to better see the overall relationships between species.

A parsimony analysis on the subset of clones (58 sequences) was run in PAUP* with *Leptosiphon floribundus* set as the outgroup using the TBR branch swapping method. Gaps were coded as missing and maxtrees was set to 20,000. A bootstrap analysis was run with 10,000 replicates using the fast-stepwise addition method.

The 58-sample subset was also run with gaps coded. SeqState was used to code the gaps using simple indel coding (Simmons & Ochoterena, 2000). In PAUP*, maxtrees was also set to 20,000 and a parsimony analysis was run using TBR branch swapping. A bootstrap analysis was run with 10,000 replicates using the fast-stepwise addition option.

The 58-sample subset of clones was run through jModelTest (Posada 2008) where likelihood computations were made and Akaike information criteria (AIC) parameters were used to estimate a model for phylogenetic analyses. jModel Test recommended the TIM2+G model which is not available in MrBayes so a similar model (GTR+G) was used. In MrBayes, gaps were coded as missing data and the subset was set to run for 20,000,000 generations in CIPRES.

The 58-sample subset was also run in MrBayes with gaps coded (simple indel coding using MrBayes desktop version; Simmons & Ochoterena, 2000) and was set to run for 2,000,000 generations with the GTR+G model.

Figures from MrBayes and PAUP* were exported or saved as tree files (.tre) which were then opened in FigTree v1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/). FigTree files were exported as a PDF and were edited using Adobe Illustrator (Adobe Systems Incorporated).

Ploidy level information for particular samples was obtained from the Ferguson lab (unpubl.; including flow cytometry and/or chromosome counts), and clones from samples inferred to be diploid are denoted with an asterisk in the 58-sample phylogenies.

Results

Sequence Data

The complete dataset included 163 clones from 53 samples (with an average of three clones per samples; see Table 2.1).

The length of the unaligned sequences was 1378-1466 base pairs long and the aligned sequence matrix was 1608 base pairs long including the gaps. There were only two regions of possible poly A/T length variation and they were short (2-3bp). Because data matrices were coded as gaps missing or with simple indel coding, these characters were not excluded during analysis. There were no parts of the alignment that could not be aligned, thus, no characters warranted exclusion.

Parsimony Analyses

The complete and 58-sample datasets resulted in a large amount of informative characters (see Table 2.2). The strict consensus trees resulting from the parsimony analyses are presented in

Figs. 2.1-2.3 with bootstrap values greater than 50 above each branch, and clones from the same samples tend to group together in the complete dataset.

When comparing the phylogenies for the 58-sample dataset (gaps missing vs. simple indel coding) there was only a minor difference with *P. longifolia* clones coming out as a monophyletic group with weak support in the simple indel coded phylogeny (see Fig. 2.2, 2.3). The remaining relationships were the same between the two trees, but due to gaps being coded as a new state there are more variable and informative characters in the tree with simple indel coding (see Table 2.2).

Table 2.2 Results from parsimony analyses in PAUP* for the entire dataset and the subset (gaps missing and simple indel coding).

Analysis	Number of sequences included	Treatment of gaps	Number of trees retained	Variable characters	Informative characters	Steps in tree	CI[1]	RI[2]
A	163	Missing data	20,000	601	442	1100	0.62	0.93
B	58	Missing data	854	466	229	803	0.55	0.83
C	58	New state	19,245	555	269	993	0.54	0.82

[1]Consistency Index (Kluge and Farris 1969) excluding uninformative characters

[2]Retention Index (Farris 1989)

Bayesian Analyses

The complete dataset resulted in a tree where clones of the same DNA and the same species tended to group together. It is curious that the complete dataset ran for a fewer number of generations (compared to the 58-sample dataset) before reaching a standard deviation of split frequencies below 0.01although having more clones with very similar sequences may make it easier for the program to determine relationships in the complete dataset. The Bayesian trees

resulting from analyses D-F (Table 2.3) are presented in Figs. 2.4-2.6 with posterior probabilities above 0.50 shown along the tops of the branches.

The 58-sample Bayesian trees (gaps missing vs. simple indel coding) resulted in only slightly different phylogenies with *P. longifolia* becoming a monophyletic group in the tree with simple indel coding (well supported by posterior probabilities and weakly supported by bootstrap values; see Fig. 2.5, 2.6). All other relationships were the same. Due to only small differences in the 58-sample trees the simple indel coded Bayesian tree (Fig. 2.6) was chosen for further discussion.

Table 2.3 The results from Bayesian analyses run on MrBayes for the complete data set and the subset of data (gaps missing and simple indel coding).

Analysis	Number of sequences included[1]	Treatment of gaps	Model	Number of generations	Standard deviation of split frequencies
D	160	Missing data	GTR+G	1,610,000	0.009855
E	58	Missing data	GTR+G	18,495,000	0.00844
F	58	New state	GTR+G	2,000,000	0.006785

[1]Only one clone of *Leptosiphon floribundus* was used in the analyses of the entire dataset resulting in 160 taxa/clones included.

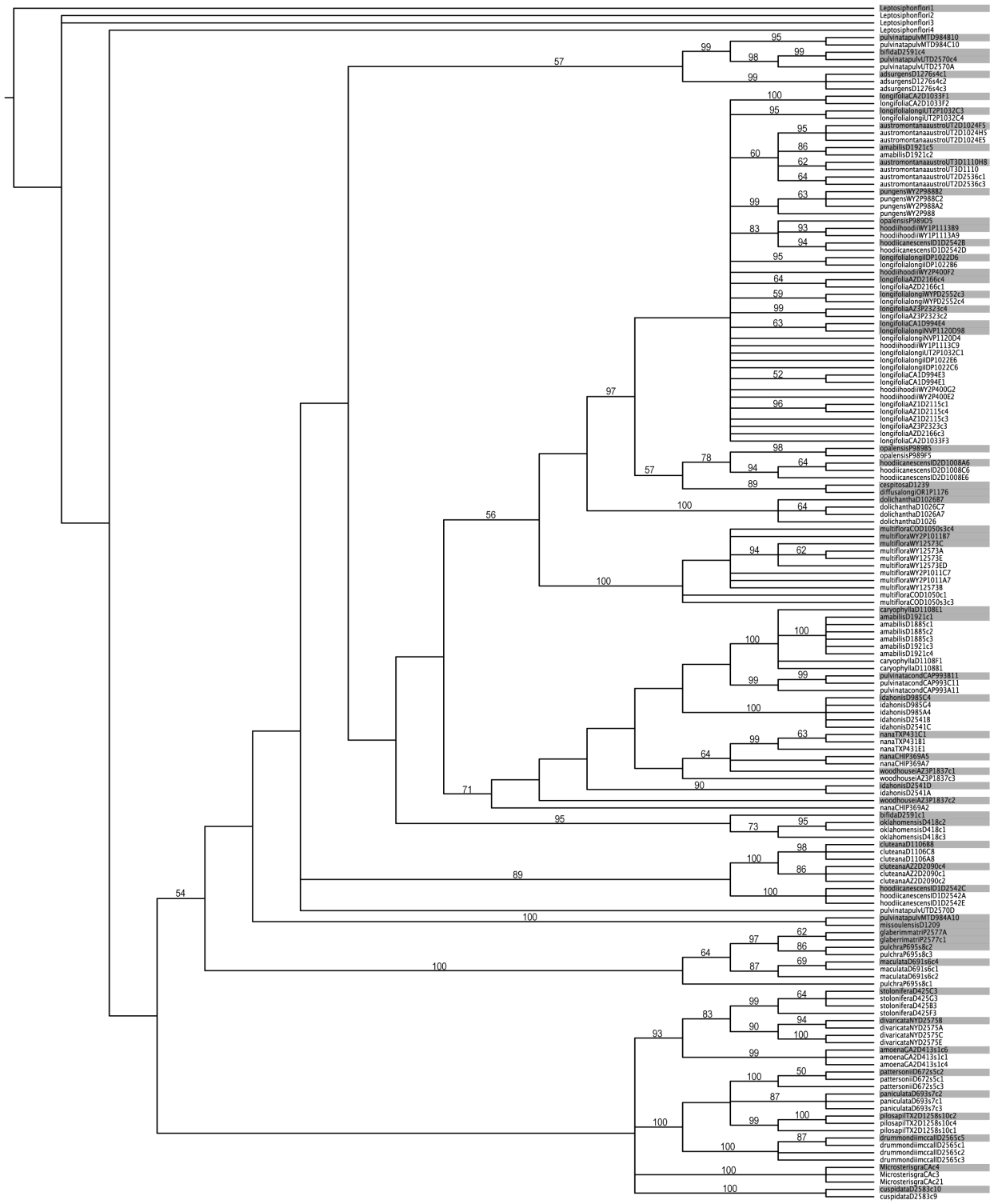

Figure 2.1 Strict consensus tree from parsimony analysis A (see Table 2.2). Clones highlighted in gray were chosen for the 58- sample subset of data used for further analysis. The same sequences were chosen for all the subset analyses including those run in MrBayes. Bootstrap values above 50 are shown above the branches.

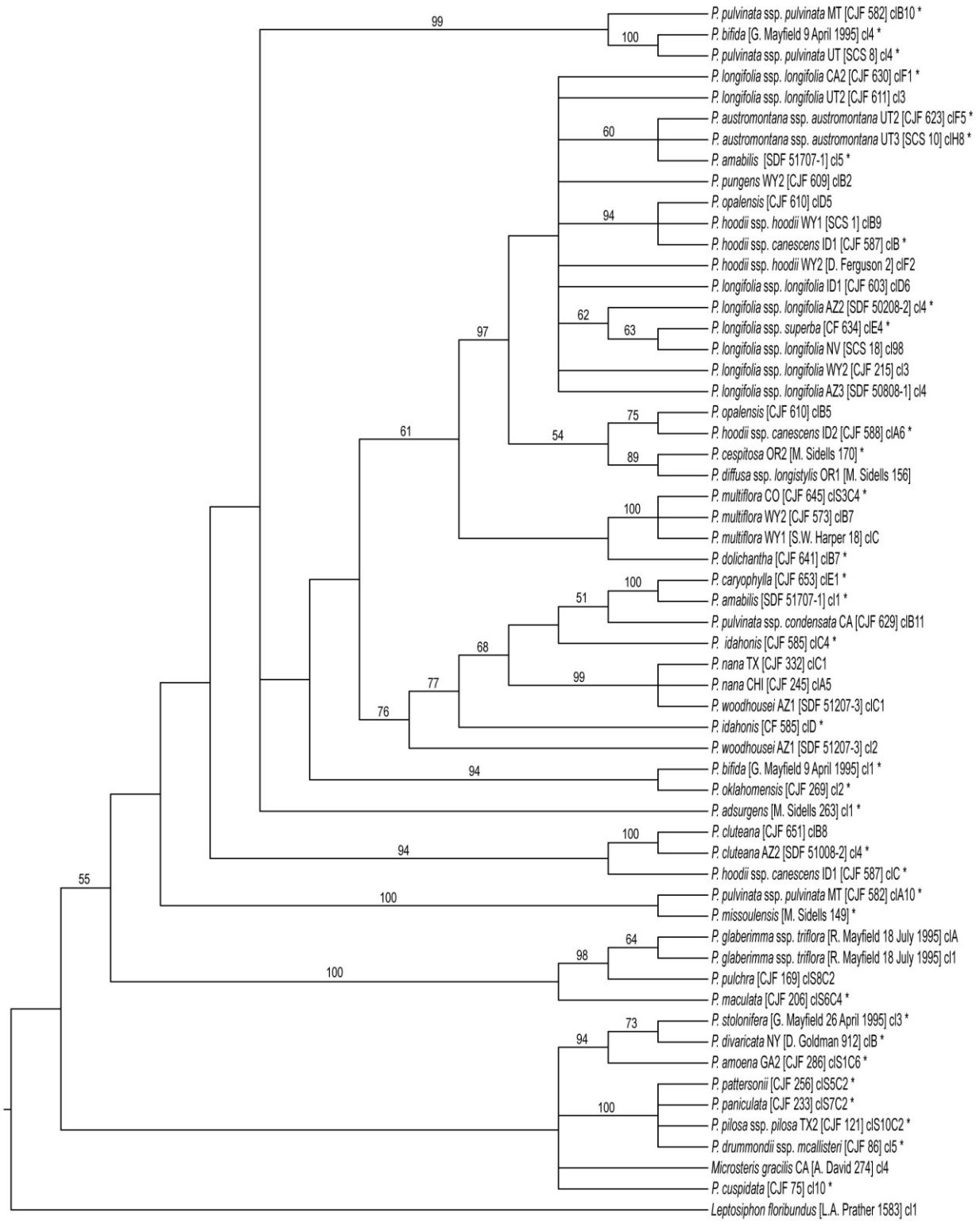

Figure 2.2 Strict consensus tree constructed from analysis B (see Table 2.2). Taxa marked with an asterisk are considered diploid. All remaining clones are polyploid. Bootstrap values above 50 are shown along the branches.

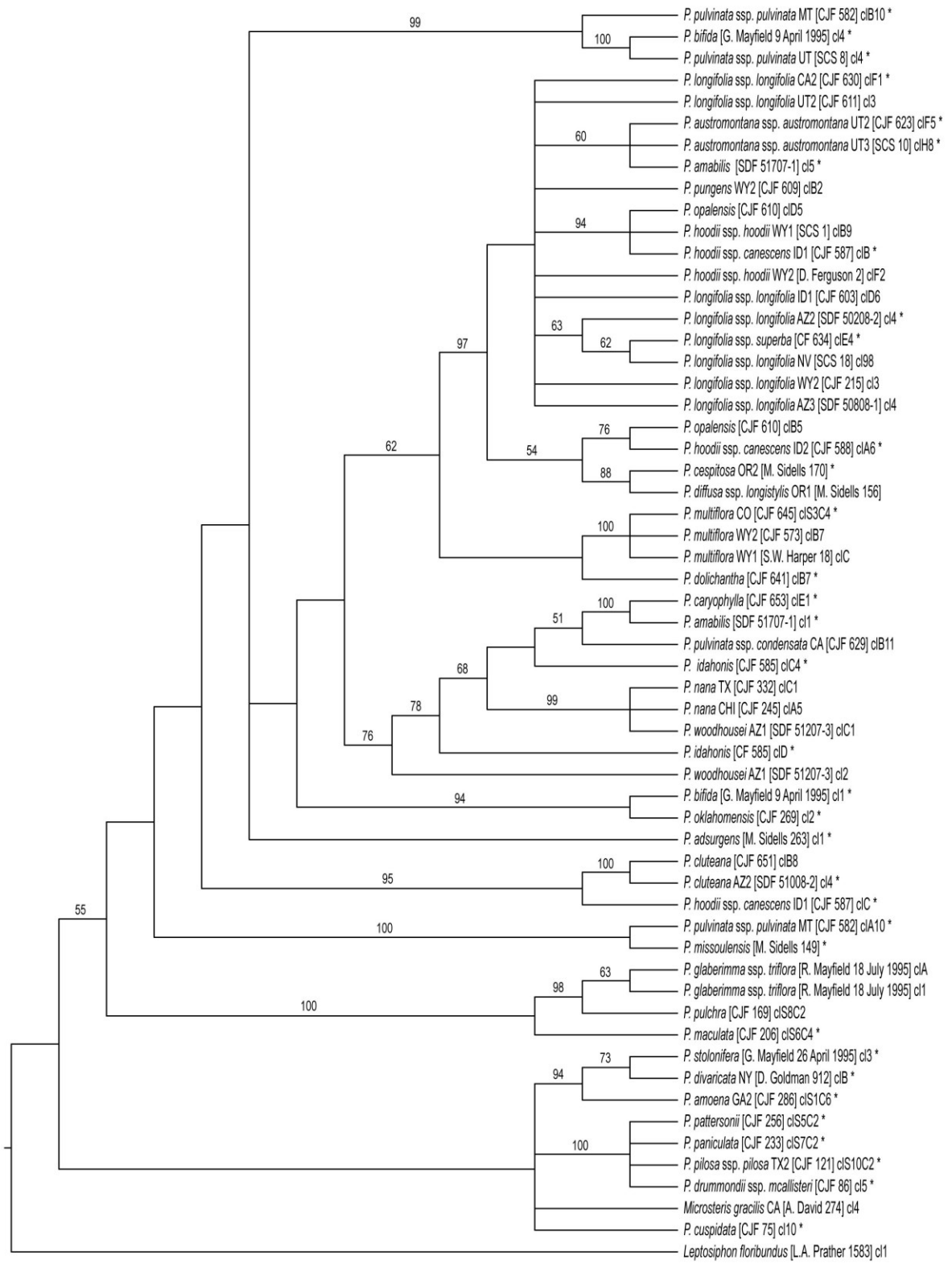

Figure 2.3 Strict consensus tree constructed from analysis C (see Table 2.2). Taxa marked with an asterisk are considered diploid. All remaining taxa are polyploid. Bootstrap values above 50 are shown along the branches.

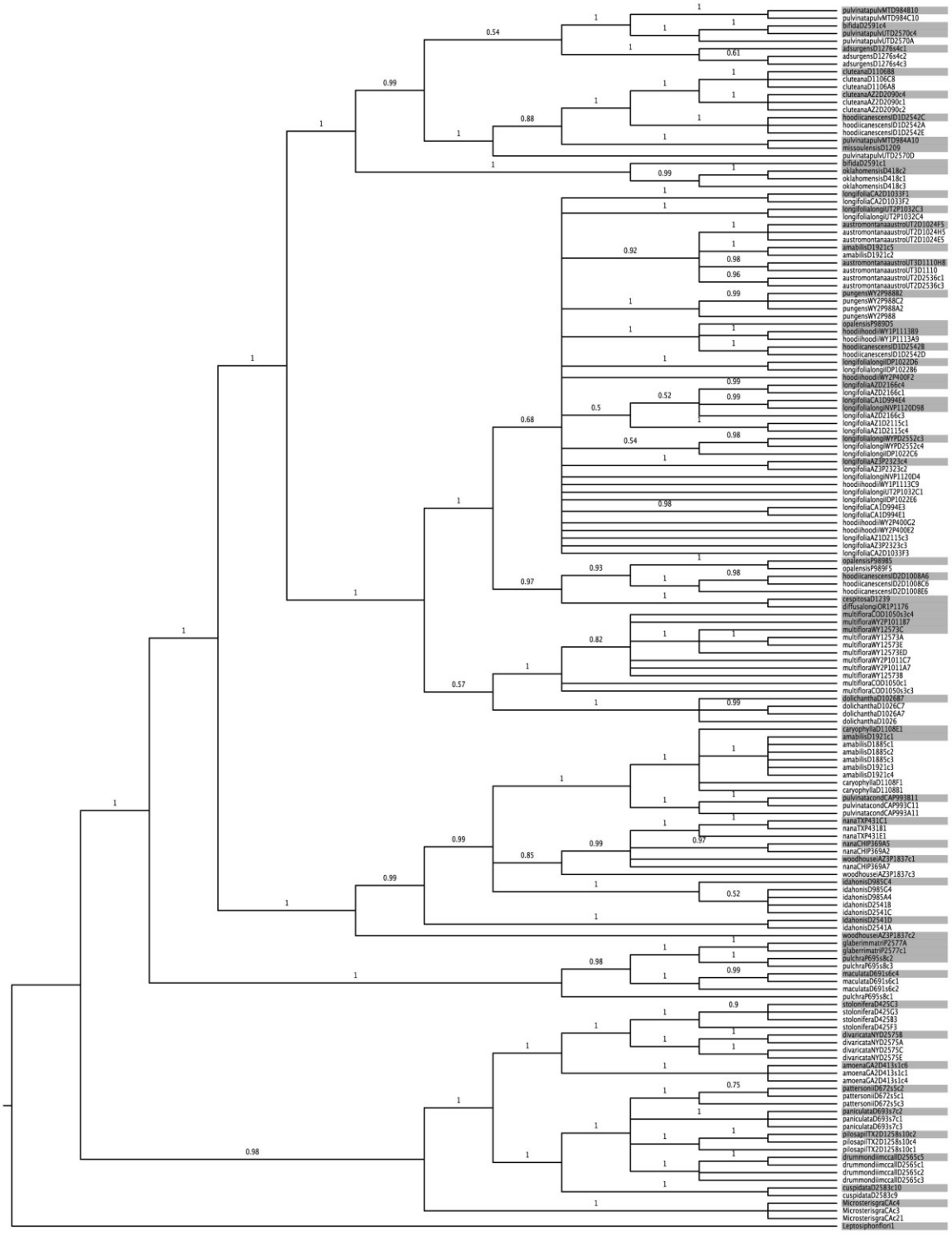

Figure 2.4 Bayesian tree constructed from analysis D (see Table 2.2). Sequences highlighted in gray were chosen for as the 58-sample subset used for further analysis. The same clones were chosen for all the subset analyses including those run in PAUP*. Posterior probabilities above 0.50 are shown along the branches.

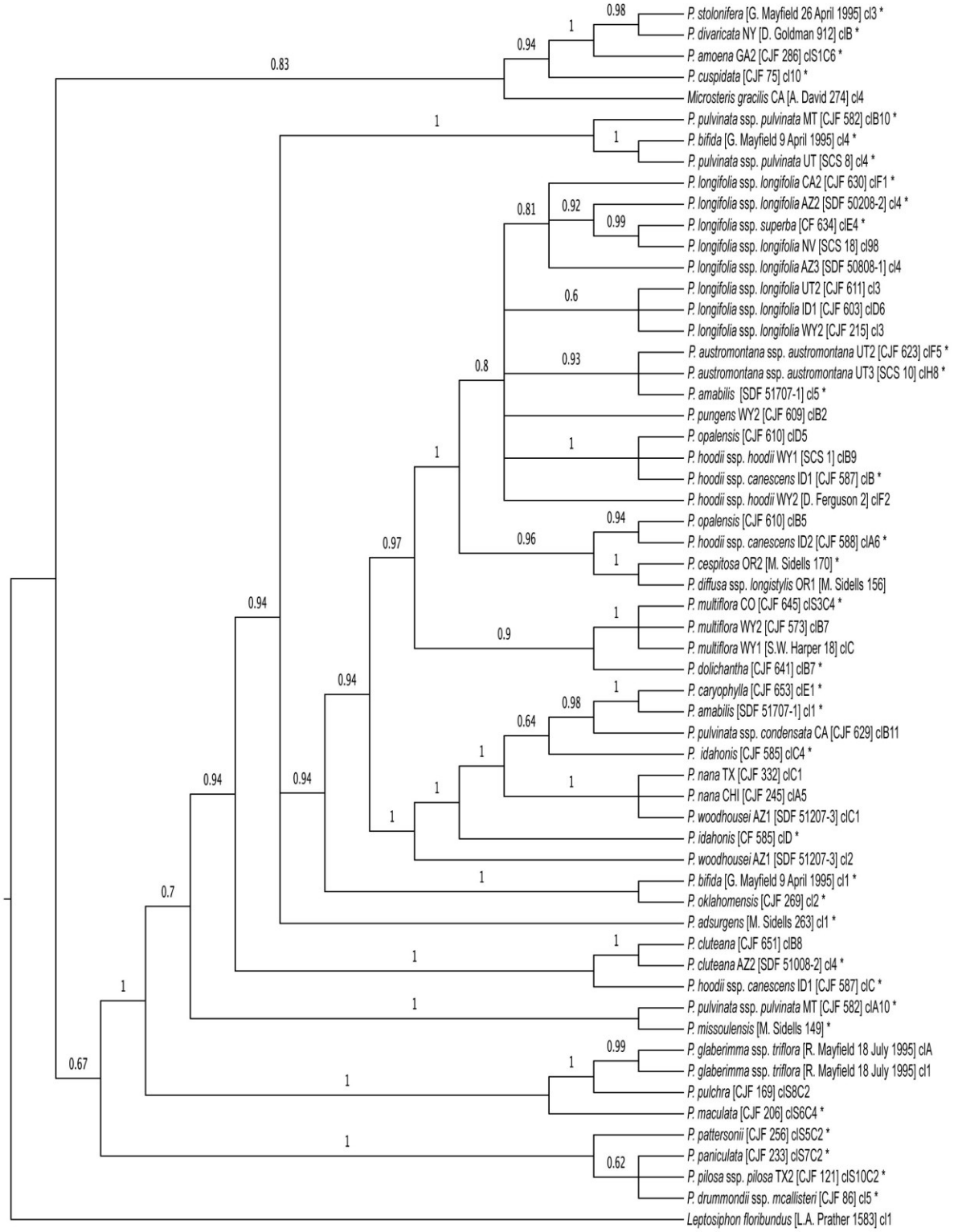

Figure 2.5 Bayesian tree constructed from analysis E (see Table 2.2).
Clones marked with an asterisk are considered diploid. All remaining taxa are polyploid.
Posterior probabilities above 0.50 are shown along the branches.

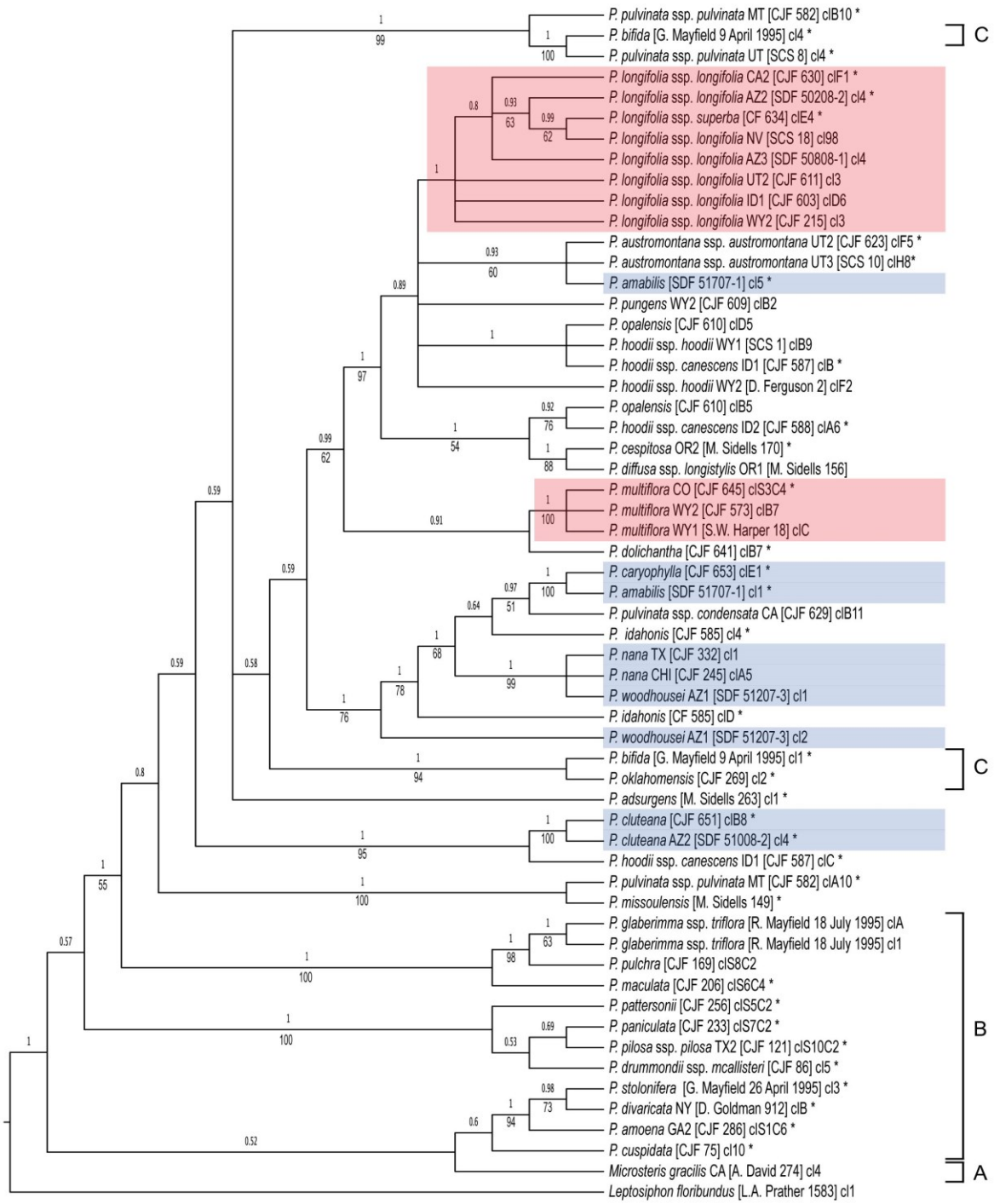

Figure 2.6 Bayesian tree constructed from analysis F (with simple indel coding; see Table 2.2).

Taxa/clones marked with an asterisk are diploid. Posterior probabilities above 0.50 are shown along the tops of the branches and bootstrap values are shown below the branches. Groups discussed in the text are indicated on the tree as follows: A. *Microsteris*; B. eastern upright taxa; C. eastern mat-forming taxa; Blue color, samples of southwestern upright taxa; red color, particular clades noted in discussion.

Discussion

Phylogeny development in Phlox using idh*B*

The *idh*B region posed some difficulty when it came to amplification and cloning. Some samples would amplify well, while others would only show a weak band on a gel or would not amplify at all. Furthermore, not all samples that would amplify would clone easily or in some cases only a few colonies were recovered. Previous researchers in the lab have easily sequenced *idh*B so the technical challenges that were experienced may just be caused by not having optimal conditions for sequencing.

Initial alignment for the region was challenging due to many variable regions (including insertion/deletion events). However, we have good confidence in the final alignment, and there were no regions of the matrix deemed problematic (no characters warranted exclusion in the phylogenetic analyses). The aligned *idh*B matrix, based on a diversity of samples, is a valuable contribution to ongoing work on *Phlox*.

The variation and the number of parsimony informative characters were greater in the *idh*B analyses compared to previous analyses on cpDNA and ITS. The complete dataset, *idh*B parsimony analyses had 96 more informative characters when compared to the complete cpDNA analyses from Ferguson and Jansen (2002) and 308 more informative characters when compared to the ITS analyses. The 58-sample *idh*B analysis (B) had 36 more informative characters (76 more characters with simple indel coding) when compared to the 55-sample cpDNA analyses and 148 more informative characters (188 more characters with simple indel coding) when compared to the 55-sample ITS analyses from Ferguson and Jansen (2002). Overall the *idh*B region has more informative characters and is more variable than previously used regions but should not be used alone when trying to resolve the relationships in the genus. Combining this

region with other nuclear and chloroplast gene regions may help better resolve relationships in Phlox, compared to using any region alone.

Phylogenetic data for Phlox

This study focused on evaluating the utility of *idh*B as well as focusing on diploid samples. Diploid samples are indicated with an asterisk in the trees constructed from a subset of the larger dataset. We find that polyploidy has arisen several times in the genus as we have not recovered a single clade of polyploids. In most cases, multiple clones of the same DNA, regardless of ploidy level, group together in the phylogeny with the exception of a few cases.

Trees resulting from different analyses are mostly in agreement with each other (see Fig. 2.1-2.6) and many aspects of the *idh*B phylogeny are in agreement with other findings on the phylogeny of *Phlox* (see Ferguson et al. 1999; Ferguson & Jansen 2002; Ferguson et al. 2008; and unpubl.) although not always in strong agreement. Aspects of the phylogeny constructed from the 58-sample Bayesian analysis, with gaps treated as a new state (see Table 2.3, analysis F and Fig. 2.6) are discussed and the phylogeny is also considered in light of broader findings.

The 58-sample Bayesian phylogenies resulted in slightly different trees with regard to the resolution of *P. longifolia*. *Phlox longifolia* is grouped in an unresolved clade with other species such as *P. hoodii* and *P. austromontana* in the tree reconstructed from analysis E(see Table 2.3 and Fig. 2.5). In the simple indel coding tree, all the samples of *P. longifolia* form a monophyletic group (see Fig. 2.6 in red), although the division of this group is weakly supported by a bootstrap value less than 50. All other relationships tend to be the same when comparing these trees. The relationships seen in the simple indel coded tree (Table 2.3, analysis F) are discussed further (see Fig. 2.6 and text below).

Placement of particular taxa or groups of taxa in the idh*B tree*

Microsteris

The genus *Microsteris* has been considered a distinct genus, closely related to *Phlox*. In previous phylogenies it has been recovered as sister to *Phlox* (e.g. Ferguson et al 1999; Ferguson & Jansen 2002) but the *idh*B phylogeny shows *Microsteris* as being nested within the eastern upright taxa but its relationship is poorly supported (see Fig. 2.6 A). Although there is poor support for this relationship, further investigation may be necessary.

Eastern upright taxa

The eastern upright taxa do not form a clade in the *idh*B phylogeny (see Fig. 2.6) although we find it as a monophyletic group in broader, unpublished phylogenies. It is here combined with *Microsteris gracilis* to form a weakly supported basal grade. Although this group does not form a clade, the interior relationships are poorly resolved and may form a clade when this data is combined with other gene regions.

Eastern mat-forming species

The eastern mat-forming taxa (*P. bifida* and *P. oklahomensis* are sampled) group as closely related but with the exception of an additional *P. bifida* clone that groups with two accessions of *P. pulvinata*, which is a western, alpine, cespitose species (see Fig. 2.6 C). This relationship is odd and has not been found based on other datasets (Ferguson Lab unpubl. data). This may reflect a close relationship between eastern mat-forming species and *P. pulvinata* or it is possible that this sample could have been mislabeled in the sequencing process. This oddity warrants further investigation to confirm the *idh*B relationships.

Southwestern upright taxa

The southwestern upright taxa, including *P. amabilis*, *P. caryophylla*, *P. cluteana*, P. *nana* and *P. woodhousei*, were not recovered as a clade in the *idh*B phylogeny (see Fig. 2.6 in blue). The taxa are of interest because a close relationship has been indicated based on other data (Ferguson Lab unpubl.), and several taxa, including *P. nana* (Chapter 1), are of particular interest in ongoing research. There is a clade that includes most of these taxa plus a sample of *P. pulvinata* and *P. idahonis*. *P. cluteana* is more distantly related in the idhB tree (Fig. 2.6). Furthermore, one clone of *P. amabilis* groups with *P. austromontana*.

Other groups of samples

The *idh*B tree does resolve some widespread, variable taxa as monophyletic. For example, all the samples of *P. longifolia* form a monophyletic group (see Fig. 2.6) and all samples of *P. multiflora* also form a monophyletic group, both with high support.

Ongoing work

The overall *idh*B gene region is an important piece of discovering relationships within the genus *Phlox* and developing a strong set of gene trees using multiple regions will further our understanding of the species tree. Ongoing work in the Ferguson lab includes utilizing additional low copy regions in conjunction with the high copy ITS region and various chloroplast regions in order to further understand the relationships in the genus. The *idh*B data presented here are a valuable contribution to our understanding of relationships in this interesting and complicated genus.

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Appendix A - Supplemental Materials for Chapter 1

Table A. 1: *Phlox paniculata* samples tested to find significant differences between picogram values and the coefficient of variations between refrigerated, frozen, silica gel dried and fresh plant material when used for flow cytometry.

Sample Number	Tissue Preservation Type	Picograms DNA	Coefficient of Variation
BW 147 2A	Refrig 1 week	10.78	2.76
BW 147 2B	Refrig 1 week	10.85	2.34
BW 147 2C	Refrig 1 week	10.16	3.42
BW 147 3D	Refrig 1 week	10.19	2.77
BW 147 2E	Refrig 1 week	10.62	2.81
BW 147 2F	Refrig 1 week	10.24	3.12
BW 147 2G	Refrig 1 week	10.29	3.63
BW 147 2H	Refrig 1 week	10.59	3.22
BW 147 2I	Refrig 1 week	10.40	2.72
BW 147 2J	Refrig 1 week	10.10	3.54
BW147 3N	Refrig. 2 wks.	12.70	2.72
BW147 3O	Refrig. 2 wks.	11.49	2.31
BW147 3P	Refrig. 2 wks.	11.98	1.87
BW147 3Q	Refrig. 2 wks.	11.17	1.77
BW147 3R	Refrig. 2 wks.	11.44	2.96
BW147 3S	Refrig. 2 wks.	11.22	1.84
BW147 3T	Refrig. 2 wks.	11.66	2.13
BW147 3U	Refrig. 2 wks.	11.49	1.99
BW147 3V	Refrig. 2 wks.	11.74	1.66
BW147 3W	Refrig. 2 wks.	11.86	1.85
BW 147 3D	Refrig 3 weeks	10.77	1.46
BW 147 3E	Refrig 3 weeks	11.02	1.55
BW 147 3F	Refrig 3 weeks	10.45	1.47
BW 147 3G	Refrig 3 weeks	10.83	2.1
BW 147 3H	Refrig 3 weeks	10.63	1.86
BW 147 3I	Refrig 3 weeks	10.47	2.8
BW 147 3J	Refrig 3 weeks	10.61	2.76
BW 147 3K	Refrig 3 weeks	11.46	1.37
BW 147 3L	Refrig 3 weeks	11.33	1.36
BW 147 3M	Refrig 3 weeks	11.94	2.06
BW147 3X	Refrig 4 weeks	10.92	1.43
BW147 3Y	Refrig 4 weeks	11.10	1.33
BW147 3Z	Refrig 4 weeks	10.90	1.31
BW147 4A	Refrig 4 weeks	11.46	1.97
BW147 4B	Refrig 4 weeks	11.49	1.28
BW147 4C	Refrig 4 weeks	12.18	2.18
BW147 4D	Refrig 4 weeks	11.60	1.39

BW147 4E	Refrig 4 weeks	11.58	1.1
BW147 4F	Refrig 4 weeks	11.36	1.91
BW147 4G	Refrig 4 weeks	11.74	1.55
BW147 4T	Frozen	10.58	1.89
BW147 4U	Frozen	10.69	1.96
BW147 4V	Frozen	10.49	1.77
BW147 4W	Frozen	10.28	1.68
BW147 4X	Frozen	10.39	1.92
BWI47 4Y	Frozen	10.44	1.81
BW147 4Z	Frozen	10.32	1.84
BW147 5A	Frozen	10.87	1.69
BW147 5B	Frozen	10.96	1.73
BW147 5C	Frozen	10.71	1.93
BW147 5D	Frozen	10.48	2.39
BW147 5E	Frozen	10.59	2.49
BW147 5F	Frozen	10.66	2.16
BW147 5G	Frozen	10.64	1.98
BW147 5H	Frozen	10.38	1.9
BW147 5I	Frozen	10.58	1.89
BW147 5J	Frozen	10.48	1.84
BW147 5K	Frozen	10.47	1.97
BW147 5L	Frozen	10.93	1.67
BW147 5M	Frozen	10.81	1.89
BW147 5N	Frozen	10.59	2.63
BW147 5O	Frozen	10.72	2.28
BW147 5P	Frozen	10.46	2.31
BW147 5Q	Frozen	10.41	1.97
BW147 5R	Frozen	10.74	1.85
BW147 5S	Frozen	10.58	2.38
BW147 5T	Frozen	10.68	2.15
BW147 5U	Frozen	10.55	2.36
BW147 5V	Frozen	10.20	2.73
BW147 5W	Frozen	10.52	2.53
BW147 5X	Frozen	11.19	1.88
BW147 5Y	Frozen	11.29	1.67
BW147 5Z	Frozen	10.45	2.35
BW147 6A	Frozen	10.73	2.08
BW147 6B	Frozen	10.30	2.45
BW147 6C	Frozen	10.92	1.94
BW147 6D	Frozen	10.84	2.18
BW147 6E	Frozen	10.82	1.87
BW147 6F	Frozen	10.38	3.38
BW147 6G	Frozen	10.63	2.74
BW 147A	Silica Gel	10.84	2.78

BW 147B	Silica Gel	10.54	2.88
BW 147C	Silica Gel	10.89	2.91
BW 147D	Silica Gel	11.02	2.66
BW 147E	Silica Gel	10.88	3.09
BW 147F	Silica Gel	11.05	3.02
BW 147M	Silica Gel	10.36	2.46
BW 147N	Silica Gel	10.47	2.72
BW 147O	Silica Gel	10.43	2.93
BW 147P	Silica Gel	10.37	2.71
BW 147Q	Silica Gel	10.17	2.91
BW 147R	Silica Gel	10.30	2.48
BW 147AA	Silica Gel	10.01	3.97
BW 147BB	Silica Gel	10.28	3.58
BW 147CC	Silica Gel	10.31	3.23
BW 147DD	Silica Gel	10.09	3.36
BW 147EE	Silica Gel	10.17	2.89
BW 147FF	Silica Gel	10.37	3.43
BW147 4H	Silica Gel	10.96	2.26
BW147 4I	Silica Gel	10.83	3.04
BW147 4J	Silica Gel	11.42	2.63
BW147 4K	Silica Gel	11.49	1.57
BW147 4L	Silica Gel	11.04	2.33
BW147 4M	Silica Gel	10.94	2.82
BW 147G	Fresh	11.53	2.94
BW 147H	Fresh	11.38	2.64
BW 147I	Fresh	11.37	2.43
BW 147J	Fresh	11.30	2.4
BW 147K	Fresh	11.37	2.26
BW 147L	Fresh	11.75	2.65
BW 147S	Fresh	10.54	2.69
BW 147T	Fresh	11.06	2.28
BW 147U	Fresh	10.72	2.37
BW 147V	Fresh	10.46	2.8
BW 147W	Fresh	11.77	2.42
BW 147X	Fresh	11.22	2.59
BW 147Y	Fresh	11.20	2.14
BW 147Z	Fresh	10.87	2.36
BW 147GG	Fresh	10.37	2.98
BW 147HH	Fresh	10.39	3.29
BW 147II	Fresh	10.21	3.58
BW 147JJ	Fresh	10.67	2.35
BW 147KK	Fresh	10.50	3.25
BW 147LL	Fresh	10.29	3
BW 147 2K	Fresh	10.52	2.99

BW 147 2L	Fresh	10.53	2.84
BW 147 2M	Fresh	10.24	2.79
BW 147 2N	Fresh	10.05	3.22
BW 147 2O	Fresh	10.45	2.77
BW 147 2P	Fresh	10.40	2.73
BW 147 2Q	Fresh	10.49	2.68
BW 147 2R	Fresh	10.48	2.72
BW 147 2S	Fresh	10.25	3.01
BW 147 2TA	Fresh	10.41	2.73
BW147 4N	Fresh	11.12	1.61
BW147 4O	Fresh	11.17	1.46
BW147 4P	Fresh	11.21	1.33
BW147 4Q	Fresh	11.14	1.58
BW147 4R	Fresh	11.34	1.98
BW147 4S	Fresh	11.20	1.85

Table A. 2: Average amount of DNA (pg) and the average coefficient of variation (and standard deviations) for each type of preservation method.

Preservation Type	Average Picogram Value	Average CV Value
Fresh (n=36)	10.83±0.48	2.54±0.52
Silica Gel Dried (n=24)	10.64±0.41	2.86±0.48
Refrigerated (n=40)	11.12±0.62	2.14±0.70
Frozen (n=40)	10.62±0.24	2.10±0.36

Table A. 3: P-values for each Kruskal-Wallis test of picogram values (p*=0.0125). A significant difference was only found between refrigerated and frozen plant material.

	Fresh vs. Silica	Fresh vs. Refrigerated	Fresh vs. Frozen	Silica vs. Refrigerated	Silica vs. Frozen	Refrigerated vs. Frozen
p-value	0.0737	0.0370	0.1586	0.0024	0.7815	0.0002