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Evidence of Ecological Speciation in *Phacelia*.

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Evidence of Ecological Speciation in *Phacelia*

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by

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ABSTRACT

Evidence of Ecological Speciation in *Phacelia*

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Phacelia purshii Buckley and *P. fimbriata* Micheaux are two species that are nearly morphologically indistinguishable. Seed germination experiments showed that the high elevation endemic, *P. fimbriata* requires lower temperatures to trigger germination. Following interspecific crosses, pollen tubes enter ovules and maternal tissue of the gynoecium matures but hybrid diploid and triploid organs fail to develop. DNA sequences from the ribosomal DNA internal transcribed region showed that *P. fimbriata* and *P. purshii* comprise a monophyletic clade but that *P. fimbriata* is more differentiated from related species. In contrast, *P. purshii* supported significantly higher levels of intraspecific polymorphism. *Phacelia fimbriata* and *P. purshii* are sister species with similar morphology but they are unable to hybridize, they are differentiated in physiological characteristics related to environment, and they inhabit different elevations. This pattern of relationship and differentiation suggests *P. fimbriata* may be the product of ecological speciation.

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CHAPTER 1

INTRODUCTION

Phacelia purshii Buckley and *P. fimbriata* Micheaux are both members of the native southern Appalachian flora where they are found as part of the herbaceous layer of moist deciduous hardwood forests that are abundant in the region. *Phacelia* is a species-rich genus traditionally assigned to Hydrophyllaceae, a family that was recently merged with Boraginaceae (Judd et al. 2002). There are approximately 200 American species in the genus *Phacelia* with the center of diversity in California. Of these 200 species, only 14 are found in the eastern United States (Constance 1949; Gilbert et al. 2005). All 14 eastern species have been classified in the subgenus Cosmanthus based on unique morphological characters (presence of corolla glands and absence of corolla scales), geographical distribution (eastern North America), and base chromosome numbers of n = 5, 8, 9, and 14 (Constance 1949). The predominant chromosome number in the remainder of the genus *Phacelia* is n = 11, while in the subgenus Cosmanthus there are no species with n = 11 (Constance 1949). Because most species of *Phacelia* occur in the western United States, most research within this genus has focused on western species. Relatively little research has been conducted on subgenus Cosmanthus species with little attention paid to either *P. purshii* or *P. fimbriata*. These two species are of interest because they are both native to the Tennessee-western North Carolina area, are not well researched, are readily available from several well-established populations, and particularly because they may represent an example of ecological speciation.

Phacelia, especially western species, has been the subject of phylogenetic studies using both morphological and molecular data. Hofmann (1999) investigated flower and fruit

development to distinguish between three closely related western species; *Phacelia ciliata* Bentham, *P. distans* Bentham, and *P. tanacetifolia* Bentham. For this species group, the shape of corolla scales (absent in subgenus Cosmanthus) and other floral features such as "orientation of two stylar lobes, the development of stigmatic papillae, the differentiation of pollen-tube transmitting tissue, and the presence/absence of a stylar canal" were all useful distinguishing characters.

In addition to morphological studies, several researchers have used molecular data to investigate species relationships within *Phacelia*. Ferguson (1999) used DNA sequence data from the chloroplast-encoded gene *ndhF* to examine the evolutionary relationship between Hydrophyllaceae and Boraginaceae. A secondary result of this analysis was the comparison of DNA sequence data within the genus *Phacelia.* Ferguson found that *ndhF* sequences are useful for analyzing relationships at the taxonomic level of family due to the combination of a high amount of variation at the 3'end of the gene and the conserved 5' end. Ferguson's analysis placed Hydrophyllaceae within Boraginaceae and placed subgenus Cosmanthus in a clade embedded among other *Phacelia* species. Although Ferguson analyzed data from only two Cosmanthus species, *P. hirsuta* Nuttall and *P. patuliflora* (Engelmann & Gray) Gray, she found the subgenus to be monophyletic and closely related to the "Franklinii" group that was located in a derived position in the clade. Although this analysis only minimally sampled Cosmanthus species, it confirmed cytogenetic and morphologic data supporting Cosmanthus as a natural phylogenetic group and placed it among the derived members of the genus *Phacelia*.

Recently Gilbert et al. (2005) performed a molecular analysis of *Phacelia* using nuclear ribosomal DNA internal transcribed spacer (ITS) sequences to clarify phylogenetic relationships within the subgenus Phacelia. Their analysis found that subgenus Phacelia is a paraphyletic

grouping that includes subgenus Cosmanthus. This analysis included two Cosmanthus species; *P. hirsuta* and *P. patuliflora*, the same species analyzed in Ferguson's study. Gilbert et al. noted that additional work, especially within Cosmanthus, was needed before the relationships among *Phacelia* species could be fully understood.

While much of the recent work has focused on western *Phacelias* there has been some research analyzing species relationships within subgenus Cosmanthus. Most of the early systematic work on the 14 Cosmanthus *Phacelias* was initiated by Constance (1949). Constance completed a monograph in which he described the morphologic differences between Cosmanthus and other *Phacelia* species and delineated each of the Cosmanthus species using cytological, morphological, and ecological data. Gillett (1964) employed a biosystematic approach to studying Phacelias of subgenus Cosmanthus by investigating genetic barriers between all but four of the Cosmanthus species using hybridization experiments. With a very limited number of crosses, Gillett suggested that *P. purshii* and *P. fimbriata* are not interfertile. This conclusion was based on data from only 20 ovules or approximately five flowers. Within Cosmanthus, only one cross resulted in hybrids, *P. hirsuta* x *P. gilioides*, leading to his conclusion that speciation in *Phacelia*, at least the Cosmanthus species studied, often accompanies reproductive isolation.

Levy (1991) conducted additional biosystematic work on reproductive isolation in Cosmanthus using varieties within *P. dubia*. Levy selected three allopatric varieties of *P. dubia* that could produce hybrids for further study. To study the reproductive barriers, Levy measured the rate of pollen tube growth, number of mature seeds per capsule, F_1 seed germination, F_1 flowering phenology, and F_1 pollen fertility. Although hybrid plants were viable and grew as well as parent plants, partial pollen sterility occurred in all hybrids as well as sterile ovules. In a later study biochemical and molecular markers were used in conjunction with hybridization

studies to gain a better understanding of the genetic controls of reproductive isolation within varieties of *P. dubia* (Levy and Malone 2001).

The Cosmanthus species investigated in this research were *Phacelia purshii* and *P. fimbriata*. Both *P. purshii* and *P. fimbriata* are winter annuals, a life cycle most prevalent in Cosmanthus Phacelias. The winter annual life cycle begins with seed germination in the fall, usually starting in late September and progressing through the later autumn, although smaller numbers of seeds germinate in early spring. Germination is triggered by changes in day- and nighttime temperatures. The seeds must experience a period of warm days and cool nights before germination can take place (Baskin and Baskin 1976). After germination, a rosette forms and overwinters until early spring when plants bolt to form several, many-flowered inflorescences. After fertilization, seeds form and the capsular fruit dehisce to passively release seeds by mid to late June. This is followed by plant senescence. The seeds that have fallen to the ground must undergo a warm after-ripening period before they can respond to the cooler fall germination cues.

Phacelia purshii and *P. fimbriata* share several morphological characteristics including a fimbriate or fringed corolla, which is unique in Cosmanthus, *Phacelia,* Hydrophyllaceae and Boraginaceae. These two species of *Phacelia* share so many morphological characteristics that they can easily be mistaken. The two species are similar in their leaf shape and leaf dissection patterns, in flower size and shape, and in growth habits. One of the key characters that differentiates these two species is corolla color; *P. purshii* has a lavender or pale lavender corolla while *P. fimbriata* typically has a white corolla (Figure 1). This character cannot be considered perfectly diagnostic because *P. fimbriata,* although typically white-flowered, has been found to

occasionally have a corolla tinged with blue or lavender, and corollas of some *P. purshii* are nearly white.

P. fimbriata	*P. purshii*
	

Figure 1 Photographs of *P. fimbriata* and *P. purshii* Plants Grown in the Lab Growth Chamber. Note the stunted growth of *P. fimbriata* compared to *P. purshii*.

Perhaps, in areas where their ranges overlap, the only reliable way to differentiate between the two species is through a combination of elevation and pubescence characters. Constance (1949) described the pubescence on stems of *P. fimbriata* as sparsely hirsute with deflexed (curved downward on the stem) spreading hairs while *P. purshii* stems are strigose with afflexed (curved upward on the stem) hairs (Figures 2 and 3).

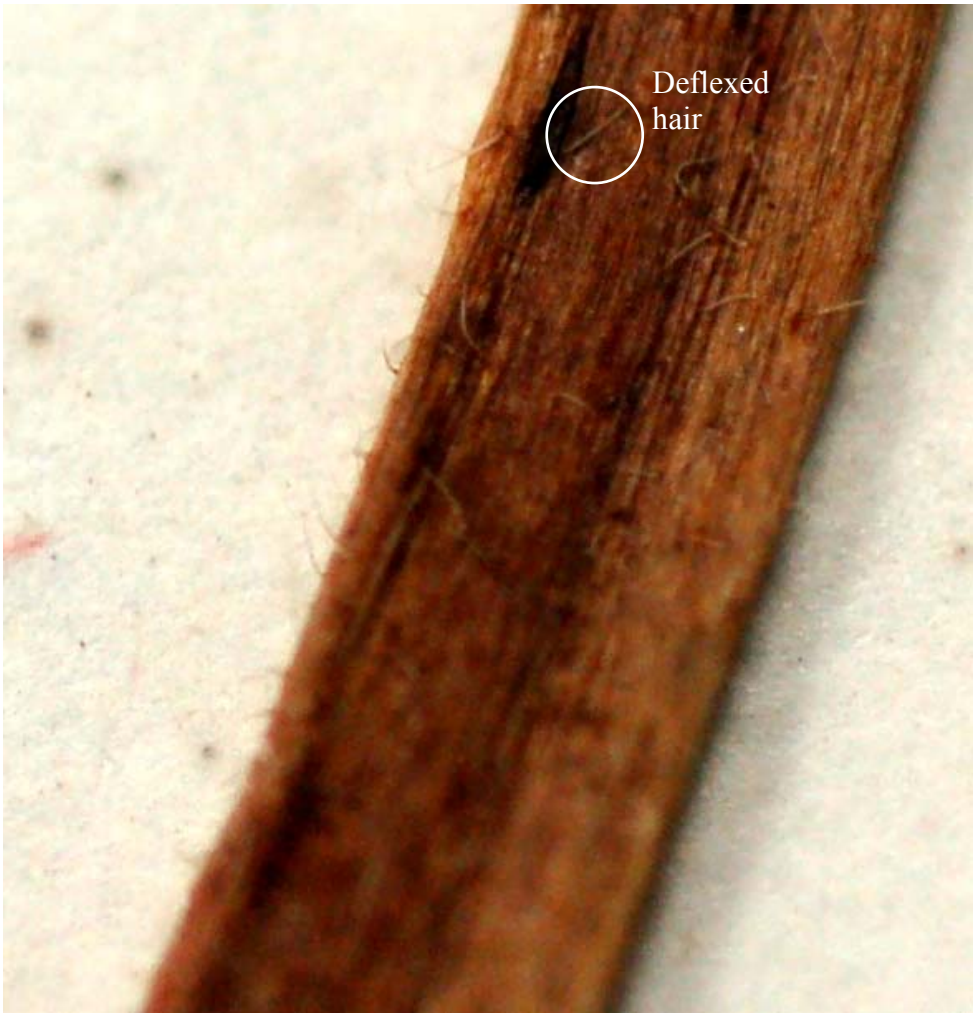


Figure 2 Photograph of *P. fimbriata* Stem Taken from an Herbarium Specimen. Note the sparsely hirsute stem. The hairs point in various directions with some hairs clearly deflexed.

Figure 3 Photograph of *P. purshii* Taken from an Herbarium Specimen. Note the strigose afflexed hairs.

Phacelia purshii occurs at relatively low elevations of usually less than 1,070 m while *P. fimbriata* is usually restricted to elevations above 1,200 m. The restricted elevational range of *P. fimbriata* provides it with limited available habitat, compared with *P. purshii*, which is widespread in the Ohio Valley region of the eastern United States. Moreover, the narrow range of *P. fimbriata* is limited to the southern Appalachians. *Phacelia fimbriata* is endemic to the southern Appalachian high mountains found only in southwestern Virginia, east Tennessee, Georgia, and western North Carolina (Constance 1949, Weakley 2006). This limited geographic range and ecological restriction to higher elevation forests makes *P. fimbriata* a species of concern for conservation programs. Currently both *P. fimbriata* and *P. purshii* are listed as

species of special concern in Georgia while in Virginia *P. fimbriata* is listed as rare and *P. purshii* is on the watch list (Weakley 2006).

The morphological similarity between these two species raises the question of their phylogenetic relationship to one another. Before proceeding, the term species must be defined. Currently, the morphological species concept supplemented by distribution restrictions is the basis for treating *P. purshii* and *P. fimbriata* as two different species. In contrast, the biological species concept (BSC) defines species as individuals from populations with gene exchange that are reproductively isolated from individuals of populations where no gene exchange occurs (Stebbins 1950). To determine if *P. purshii* and *P. fimbriata* are biological species according to the BSC, a series of controlled crosses were completed to assess their reproductive isolation. This analysis was an extension of the skeletal study by Gillett (1964). Larger sample sizes that included plants from several populations of each species were used in the current analysis.

Other species concepts may apply to these two taxa. One such species concept is the genotypic cluster species concept (GCSC), which describes a species as a group of morphologically distinguishable populations that has no or few intermediates when in contact with another such group (Mallet 1995). Another species concept is the cryptic species concept, which is defined as two closely related biological intersterile species that are morphologically identical (Collins and Paskewitz 1996). In this case molecular analysis the primary tool used to identify cryptic species. Molecular analysis can also be used to identify phylogenetic species based on the phylogenetic species concept, which is described as an irreducible, monophyletic group whose members are more closely related to each other than to any outside group and those members have a shared ancestry (Coyne and Orr 2004). To determine whether or not *P. purshii* and *P. fimbriata* are phylogenetic species, a phylogenetic analysis of these two species along

with other members of Cosmanthus was conducted and a phylogenetic tree was constructed. If *P. purshii* and *P. fimbriata* are found to comprise a single species according to any of the species concepts listed above, they may represent ecological variants. Ecological variants, or ecotypes, are members of the same species that are differentiated based on unique reactions to their environment with or without associated morphological differences between ecotypes (Stebbins 1950). If *P. fimbriata* and *P. purshii* are ecotypes of a single species, at most partial reproductive isolation may be observed as was the case when different ecotypes of *P. dubia* were crossed (Levy and Malone 2001).

According to the biological species concept, speciation occurs when divergent populations become reproductively isolated as a result of either a pre- or post-zygotic barrier. This reduction of gene flow can be the result of many different events that can impact gene flow either by acting directly on breeding characters or indirectly as a by-product of selection for other characters. For example, in allopatry, reductions in gene flow that accompany adaptation to different environments are considered by-products of the selection process. Ecological speciation is the result of a reduction in gene flow that accompanies adaptation to different habitats. This process is hypothesized to occur in allopatric, parapatric or sympatric conditions. To date, evidence for ecological speciation comes largely from studies of hybrids in the habitats of both parentals. Hybrids exhibiting characteristics intermediate to the two parents are often less fit than either parent in their respective habitats. For instance, Hatfield and Schluter (1999) tested for evidence of ecological speciation in a pair of sympatric stickleback (*Gasterosteus aculeatus* complex) species by placing hybrids in the wild and comparing their fitness to the parental types. While sympatric, each species is differentiated based on morphology and ecology (limnetic and benthic). Morphologically intermediate hybrids placed in the field grew 25% more slowly

compared to the parent in the habitat of that parent. This result suggests that divergent selection is acting on the intermediate hybrids contributing to postmating reproductive isolation and supports the hypothesis of ecological speciation in sticklebacks.

The purpose of this study was to investigate the biosystematic and molecular aspects of the relationship and nature of the differences separating *P. purshii* and *P. fimbriata*. A combination of germination experiments, experimental hybridizations, and DNA-based phylogenetic analyses were used. The specific questions addressed were:

- Do *P. purshii* and *P. fimbriata* use different environmental cues for germination?
- Can *P. purshii* and *P. fimbriata* hybridize?
- Is there a difference in pollen tube growth rate in intraspecific crosses compared to interspecific crosses?
- Is the reproductive isolation, if present, pre- or post-zygotic?
- Do the species differ in levels of genetic variation?
- What are the phylogenetic relationships and degrees of genetic divergence among these two species and other members of the subgenus Cosmanthus?

CHAPTER 2

METHODS

Ecological Analysis

Based on the differences in elevation of their respective habitats, *P. purshii* and *P. fimbriata* would be expected to receive different day and nighttime temperatures throughout the year (Figure 4). To investigate how this ecological difference may translate into differences in their timing of germination, seeds from three different populations for each species were germinated both under controlled laboratory conditions and in a common field environment.

The purpose of the field component was to estimate germination rates using natural cues, while the laboratory component compared germination under controlled temperature and light conditions. Both experiments used seeds collected in the field by Michele Glass, Foster Levy, and Elaine Walker, from three different populations for each species (Table 1). The seeds were collected in early and mid June 2005, placed in paper envelopes, and allowed to after-ripen for two months at room temperature.

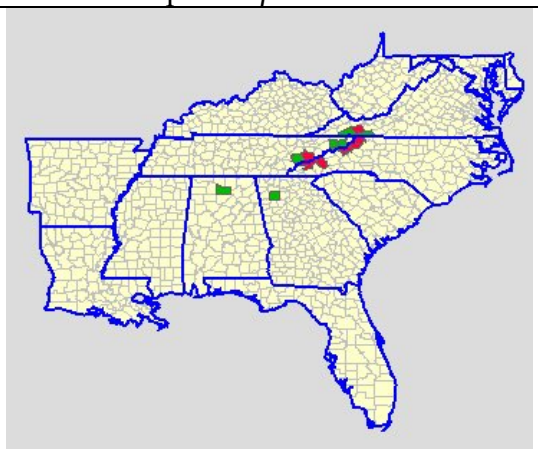
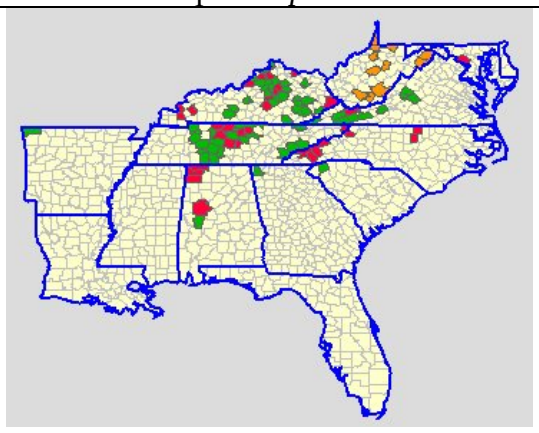
Map of *P. fimbriata*	Map of *P. purshii*
	

Figure 4 Map of the Southern United States Showing *P. fimbriata* and *P. purshii's* Distribution (Color Corresponds to Different Voucher Collections; NCU Flora of the Southeast)

Table 1 *Phacelia fimbriata* and *P. purshii* Populations Used for the Germination and Hybridization Studies

Species	Population	County	State	Location	Approximate Elevation (m)	Population size (visual estimate)
Phacelia fimbriata	Big Bald (BB)	Yancy	NC	~12 km off US-19, access through Wolf Laurel Country Club, in wooded area north of Bald along Appalachian Trail	1,681	10,000+
	Ripshin (R)	Carter	TN	½ km east on public road and 1 ½ km south on shore of Ripshin Lake, along private road on east side of Ripshin Lake	1,036	100-300
	Roan Mountain State Park (RM)	Carter	TN	Along trail after crossing Doe River in Roan Mountain State Park	1,036	300-1,000
Phacelia purshii	Paddle Creek Road (PC)	Unicoi	TN	Along the south side of Whispering Pines Road at the junction with Paddle Creek Road.	549	300-1,000
	Red Hill (RH)	Mitchell	NC	On NC-226, in growth along RR tracks that parallel Toe River	665	1,000+
	Rocky Fork (RF)	Unicoi	TN	Approximately 1.5 km W of TN 23, then approximately 200 m N of Rocky Fork Rd. along WMA access road that parallels Rocky Fork Creek	732	200-500

The seeds in the field experiment were planted in a total of 24 round, plastic, 15.24 cm x 15.24 cm (6" x 6") pots filled with Fafard 3B mix. From each population a total of 128 seeds for each species were planted in four pots with 32 seeds per pot. These pots were set outside in Bluff City, TN (approximate elevation 436 m) and watered as needed. Pots were not allowed to

dry out. All the pots with seeds were covered with wire mesh to minimize damage and predation by medium to large herbivores (i.e., squirrels and rabbits) (Figure 5). Pots were checked for germination and the total number of germinated seeds was counted each week. Population and species means were compared using one-way ANOVA as implemented using the GLM procedure in SAS (SAS institute, 1990).

Figure 5 Field Germination Experiment. All 24 pots were placed under a wire mesh to minimize predation by medium to large herbivores. The position of each pot was moved during the experiment so that all pots would have similar exposure to the edge of the array.

At the time the field experiment was conducted, seeds were also germinated in the laboratory under controlled conditions. In the laboratory, two sheets of filter paper moistened with dH_2O and fungicide (0.1% Captan dissolved in dH_2O) solution was used to germinate a total of 30 seeds per population. Two petri dishes were used for each population with 15 seeds in each. Throughout the germination period dH_2O was used to keep filter papers moist and fungicide was used once each week. Petri dishes with seeds were placed in a growth chamber with 12-hour day and 12-hour night and temperatures of 20^{o} C day and 14^{o} C night (Baskin and Baskin 1976). Species and population germination means were compared using one-way ANOVA.

Hybridization Experiment

To determine whether *P. purshii* and *P. fimbriata* are reproductively isolated, experimental hybridizations were conducted by controlled pollinations. If *P. fimbriata* and *P. purshii* are not valid species sensu BSC, then hybridization would result in the production of viable offspring. If, however, they are distinct biological species, some level of reproductive isolation is expected. The plants used in this experiment were grown from the seeds germinated in the laboratory. Once seedling cotyledons expanded they were transferred to potting mix (Farfard 3B) and grown in a growth chamber. One of the problems that arose with this design was that when seeds were germinated under the same conditions *P. purshii* and *P. fimbriata* did not demonstrate the same vigor either in seed germination or plant growth. The conditions used during seed germination and in the growth chamber, where the maturing plants were grown, appeared more favorable to *P. purshii*. As a result, fewer *P. fimbriata* seeds germinated, the seeds that did germinate were started later in the growth chamber, and they did not grow as vigorously. Nevertheless, some healthy *P. fimbriata* plants were available for use in the hybridization experiments as both pollen and seed parents.

Upon flowering, pollen viability was tested by staining pollen obtained from two flowers with lactophenol-analine blue and observing under a light microscope (Levy 1991). The freshly dehisced anthers were touched to a clean slide in order to transfer pollen grains. One drop of lactophenol-analine blue stain was applied and the pollen was covered with a cover slip. Fertile pollen takes up the stain and appears larger, rounder, and slightly darker against the background (Levy 1991). Plants with pollen viability of greater than or equal to 90% were considered fully fertile and used for the crossing experiments. In addition, two typical flowers from each plant were preserved for morphological comparison. The plants that were to become seed parents

were emasculated in the bud to prevent unwanted pollen contamination. The anthers from the pollen parents were removed with sterile forceps and the receptive stigmatic regions of the seed parent were manually pollinated daily for several days until the styles began to turn brown.

For each seed parent, several flowers on one or more inflorescences were not pollinated to serve as negative controls for pollen contamination. In addition, crosses to *P. dubia*, another member of the subgenus Cosmanthus, were performed (Table 2). Plants were assessed for the presence of swollen seed capsules and resultant seeds were collected. Seven different seed-pollen parent combinations were completed for interspecific crosses with *P. purshii* as the seed parent and *P. fimbriata* as the pollen parent. For each species, plants from two different populations were represented (Table 3). Four different seed-pollen parent combinations were used for the reciprocal crosses. Four *P. fimbriata* seed plants from two different populations were crossed with three pollen plants representing all three *P. purshii* populations (Table 4).

Table 2 Crossing Design for *P. purshii*, *P. fimbriata*, and *P. dubia* Hybridizations

Ovule Parent	**Pollen Parent**		
	fimbriata	*purshii*	*dubia*
fimbriata	✓ (3)	✓ (4)	x
purshii	✓ (7)	✓ (10)	✓ (7)
dubia	x	x	x

✓ = crosses conducted; number in parentheses are the number of different parental combinations.
x = crosses not conducted

Table 3 Crossing Design for *P. purshii* ♀ x *P. fimbriata* ♂ Interspecific Crosses

		P. fimbriata ♂ Populations		
		BB2	RM1	RM2
P. purshii ♀ populations	PC2	x	✓	x
	PC3	x	x	✓
	PC5	✓	x	x
	PC6	✓	x	x
	PC7	x	x	✓
	RH1	x	✓	x
	RH3	x	x	✓

✓ = crosses conducted
x = crosses not conducted
P. fimbriata populations: BB = Big Bald; RM = Roan Mountain
P. purshii populations: PC = Paddle Creek, RH = Red Hill
number beside the population code represents different individuals from each population

Table 4 Crossing Design for *P. fimbriata* ♀ x *P. purshii* ♂ Interspecific Crosses

		P. purshii ♂ Populations		
		PC1	RH1	RF1
P. fimbriata ♀ populations	BB2	x	✓	x
	RM1	✓	x	x
	RM4	x	x	✓
	RM9	x	x	✓

✓ = crosses conducted
x = crosses not conducted
P. fimbriata populations: BB = Big Bald; RM = Roan Mountain
P. purshii populations: PC = Paddle Creek, RH = Red Hill, RF = Rocky Fork
number beside the population code represents different individuals from each population

Pollen Tube Visualization

To determine the developmental stage at which a reproductive barrier was manifested, additional controlled pollinations were conducted between *P. purshii* and *P. fimbriata* to determine whether pollen tubes emerged from pollen grains, estimate rates of pollen tube growth and to determine whether pollen tubes entered ovules. A pollen tube entering an ovule was considered evidence that fertilization was likely.

The crossing design for this pollen tube assay consisted of intra- and interspecific crosses using two seed plants and two pollen parents for each species. For each combination (two intraspecific; two interspecific), four replicates were conducted (Table 5). In order to assess differences in growth rates, one set of 36 pollen tubes were allowed to grow for 4 hours after pollination. The other set of 36 pollen tubes were allowed to grow for 24 hours to determine whether pollen tube entered ovules.

Table 5 Crossing Design for *P. fimbriata* and *P. purshii* Pollen Tube Assay

Seed Plant	Pollen Plant Time prior to style fixation	
	4-hour	24-hour
P. purshii (2)	*P. purshii* (2)	*P. purshii* (2)
	P. fimbriata (2)	*P. fimbriata* (3)
P. fimbriata (3)	*P. purshii* (2)	*P. purshii* (2)
	P. fimbriata (2)	*P. fimbriata* (2)

The number in parentheses indicates the number of individual plants used. All combinations had four replicated pollinations for a total of 64 pollinated flowers.

Plants were grown as described in the hybridization experiments. Pollen fertility was assayed for all plants involved in the crosses, seed parents were emasculated in the bud, and the receptive stigmatic regions were pollinated. Pollen fertility was > 90% for all plants included in this experiment. After pollination, pollen grains were allowed to germinate and grow pollen tubes for either 4-hours or 24-hours, after which the gynoecia were removed from the plant and placed in FAA (5 parts stock formalin, 5 parts glacial acetic acid, and 90 parts 50% ethanol) until preparation for viewing pollen tubes using fluorescence microscopy. Before staining and viewing, the gynoecial tissue was rinsed in dH_2O then cleared in 8N NaOH for 12 hours. The

NaOH was rinsed from the cleared gynoecia in a 1-hour soak with several changes of dH_2O, then stained with 0.1% aniline blue dissolved in 0.1 M of K_3PO_4 for 30 minutes (Kho & Baer 1968). Once stained the gynoecia were gently squashed and viewed using fluorescence microscope model Leitz Ortholuix with a Xenon source and a 510 barrier for the fluorescence. An excitation wavelength of 365 μm was used to excite aniline blue. All stained gynoecia were observed to determine the number of pollen grains at the stigma as well as to count the number of pollen tubes that traversed each portion of the style. For data collection, the style was divided into stigma, stigma to fork, fork to ovary, and ovary regions and then scored for numbers of pollen tubes present. These data were then compared using heterogeneity chi-square analysis. All ovaries were viewed to determine whether or not the pollen tube had entered an ovule.

Phylogenetic Analysis

DNA sequencing was conducted to test assumptions based upon the ecological speciation hypothesis, to assess genetic diversity within and between species, and to clarify the relationship of *P. fimbriata* to *P. purshii*. DNA was extracted using a Puregene DNA Purification Kit from fresh, young, leaf tissue from the plants grown for the hybridization experiments. The resultant DNA product contained large insoluble precipitates. This extract resulted in poor quality DNA for polymerase chain reaction. DNA was extracted a second time using a hexadecyltrimethylammonium bromide (CTAB) procedure with two chloroform isoamyl alcohol (CIA) extractions (Doyle and Doyle 1987). This procedure yielded high quality DNA. An additional DNA isolation method was also performed using DNeasy Plant Mini Kit (Qiagen, Valencia, CA), which also yielded high quality DNA. DNA judged to be high quality showed

single tight high molecular weight DNA fragments with virtually no smearing in 0.8% agarose gels.

Polymerase chain reaction (PCR) was performed on these DNAs using primers for the internal transcribed spacer ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') and ITS5 (5'GAA AGT AAA AGT CGT AAC AAG G 3') (Gilbert et al. 2005). The PCR cycle consisted of an initial 10 minute denaturation at 94° C followed by 35 cycles of denaturation at 94° C for 30 seconds, annealing at 55° C for 60 seconds, and extension at 72° C for 40 seconds. These 35 cycles were followed by 7 minutes at 72 ° C to allow complete extension. A 4° C soak followed. ITS4 and ITS5 primers amplify a region in the nuclear genome referred to as the internal transcribed spacer (ITS). The ITS region is located between the 16S and 28S ribosomal subunit genes (Figure 6).

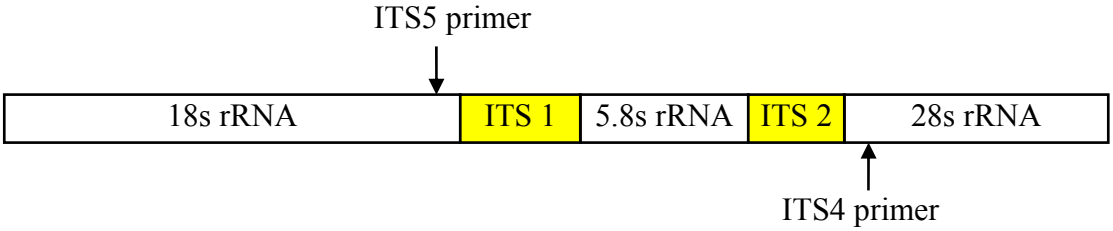


Figure 6 Internal Transcribed Spacer (ITS). The ribosomal RNA genes are shown in white and the subunit density is indicated. The ITS sequences are shown in yellow. The arrows indicate the location of the two primers used in the PCR reactions and in sequencing.

The primers were both located within the ribosomal DNA (rDNA) sequences, which are highly conserved. Unlike the rDNA sequences, the ITS regions are spliced from the transcripts and are therefore more subject to mutational variation. Consequently, ITS sequences display relatively rapid evolution and differences can be detected at the species and even at the population level (White et al. 1990).

The PCR products were purified using the ChargeSwitch PCR Clean-Up Kit (Invitrogen Corporation, Worldwide) and then sequenced at the ETSU/JHQCOM Molecular Biology Core Facility. Sequences were aligned using Multalin (Corpet 1988) and ClustalW (Chenna et al. 2003) and were manually corrected for obvious errors such as frame shifts. Sequence regions that could not be unambiguously aligned were excluded from the analysis. Additional sequences were downloaded from the NCBI GenBank database for other *Phacelia* species as well as representatives from all available genera in the Hydrophyllaceae. These sequences were used to construct a phylogenetic tree for the family using PAUP* version 4 (Swofford 2002).

CHAPTER 3

RESULTS

Germination Experiments

Seed germination in the field began at a later date compared to the seeds germinated in the laboratory. The field-germinated seeds did not begin to show activity until mid October which was after a series of days with cool temperatures (Figure 7). Temperature records from the National Weather Service forecast center in Morristown, TN showed daytime temperatures of 20-25^{o} C and nighttime temperatures of 7-12^{o} C when the germination bursts began.

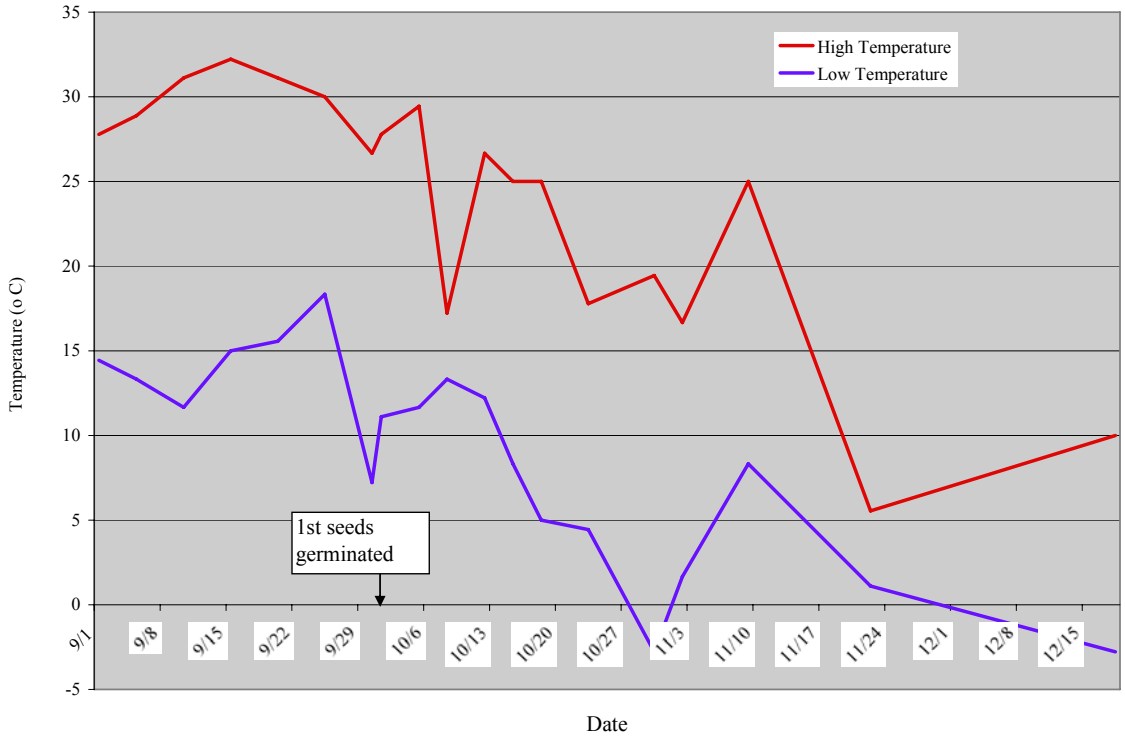


Figure 7 High and Low Temperatures for the Tri-Cities, TN Region From 9/08/05 to12/15/05. Data from the National Weather Service forecast office in Morristown, TN.

As the field germination data were collected, it was noted that some seedlings were lost

and additional seeds continued to germinate throughout the monitoring period. To compensate for these losses (which probably resulted from an inability to exclude invertebrates from the wire cage) and gains, the total number of seeds that germinated for each pot was tallied and that cumulative value was used to calculate percent germination and *P. purshii* population germination means (Figure 8).

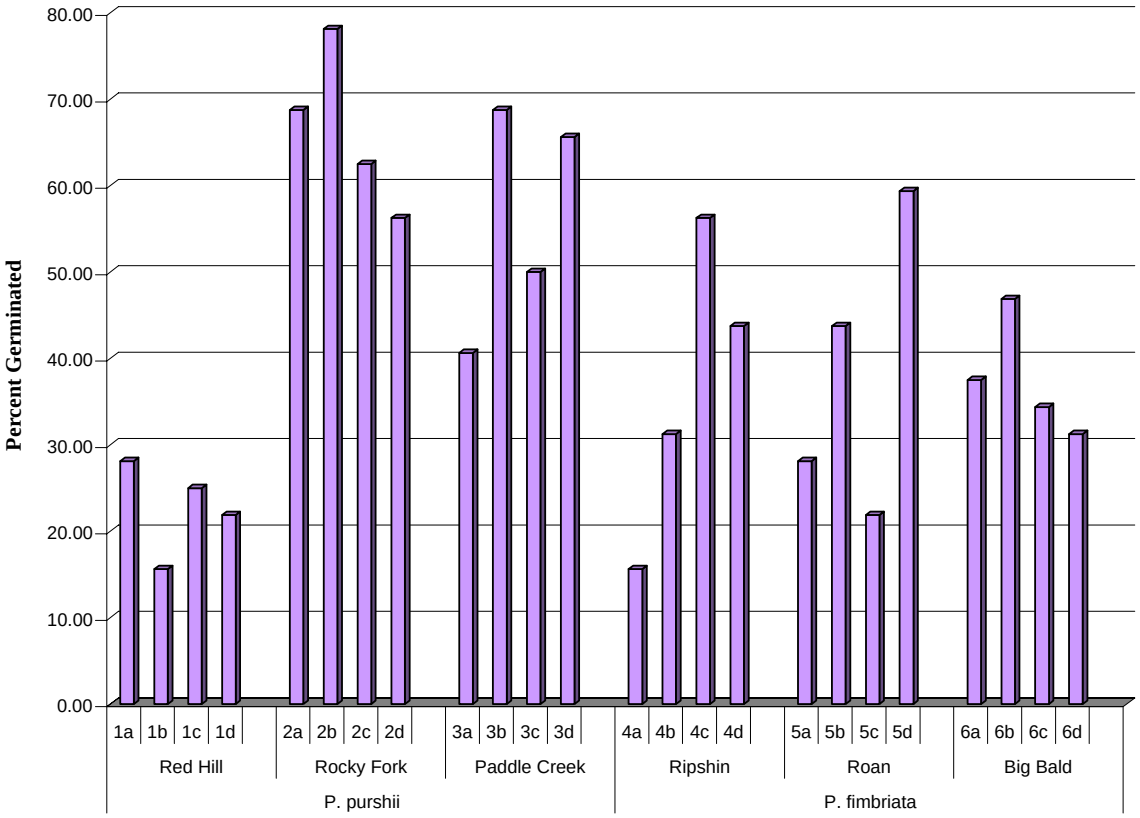


Figure 8 Percent Germination for Field-Germinated Seeds of *P. purshii* and *P. fimbriata* Shown by Species and Population. Four pots were used for each population, each with 32 seeds. These replicates are indicated by "a", "b", "c", and "d".

Percent germination was arc-sine transformed and population and species means were compared using a nested ANOVA. A comparison was made both between species and among populations of species (Tables 6 and 7). The only significant difference was between populations of *P. purshii* (P = 0.0015). An LSD *a posteriori* comparison of means showed the Red Hill population

had significantly lower germination ($\bar{x}$ = 7.25; sd = 1.71) compared to the Rocky Fork population ($\bar{x}$ = 21.25; sd = 2.99) and Paddle Creek population ($\bar{x}$ = 18.00; sd = 4.24) (Figure 9 and Appendix A).

Table 6 Results of Nested ANOVA Comparing Numbers of Field-Germinated Seeds Between Species and Populations of *P. fimbriata* and *P. purshii*

Source	**df**	**MS**	**F**	**P**
Between species	1	80.67	0.90	0.400
Between populations (within species)	4	89.95	5.66	0.004
Error	18	15.89		

Table 7 Results of One-Way ANOVAs Comparing Numbers of Field-Germinated Seeds Among Populations Within Species

A. *Phacelia fimbriata*

Source	**df**	**MS**	**F**	**P**
Between populations	2	2.33	0.12	0.89
Error	9	15.89		

B. *Phacelia purshii*

Source	**Df**	**MS**	**F**	**P**
Between populations	2	177.58	14.56	0.002
Error	9	19.58		

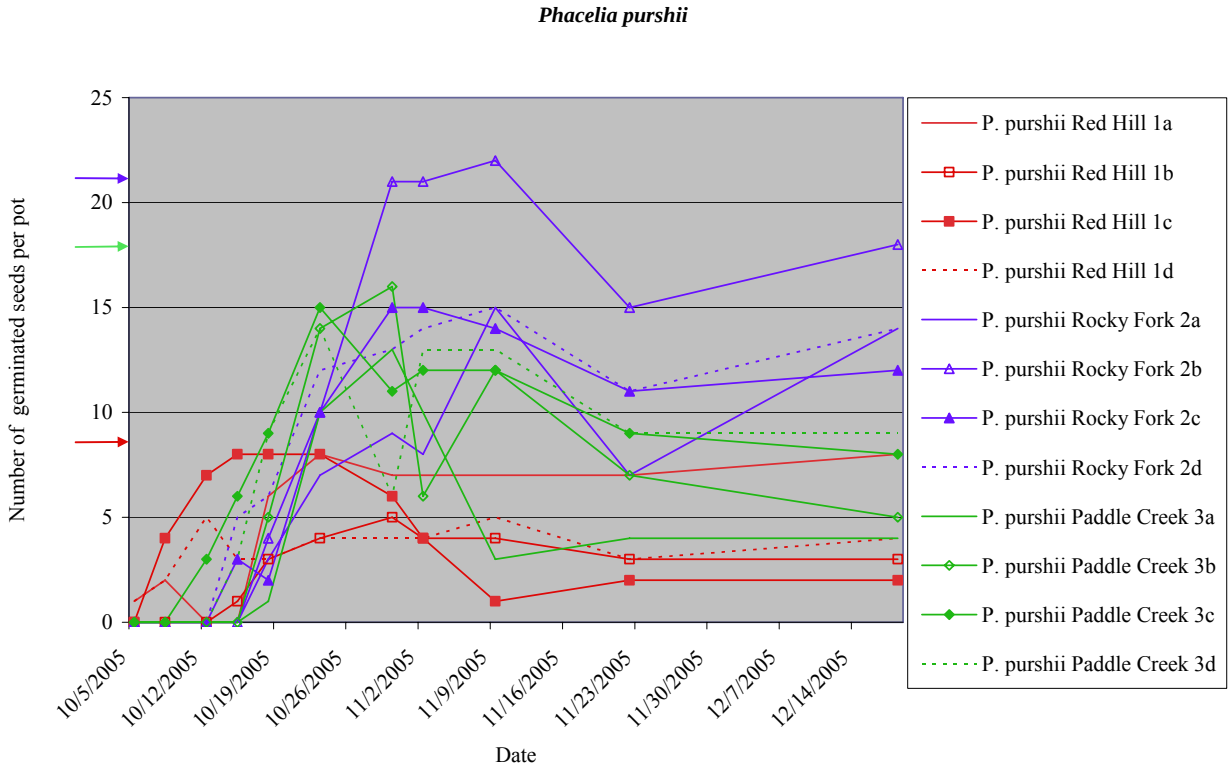


Figure 9 Timeline of Field Germination of *P. purshii* Seeds. The arrows on the sides mark the mean number of seeds germinated for each population based on the cumulative numbers of germination seeds per pot (Appendix A). Population color codes: Red Hill = red; Rocky Fork = purple; Paddle Creek = green

Seeds in the lab started germinating on September 19th after only 13 days in the growth chamber (Figure 10). Percent germination was arc-sine transformed and population and species means were compared using a nested ANOVA. A comparison was made both between species and among populations within species. In the laboratory there was a significant difference in seed germination between species, but not between populations within species (Table 8). Except for the Rocky Fork population, *P. purshii* seeds germinated and had high rates of germination (71-100%) in the growth chamber compared to seeds of *P. fimbriata* (Appendix B).

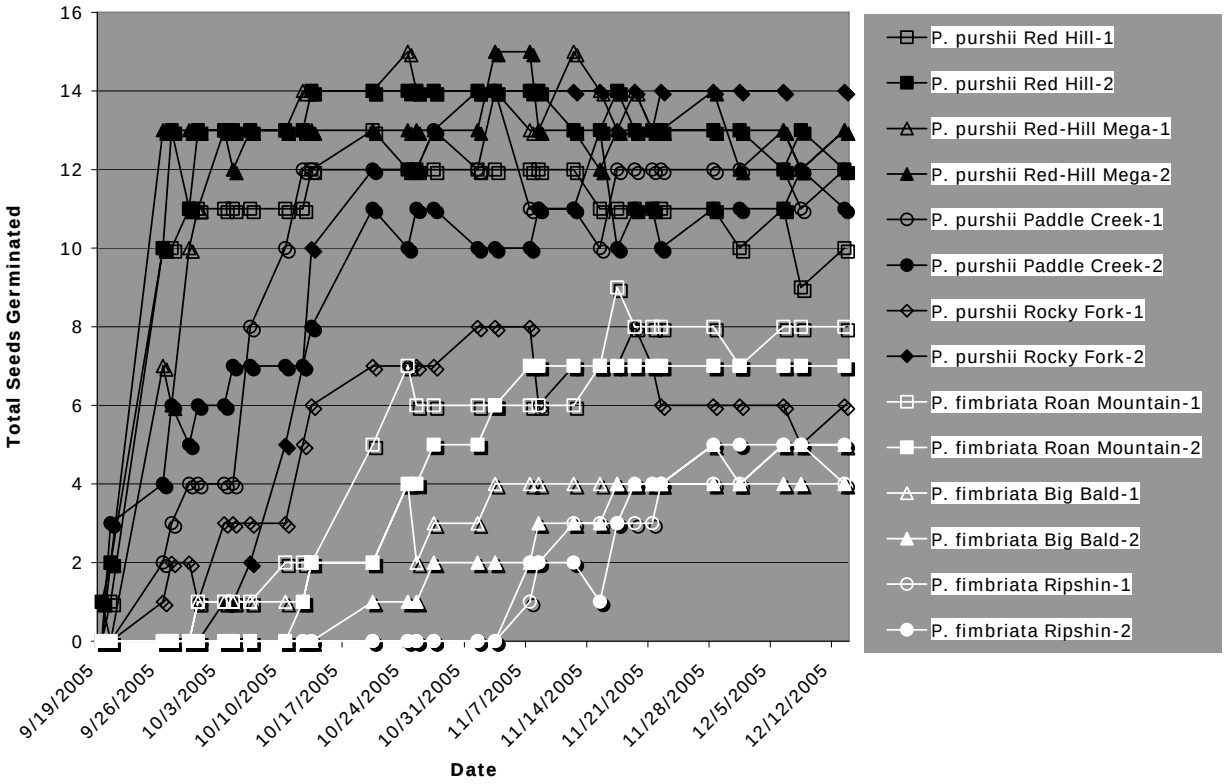


Figure 10 Timeline of Seed Germination in the Lab by Species, Population and Replicate Germination Dish. Species color codes: *P. fimbriata* = white; *P. purshii* = black.

Table 8 Nested ANOVA Comparison of Numbers of Laboratory-Germinated Seeds Between Species and Populations of *P. fimbriata* and *P. purshii*

Source	df	MS	F	P
Between species	1	0.550	29.46	0.003
Between populations (within species)	5	0.019	0.68	0.650
Error	7	0.028		

Hybridization Experiment

A total of seven different combinations of interspecific crosses were made using seven *P. purshii* seed parents from two different populations and three *P. fimbriata* pollen parents from

two populations. Four different seed-pollen plant combinations were used for the reciprocal crosses with four seed parents from two populations and three pollen parents from three populations (Table 9). Flowers on a minimum of two inflorescences were pollinated on each seed plant.

Table 9 Interspecific and Intraspecific Crossing Combinations Sorted by Species and Population

Pollen Plant			*P. purshii*				*P. fimbriata*		
			PC1	PC10	RF1	RH1	BB2	RM1	RM2
Seed Plant	*P. purshii*	PC2	✓					✓	
		PC3	✓						✓
		PC4		N = 7			N = 7		
		PC5		✓			✓		
		PC6		✓	✓		✓		
		PC7							✓
		RH3			✓				✓
		RH0	✓					✓	
	P. fimbriata	BB2				✓		✓	
		RM1	✓	N = 4				✓	
		RM4			✓		N = 2		
		RM9			✓				

Phacelia purshii populations: PC = Paddle Creek; RF = Rocky Fork; RH = Red Hill
Phacelia fimbriata populations: BB = Big Bald; RM = Roan Mountain
✓ = crossed
shaded boxes are interspecific crosses
numbers in boxes are the total number of cross combinations

After completing these crosses, observations were made regarding the seed capsule and the hybrid seeds produced (Table 10).

Table 10 Qualitative Summary of Results From Controlled Crosses

	Control (no pollination)	Intraspecific	Interspecific
Capsule	Not enlarged	Enlarged	Enlarged
Seed coat	No sculpturing	Sculpturing	Sculpturing
Seed coat color	White/cream	Brown	Brown
Seed size	Undeveloped	Large (full size)	Small to Large
Seed contents	Undeveloped	Embryo & Endosperm	Empty
Seed viability	Inviable	Viable	Inviable

Almost all pollinated flowers gave rise to swollen ovaries, while the ovaries of the no-pollen control flowers did not enlarge (Figures 11, 12, and 13). Furthermore, interspecific crosses yielded inviable seeds that lacked contents (embryo; endosperm). However, the size of the inviable seeds was nearly normal, the seed coat had the rich brown color characteristic of these species, and the seed coat had the species-specific sculpturing (Figure 14). In contrast, viable seeds were produced as a result of intraspecific crosses as evidenced by the full seed contents and ability of these seeds to germinate and produce viable and fertile offspring. Seeds from intraspecific crosses were the source of plants for the pollen tube study.

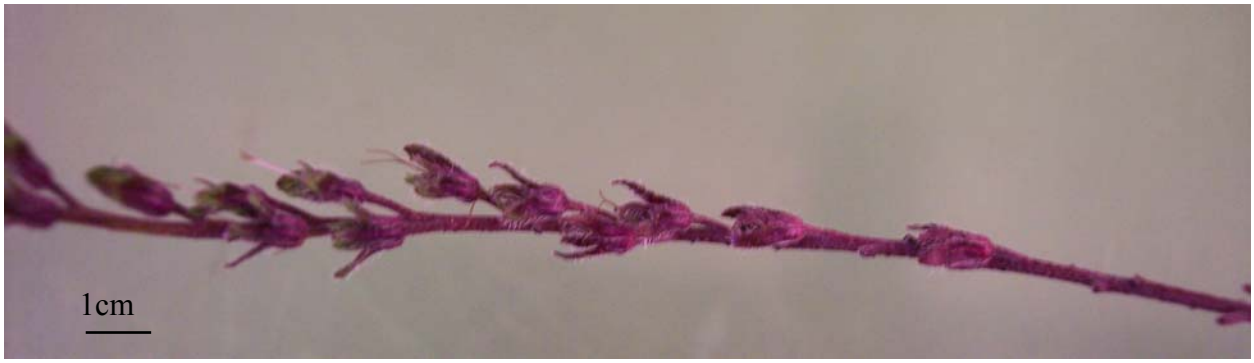


Figure 11 Photograph of *P. purshii* Control Inflorescence (No Pollination). Note the ovaries are not swollen.

Figure 12 Photograph of an Intraspecific Cross *P. purshii* x *P. purshii*. Note swollen ovaries.

Figure 13 Photograph of an Interspecific Cross *P. purshii* x *P. fimbriata*. Note swollen ovaries.

While seed capsules derived from interspecific crosses developed to the full size expected for a capsule with mature seeds, the enclosed "seeds" were obviously inviable. Many of the seeds had the characteristic brown color and the sculptured seed coat of a viable seed, but the seeds were empty and disintegrated when light pressure was applied.

A maternal influence on seed size was noted. Seeds of *P. fimbriata* are larger than those of *P. purshii*. Similarly, although inviable, interspecific hybrid "seeds" derived from crosses with *P. fimbriata* as the ovule parent were larger than those of the reciprocal cross combination (Figure 14.)

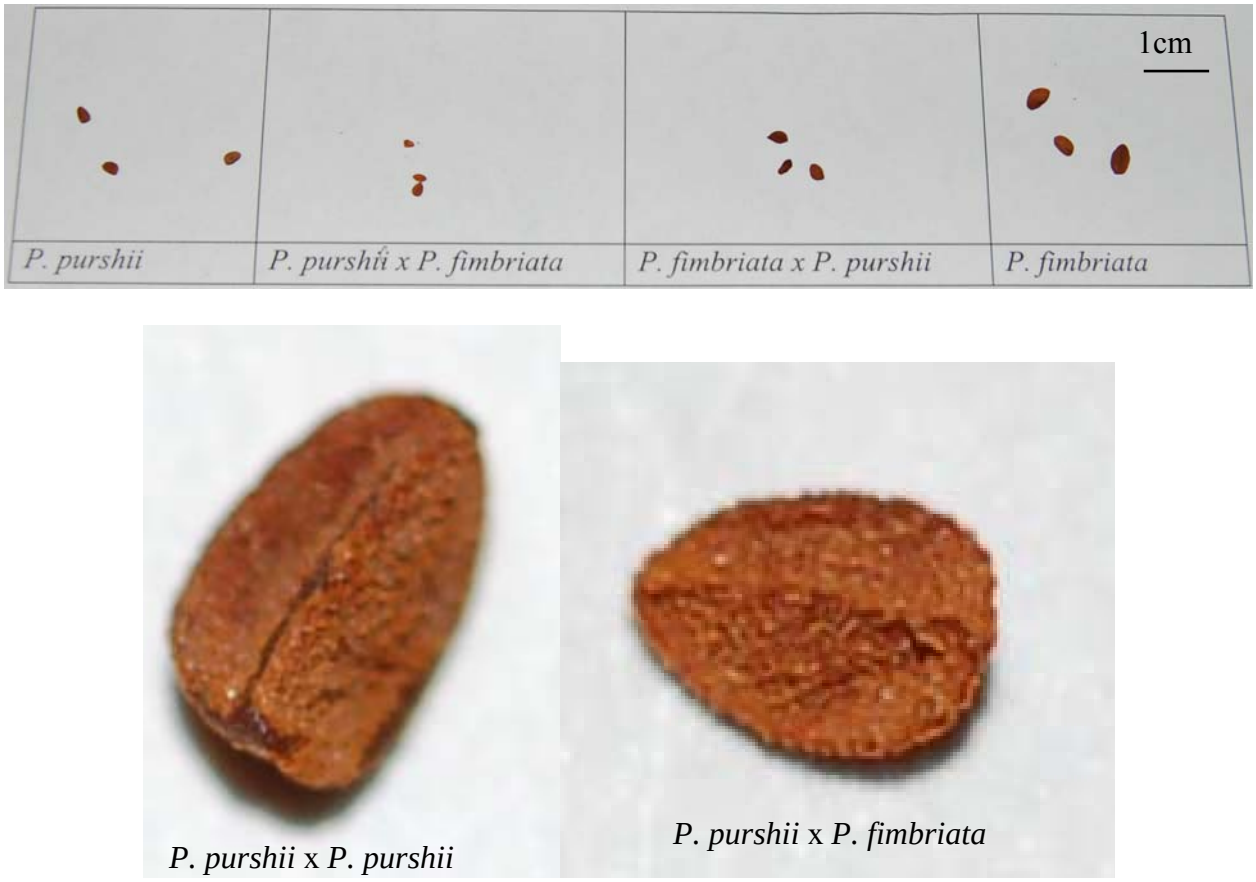


Figure 14 Photographs of *Phacelia* Seeds Resulting From Controlled Crosses. Note the maternal influence on the seed size and shape in seeds from interspecific crosses.

In intraspecific crosses, 64.1% of 248 pollinated ovaries were swollen in *P. purshii* ♀ x *P. purshii* ♂ crosses and 74.1% of 35 ovaries were swollen in *P. fimbriata* ♀ x *P. fimbriata* ♂ crosses, compared to 62.8% of 592 pollinated ovaries swollen in *P. purshii* ♀ x P. *fimbriata* ♂ crosses and 33.2% of 250 pollinated ovaries swollen in *P. fimbriata* ♀ x *P. purshii* ♂ crosses (Figure 15). Differences in percentage of swollen ovaries were observed when different researchers conducted crosses with the exception that all intraspecific *P. fimbriata* crosses were conducted by a single researcher (FL). To compare differences between researchers, analysis of frequencies and heterogeneity chi-square tests were conducted (Table 11).

Table 11 Test for Heterogeneity Among Researchers in Percentage of Swollen and Not Swollen Ovaries

A. Intraspecific *P. purshii* crosses.

Researcher	Swollen (%)	Not Swollen (%)	Total
Michele Glass	48 (39.3)	74 (60.6)	122
Foster Levy	111 (88.1)	15 (11.9)	126
Total	159 (64.1)	89 (53.8)	248

$\chi^2 = 65.43$; df = 3; P <0.0001

B. Intraspecific *Phacelia fimbriata* crosses.

Researcher	Swollen (%)	Not Swollen (%)	Total
Michele Glass	0	0	0
Foster Levy	25 (71%)	10 (29%)	35
Total	25 (71%)	10 (29%)	35

C. *Phacelia purshii* ♀ x *P. fimbriata* ♂ crosses.

Researcher	Swollen (%)	Not Swollen (%)	Total
Michele Glass	145 (48.5)	154 (51.5)	299
Foster Levy	227 (77.5)	66 (22.5)	293
Total	372 (62.8)	220 (37.1)	592

$\chi^2 = 53.20$; df = 3; P <0.0001

D. *Phacelia fimbriata* ♀ x *P. purshii* ♂ crosses.

Researcher	Swollen (%)	Not Swollen (%)	Total
Michele Glass	30 (17.8)	139 (82.2)	169
Foster Levy	53 (65.4)	28 (34.5)	81
Total	83 (33.2)	167 (66.8)	250

$\chi^2 = 56.14$; df = 3; P <0.0001

In each analysis the calculated chi-square value is much higher than expected; therefore, the pollination success of the researcher conducting the crosses was different. Despite the differences due to pollinator experience, there remained a clear pattern between pollination and ovary swelling. For each type of cross combination the ovary would swell as a result of pollination. Ovary swelling was observed in both intra- and interspecific crosses but swelling was not observed in unpollinated controls (Figure 15).

When comparing the amount of swelling in intraspecific crosses to interspecific crosses there was a significantly higher proportion of swelling in the intraspecific crosses (Table 12).

Table 12 Analysis of Frequencies for Intra- and Interspecific Crosses Comparing Swollen and Not Swollen Ovaries

Cross	Swollen (%)	Not Swollen (%)	Total
Intraspecific	184 (65.0)	99 (34.9)	283
Interspecific	455 (54.0)	387 (46.0)	842
Total	639 (56.8)	486 (43.2)	1,125

$\chi^2 = 10.19$; df = 3; P = 0.0014

This is probably not a biologically significant result but may reflect a combination of the lowered pollination success rate of the inexperienced researcher (MG) and the difficulty in pollinating the *P. fimbriata* plants grown in the growth chamber. *Phacelia fimbriata* were more difficult to use

as ovule parents because their inflorescences were somewhat stunted and grew close to the base of the plant rather than growing away from the base of the plant as in *P. purshii*.

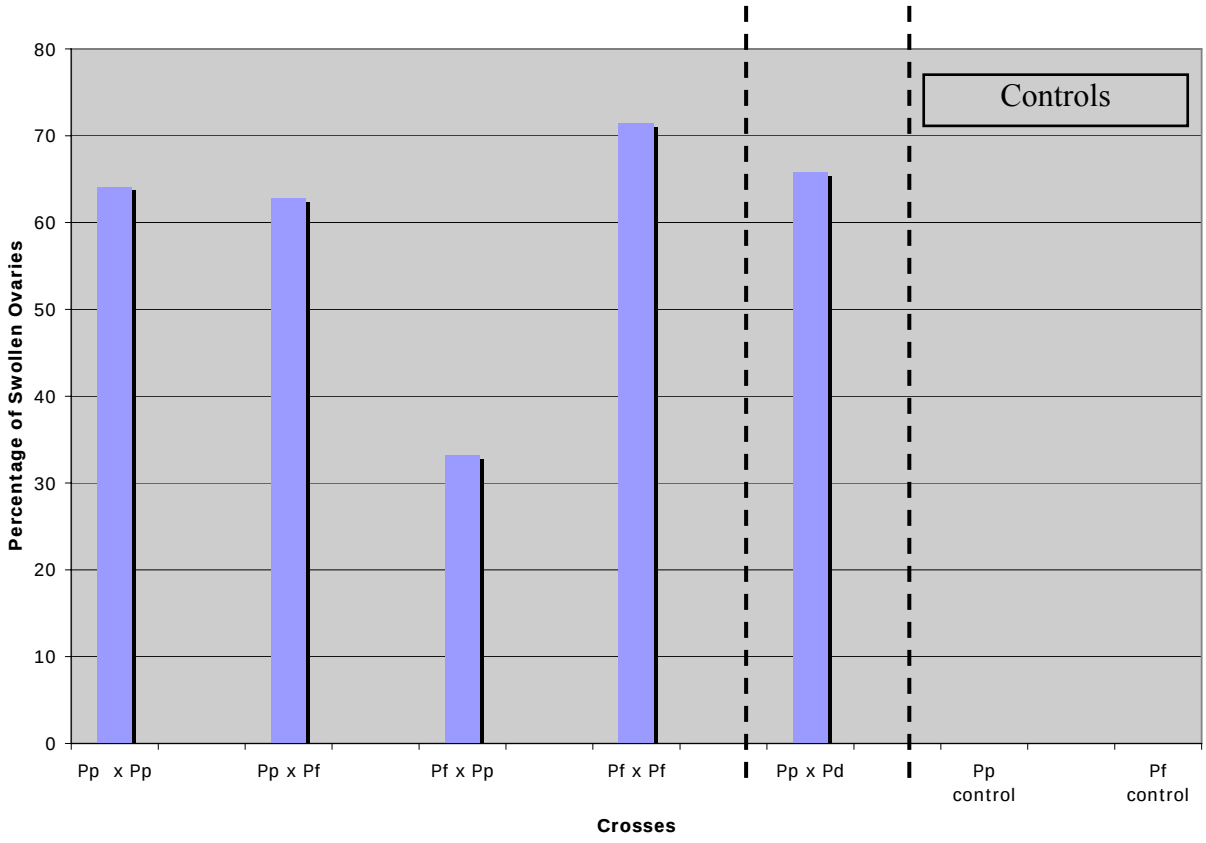


Figure 15 Percentages of Swollen Ovaries for Each Cross Combination. Pp = *P. purshii*; Pf = *P. fimbriata*; Pd = *P. dubia*; controls were unpollinated flowers. Female parents listed first.

Pollen Tube Experiments

No significant difference was observed in the rate of pollen tube growth or the frequency of fertilization between intraspecific and interspecific crosses (Appendix C). Pollen tubes were observed germinating and growing in the style, growing down to the ovary, and entering the ovule for both intra- and interspecific crosses and their reciprocals. Although no differences were observed between crosses, there were differences observed in the frequency of fertilization

for flowers that were preserved at 4 hours after pollination and those preserved at 24 hours after pollination. For the most part, 4 hours was sufficient for pollen tubes to germinate and begin to grow in the style from the stigmatic region to the style fork. However, after 4 hours few pollen tubes traveled beyond the style fork (Figure 17).

Although in a few cases a pollen tube entering an ovule was observed within the 4-hour time span, it was much more likely to occur after 24 hours (Figure 16). There was a significantly higher frequency of presumed fertilization after 24 hours compared to 4 hours (Table 13).

Table 13 Comparison of Frequencies of Fertilization in Crosses Preserved at 4-Hours and 24-Hours Using Fisher's Exact Test.

Hour	Fertilized (%)	Not Fertilized (%)	Total
4	2 (15.4)	11 (84.6)	13
24	13 (72.2)	5 (27.8)	18
Total	15 (48.4)	16 (51.6)	31

$\chi^2 = 9.81$; df = 3; P = 0.0032

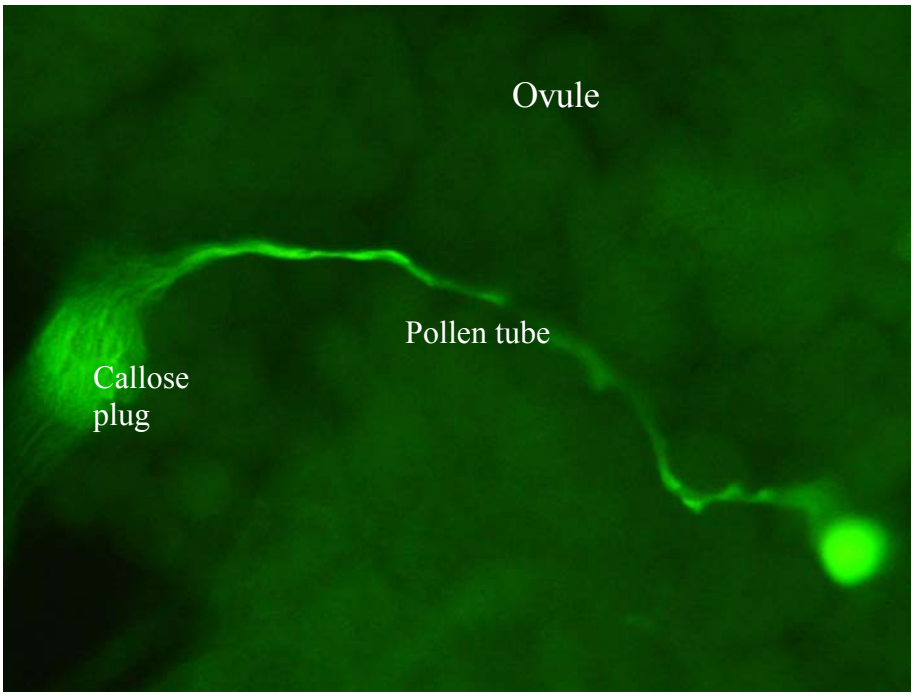


Figure 16 Photograph of *P. purshii* Intraspecific 24-Hour Cross; Pollen Tube Entering an Ovule.

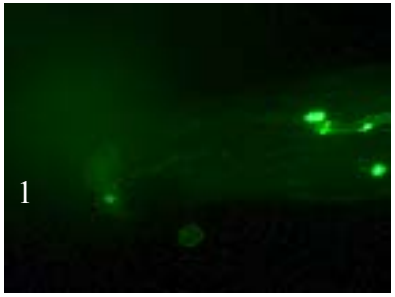
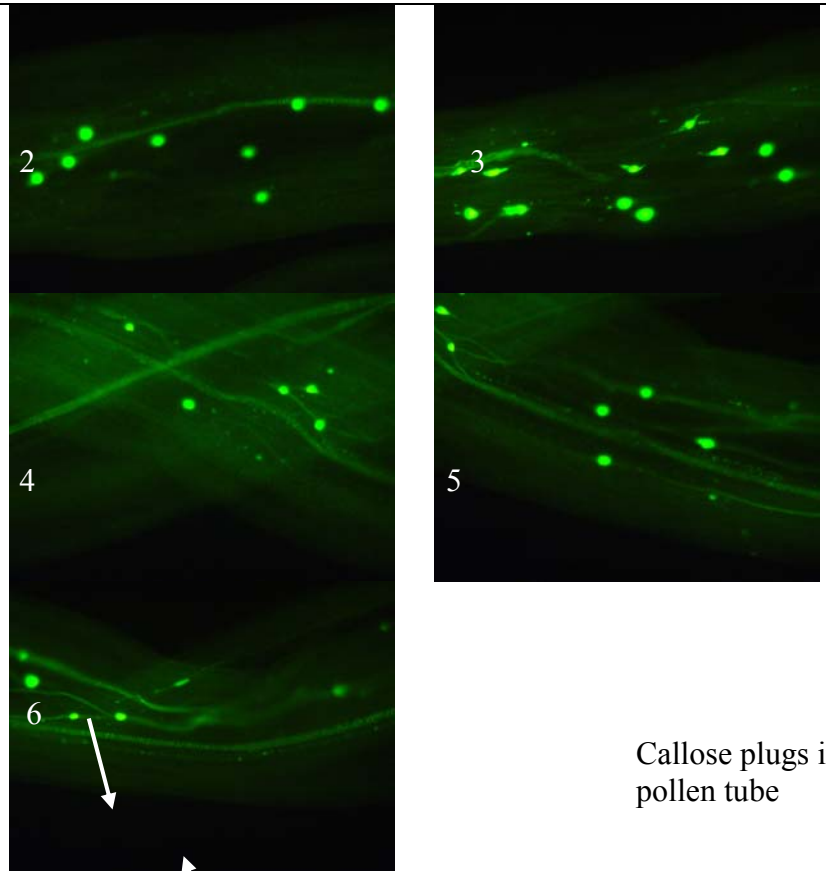
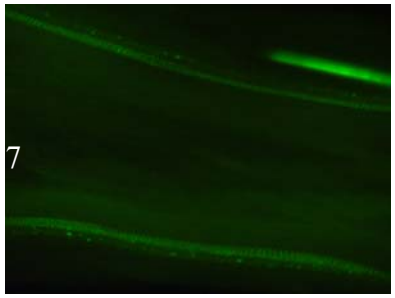
Germinated pollen grains at the stigma.	Pollen tubes growing from right below just below stigma (2) to the style distal to the style fork (6). Photograph numbers indicate a progression down the style	At style fork—note the absence of pollen tubes.
 Pollen grains	 Callose plugs in pollen tube Pollen tubes	 Vascular tissue Style fork

Figure 17 Photographs of Pollen Tubes From *P. purshii* Intraspecific 4-Hour Cross

When considering possible manifestations of reproductive barriers isolating *P. fimbriata* from *P. purshii* it is likely that these two species have similar pollinators. However, based on their different elevational ranges, it is unlikely that individual pollinators travel sufficient distances to effect cross-pollination. Nevertheless if interspecific pollination were to occur in nature, the resultant pollen tubes could grow into the ovule, but the hybrid tissue would fail to develop (Table 14).

Table 14 Potential Reproductive Barriers and Evidence for Their Applicability to Hybridization of *P. fimbriata* and *P. purshii*

Type	Barrier	Data to support	Speculation
Pre-Pollination	Pollinator	Casual observation: both visited by halactid and andrend bees (Levy, personal communication)	Probably similar, but unlikely to travel between populations of different species
	Temporal	Flowering time overlaps as observed in the field during seed collection	
	Spatial	No evidence of sympatry from field or herbarium specimens.	
Post-Pollination	Pollen germination	Yes, observed	
	Pollen tube growth	Yes, observed	
	Fertilization	Likely, observed pollen tubes entering ovules	
	Zygotic development	Embryo fails to develop, "empty" seeds (no embryo or endosperm)	
	Hybrids	Not applicable	

Molecular Evidence of Phylogenetic Relationships and Differentiation

After ITS sequences from *P. fimbriata* and *P. purshii* were aligned with sequences of other *Phacelia* species and genera from Hydrophyllaceae (Tables 15 and 16), several patterns became apparent. Both insertions and deletions (indels) and point mutations were found upon sequence alignment but, the indels appeared to have no phylogenetic importance. As a result, this analysis was based on point mutations.

Table 15 Summary of the *P. fimbriata* and *P. purshii* Sequences Used in the DNA Phylogenetic Analysis

Species	Population	Individual ID
P. fimbriata	Big Bald	BB2
		BB3
		BB4
	Roan Mountain	RM1
		RM6
P. purshii	Paddle Creek	PC1
		PC2
		PC8
	Red Hill	RH3
		RHM1

Table 16 A List of the Sequences Downloaded From NCBI Database. All are members of Hydrophyllaceae.

Species	**NCBI database number**
Phacelia hirsuta (subgenus Cosmanthus)	AF091193
Phacelia ranunculacea (subgenus Cosmanthus)	DQ005986
Phacelia hastate (subgenus Phacelia)	AY630298
Phacelia heterophylla sub. Virgata (subgenus Phacelia)	AY630300
Phacelia parryi (subgenus Phacelia)	AY653742
Phacelia rotundifolia (subgenus Phacelia)	AF091200
Phacelia grandiflora (subgenus Phacelia)	AF091190
Phacelia tetramera (subgenus Phacelia)	AY630333
Draperia systyla	AF091155
Ellisia anycetelea	AF091157
Emmenanthe penduliflora	AF091158
Eriodictyon trichocalyx var. *trichocalyx*	AF091164
Eucrypta micrantha	AF091166

Table 16 (continued)

Hesperochiron pumilis	AF091167
Hydrolea spinosa var paraguayensis	AF091168
Hydrophyllum tenuipes	AF091170
Pholistoma auritum	AF091204
Romanzoffia californica	AY630334
Tricardia watsonii	AF091209
Turricula parryi	AF091210

Parsimony analysis revealed *Phacelia purshii* and *P. fimbriata* are closely related sister species that form a monophyletic clade defined by a single point mutation synapomorphy (Figure 18). With three autoapomorphic point mutations *Phacelia fimbriata* was more divergent than *P. purshii* from all other *Phacelia* species. Despite this divergence, *P. fimbriata* supported less intraspecific variation; *P. fimbriata* had 4 variant sites compared to *P. purshii*'s 12 variant sites (Tables 17 and 18). Also when comparing intraspecific polymorphisms, *P. purshii*'s Paddle Creek population was found to have eight population-specific polymorphisms found in all members of the population. All other intraspecific polymorphisms were unique to individuals rather than to populations (Table 18). *Phacelia hirsuta,* another member of the subgenus Cosmanthus, is closely related to *P. fimbriata* and *P. purshii. Phacelia dubia* and *P. ranunculacea* formed a separate clade within Cosmanthus (Figure 18).

Table 17 Analysis of Frequencies for ITS DNA Sequence Similarities for *P. fimbriata* and *P. purshii*

Species	Unique Polymorphisms	Intraspecific Polymorphisms	Total
P. fimbriata	3	4	7
P. purshii	0	12	12
Total	3	16	19

$\chi^2 = 6.14$; df = 3; p = 0.036

Table 18 Summary of All Variable Sites (Excluding Insertions and Deletions) in *Phacelia* Subgenus Cosmanthus

Position	Cosmanthus	Pf	Pp	Pd	Pr	Ph	Polymorphisms (within species)
131	G→A						
134							PpPC1
137		G→A					
138					C→A		
141							PpPC2 & PpPC8
145							PpPC8
163		G→A					
178							PpPC2 & PpPC8
181							PpRH
182		C→A	C→A				
190							PpPC2
192							PpPC8
194	T→C						
197		G→A					
201							PpPC1
210							PpPC8
218							PpPC2 & PpPC8
224							PfBB3
229	G→A (except Ph)						
232				A→G			
234							PpPC1
252				A→C			
257					T→C		
284				T→G			
484					T→C		
493	G→T						
495				C→A			
500				C→A			
510					C→T		
511				G→A			
512					C→A		
518	G→A						
528					G→A		
532					T→C		
545				C→T			

Table 18 (continued)

Position	Cosmanthus	Pf	Pp	Pd	Pr	Ph	Polymorphisms (within species)	
640		G→A	G→A			G→A		
658	G→T							
670				G→T				
676				T→C	T→C			
683							PpPC1	
687	C→A							
689				G→A				
692	C→T							
701				G→A				
707		G→A	G→A			G→A		
712				A→T				
718	G→A							
741							PfBB3	
745							PfBB3	
746							PfBB3	
TOTAL (unshared)	n = 9	n = 3	n = 0	n = 11	n =8	n =0	Pp	n =12
							Pf	n = 4

The Cosmanthus point mutations are compared to *Phacelia* subgenus Phacelia species
Entries reflect a presumed taxon specific point mutation.
Pf = *P. fimbriata*; Pp = *P. purshii*; Pd = *P. dubia*, Pr = *P. ranunculacea*; Ph = *P. hirsuta*;
PC = Paddle Creek Population; RH = Red Hill population; BB = Big Bald population.
The numbers beside the population indicate a particular individual.

When comparing the point mutations unique to each subgenus within subgenus Cosmanthus (Tables 19-23) there was a significant transition bias with a ratio of almost two transitions for each transversion when the expectation was a 2:1 bias towards transversions (Table 24).

Table 19 Point Mutations for *Phacelia* Subgenus Cosmanthus When Compared to Western *Phacelia* Species (See List From Table 17)

Position	Point Mutation	Type	
131	G→A	S	
194	T→C	S	
229	G→A (not *P. hirsuta*)	S	
493	G→T	V	
518	G→A	S	
548	T→C	S	
658	G→T	V	
687	C→A	V	
692	C→T	S	
718	G→A	S	
TOTAL	n =10	S	n = 7
		V	n =3

S = transition; V = transversion

Table 20 Synapomorphies for *P. fimbriata* and *P. purshii*

Position	Point Mutation	Type		Shared with *P. hirsuta*	
182	C→A	V		x	
707	G→A	S		✓	
640	G→A	S		✓	
TOTAL	n = 3	S	n = 2	x	n = 1
		V	n =1	✓	n = 2

✓ = indicates shared with *P. hirsuta*; x = not shared with *P. hirsuta.*

Table 21 Point Mutations Unique to *P. fimbriata*

Position	Point Mutation	Type	
137	G→A	S	
163	G→A	S	
177	G→A	S	
TOTAL	n = 3	S	n = 3
		V	n = 0

Table 22 Point Mutations Unique to *P. dubia* With the Exception of Position 676 Shared With *P. ranunculacea*

Position	Point Mutation	Type	
232	T→G	V	
252	A→C	V	
284	T→G	V	
495	C→A	V	
500	C→A	V	
511	G→A	S	
545	C→T	S	
676	**T→C**	**S**	
670	T→G	V	
689	G→A	S	
701	G→A	S	
712	A→T	V	
TOTAL	n = 11	S	n = 8
	n = 1 (shared)	V	n = 4

Table 23 Point Mutations Unique to *P. ranunculacea*

Position	Point Mutation	Type	
138	C→A	V	
257	T→C	S	
484	T→C	S	
510	C→T	S	
512	C→A	V	
528	G→A	S	
532	T→C	S	
624	A→G	S	
TOTAL	n = 8	S	n = 6
		V	n = 2

Table 24 Chi-square Goodness-of-Fit Analysis of the Total Number of Transitions and Transversions in *Phacelia* Subgenus Cosmanthus Species

	Observed	Expected
Transition (S)	23	12
Transversion (V)	13	24
Total	36	36

$\chi^2 = 15.14$; df = 1; p = 0.0002

The most parsimonious tree in Figure 18 shows *P. fimbriata* and *P. purshii* among the other subgenus Cosmanthus species with subgenus Phacelia species as an outgroup. There is some support (bootstrap = 76) for *P. fimbriata* and *P. purshii* as sister species closely related to *P. hirsuta*. There is weaker support (bootstrap = 69) for a clade with these three species together: *P. hirsuta, P. fimbriata*, and *P. purshii.* However, when *P. fimbriata* and *P. purshii* sequences are compared in a larger database that included species from subgenus Cosmanthus, representatives from subgenus Phacelia and other genera from Hydrophyllaceae, the relationship between *P. fimbriata, P. hirsuta,* and *P. purshii* was unresolved (Figure 19). There was bootstrap support for the relationship between *P. hirsuta, P. fimbriata*, and *P. purshii* (bootstrap = 80; decay = 2). Nevertheless, in this more comprehensive analysis Cosmanthus forms a strongly supported monophyletic clade (bootstrap = 99; decay = 7) within the genus.

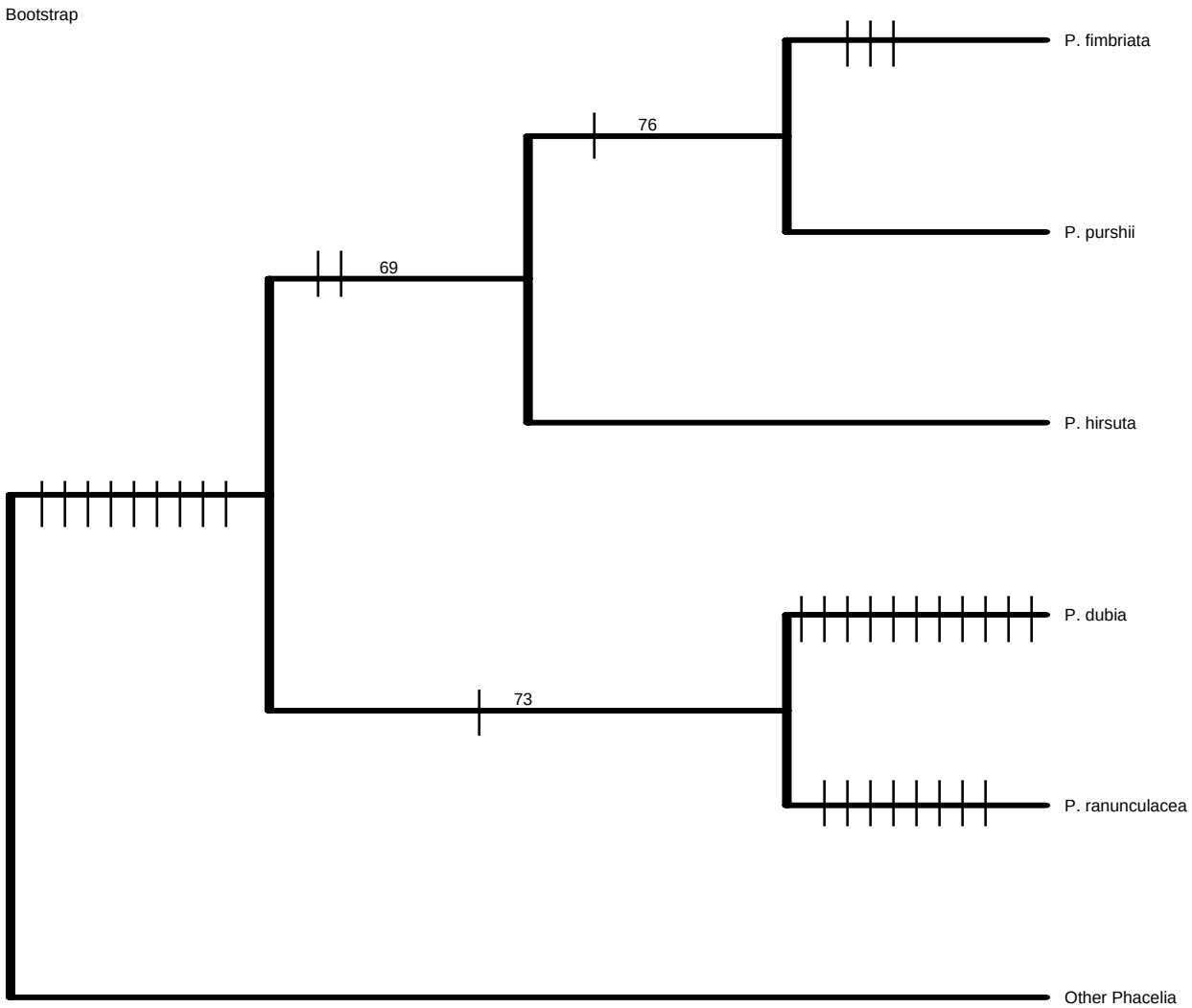


Figure 18 Most Parsimonious Phylogenetic Tree Showing *Phacelia* Subgenus Cosmanthus Species With Western *Phacelia* From Subgenus Phacelia as an Outgroup. Tree length is 36. Bootstrap based on 1,000 replicates. Vertical lines show the synapomorphies for each group. Prepared using PAUP* (Swofford 2002).

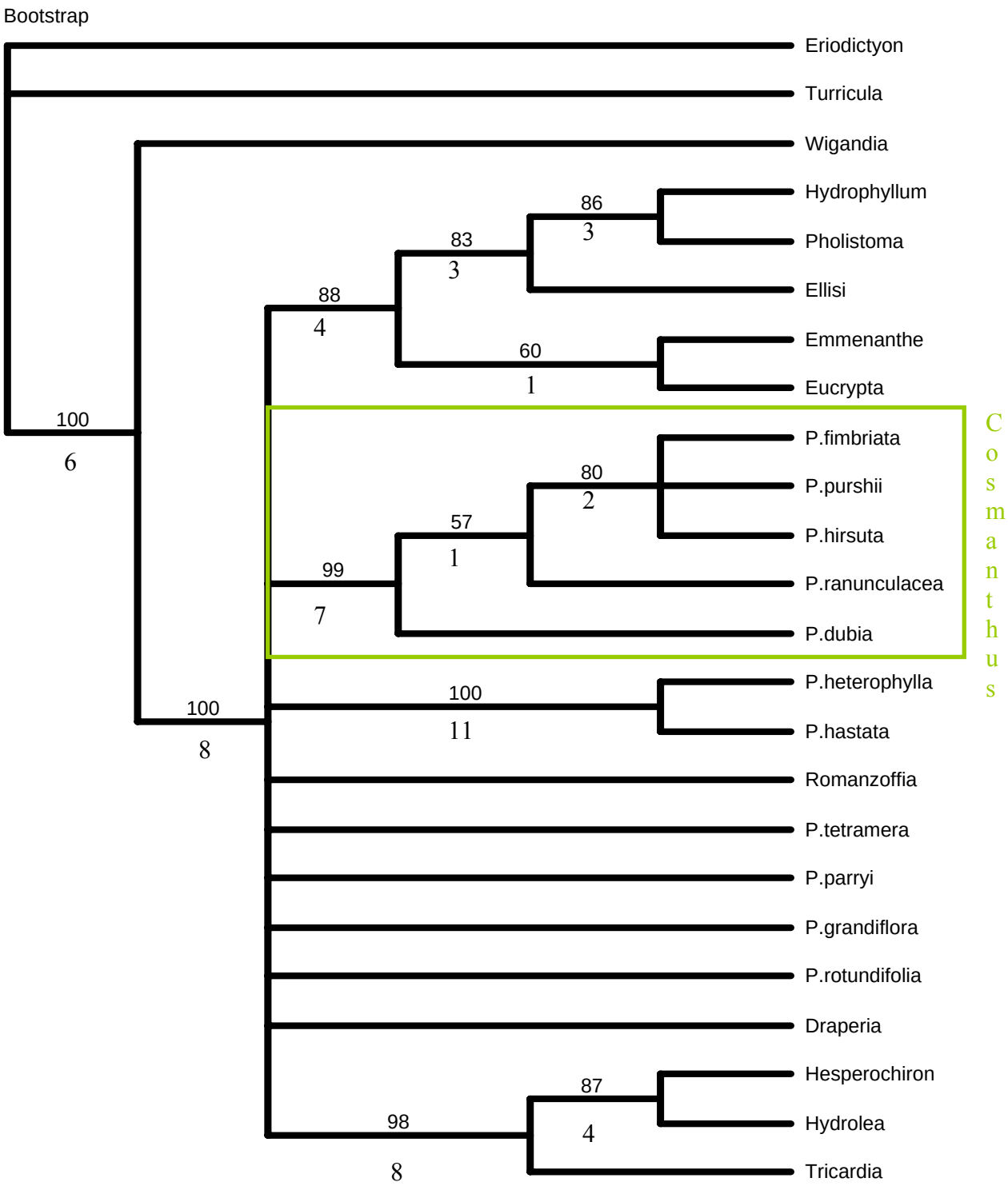


Figure 19 Most Parsimonious Phylogenetic Tree Showing *Phacelia* Cosmanthus Among Other *Phacelia* and Genera From Hydrophyllaceae. Tree length is 430. Bootstrap based on 1,000 replicates. Bootstrap values are above the branch and decay values are below. Prepared using PAUP* version 4 (Swofford 2002) with decay values using Autodecay (Eriksson 1998).

CHAPTER 4

DISCUSSION

Germination Analysis

The outcomes of the germination experiments differed depending on the germination conditions. In the field, there was no species-specific difference in germination between *P. purshii* and *P. fimbriata,* but there was a significant difference between populations in *P. purshii.* In the laboratory, there was a significant difference between *P. purshii* and *P. fimbriata*; however, there were no significant differences between populations within species. One explanation for this apparent contradiction between the two germination experiments is that the temperature in the laboratory was held constant at 20^{o} C/14^{o} C day/night temperatures while the temperature outside varied from day to day and showed a decreasing trend throughout the months of October-December. The colder temperatures outside where the temperature dropped to below zero (^{o} C) coupled with a temperature decline throughout the experiment, would likely better mimic the colder temperatures found in the high elevation habitats of *P. fimbriata*. When held at constant temperatures the growth chamber mimicked only one set of conditions. These conditions favored *P. purshii* seed germination rather than *P. fimbriata* as evidenced by the higher germination rate of 64% versus 37%, respectively. *Phacelia fimbriata* began germinating in the field experiment after nighttime temperatures dropped below 10^{o} C, indicating that this may be the temperature cue required for seed germination. Regardless of the proximal cue, the differential response to a common temperature indicates there is an intrinsic factor underlying ecological differentiation of these two species.

Hybridization Analysis

The hybridization experiment demonstrated that *P. fimbriata* and *P. purshii* are reproductively isolated and thus should be considered "good" species based on the biological species concept. Two noteworthy observations derived from the hybridization crosses were that ovaries from pollinated flowers enlarged compared to unpollinated control flowers and that the seeds from the interspecific hybrids had a recognizable species-specific and characteristic, sculptured seed coat despite the apparent lack of embryo and endosperm development. The seed coat resembled the maternal seed in each case of interspecific hybridization indicating that the maternal tissue developed as a result of pollination while the diploid embryo tissue and triploid endosperm appeared not to. There were also differences observed in the percentage of ovary swelling dependent on the researcher conducting the crosses. This difference in pollination can most likely be attributed to differential experience working with these plants. In order to successfully pollinate these flowers, the stigma has to be receptive to the pollen grains, which occurs 2-3 days after anthesis. Far higher rates of swollen ovaries were observed by the more experienced pollinator (FL) who was better able to judge the timing for optimal pollination. Nevertheless, the pattern of swollen ovaries as a result of pollination was consistent between researchers. The inability to generate interspecific hybrids raised the question of the cause of the reproductive barrier. The observation that maternal tissue developed following interspecific pollination suggests a post-pollination barrier that is likely expressed at zygote formation or during early post-zygotic development.

Pollen Tube Analysis

When given sufficient time (24 hours) pollen grains germinated and grew into the ovule at similar speeds and frequencies in both intra- and interspecific crosses. The growth of pollen tubes into the ovule in both intra- and interspecific crosses supports the conclusion that a zygotic or early post-zygotic barrier prevents the development of viable seeds. The ability of heterospecific pollen to presumably fertilize the ovule further supports the conclusion that pollination triggers the development of the maternal seed tissue despite the fact that the hybrid tissue fails to develop into either an embryo or endosperm. To further understand the nature of the species barrier requires further research.

Phylogenetic Analysis

As was expected, analysis of DNA ITS sequence data showed *Phacelia* subgenus Cosmanthus is a monophyletic group distinct from other *Phacelia* species included in the analysis. Within subgenus Cosmanthus *P. purshii* and *P. fimbriata* are closely related species. However, analysis of sequence data in a larger data set (Figure 19) placed *P. fimbriata, P. hirsuta,* and *P. purshii* together in a clade with moderate support (Bootstrap = 80; decay = 2), indicating that additional data may be needed to better understand the relationship between these species. Based on *P. fimbriata* and *P. purshii*'*s* shared unique morphology, fringed corolla, single DNA synapomorphy and other similarities it seems likely that additional sequence analysis would confirm these two to be sister species.

Genetic Diversity

When analyzing the intraspecific diversity of *P. purshii*, many (12) polymorphic base positions were found in the ITS region. *Phacelia fimbriata* demonstrated low intraspecific variation (4 polymorphic sites), despite being more divergent from the other *Phacelia* species (3 autapomorphies for the species). *Phacelia purshii*, the more widespread species of the two, has more genetic variation perhaps as a result of genetic hitchhiking with adaptations to a more diverse range of habitats or as a selectively neutral consequence of larger and more constant population sizes.

Among the sequences gathered for this study, there was a significant transition bias in point mutations. Because the ITS region does not code for protein there can be no constraint on amino acid substitutions. However, the secondary and tertiary structure of the encoded rRNA may impose constraints. Transition mutations may be more likely to persist if they preserve the secondary and tertiary structure of rRNA. For instance, specific folding of the ITS region is instrumental in determining where to cut out the spacer and a transversion could interrupt the recognition of the splice site. Another possible explanation for this transition bias may be that it is easier to substitute like nucleotides (purine for purine or pyrimidine for pyrimidine).

Systematic Implications

There may be multiple manifestations of an interspecific reproductive barrier: pre-pollination barriers, post-pollination barriers, pre-zygotic barriers, post-pollination zygotic or post-zygotic barriers (Riesberg and Willis 2007). Hybrid inviability and hybrid sterility may also present a barrier to gene flow across species. In the case of *P. fimbriata* and *P. purshii*, heterospecific pollination resulted in pollen germinating and growing pollen tubes into the ovary

and triggering the development of the seed capsule and seed coat. Despite the development of the maternal seed coat, the hybrid seed tissues (embryo and endosperm) failed to develop. Thus in controlled conditions, a post-pollination barrier prevents hybridization between these two species although in nature pollination may never occur due to habitat isolation. Additionally, based on the observation that interspecific pollen tubes enter ovules, this barrier is most likely zygotic or early post-zygotic. In research conducted among intraspecific varieties of a close relative, *Phacelia dubia*, Levy (1991) concluded that the reproductive isolation between varieties was post-zygotic and nuclear-based. This may also be the case between *P. fimbriata* and *P. purshii*. Despite the production of some sterile gametes as a result of inter-varietal *P. dubia* crosses, in backcrosses a range of fertility was observed with some plants achieving full fertility. These data suggest that the causes of hybrid sterility in *P. dubia* varieties could be the result of a difference in a single locus controlling meiotic pairing or due to structural differences among chromosomes such as inversions or reciprocal translocations.

The Bateson-Dobzhansky-Muller (BDM) model of speciation posits that geographically isolated species can develop different suites of mutations or chromosomal rearrangements without impacting the native genome. The reunion of these divergent genotypes, which are no longer compatible, results in reproductive isolation (Rieseberg and Willis 2007). Research conducted with *Mimulus guttatus* and *M. nasutus* demonstrated the applicability of the BDM model for the development of a single locus mutation causing reproductive isolation. Sweigart et al. (2006) found that a single locus was responsible for complete male sterility in *Mimulus* hybrids. Hybrid plants heterozygous for the *M. guttatus* allele for *hybrid male sterility 1* in an *M. nasutus* genetic background were found to have complete male sterility and a significant effect was also found on female fertility.

The complete reproductive isolation of these two *Phacelia* species came somewhat as a surprise due to the large number of shared characteristics. *Phacelia purshii* and *P. fimbriata* are both members of the subgenus Cosmanthus. As members of this group, their range is limited to the eastern part of the United States and they have the same chromosome number (n = 9), which is typical of subgenus Cosmanthus. In addition, *P. purshii* and *P. fimbriata* share a unique characteristic found only in these two species and nowhere else in the family Boraginaceae, a fringed corolla. This morphologically distinctive characteristic strongly suggests a close relationship between these two species especially when considering their overall similarity in morphology. The species have similar leaf size, shape, and dissection, a similar growth habit, and similar flower size, shape, and sometimes color (Constance 1949).

Despite the similarities mentioned above, there is sufficient non-reproductive evidence for separating *P. fimbriata* and *P. purshii*. While it is difficult to differentiate between the two species morphologically, stem pubescence appears to be a reliable characteristic and flower color can be used in most cases. Physiological differences in germination and growth were observed when these species were grown in the same controlled conditions. Molecular evidence from ITS sequence data also showed that these two species have differentiated at many base pairs in their nuclear DNA. *Phacelia fimbriata* and *P. purshii* initially seemed to be good candidates for the cryptic species concept, which describes species as morphologically identical, intersterile, but genetically divergent. However, several forms of evidence negate application of the cryptic species concept. *Phacelia fimbriata* and *P. purshii* are not cryptic species due to differences in morphology and other traits, and these species are reproductively isolated.

Although morphologically similar, *P. fimbriata* and *P. purshii* are not morphologically identical. Two characters can be used to differentiate these species: flower color, which is not

completely reliable, and orientation of the stem hairs. *Phacelia fimbriata* tends to have an all white corolla although flowers occasionally are tinged with blue or lavender (Figure 20). Corolla color in *P. purshii* is more variable. While most often a pale to medium lavender, it can range from dark lavender to completely white (Constance 1949).

Figure 20 Photograph of *P. fimbriata* Herbarium Specimen. Note the lavender corolla edge.

The second diagnostic character, stem pubescence, may be more reliable. When viewed with 10x magnification, *P. fimbriata* stem hairs appear sparse and deflexed (pointed toward the stem base) while *P. purshii*'s are afflexed (pointed toward the apex) (Constance 1949). When trying to identify a plant in the field, rather than focusing on the morphological differences, a simpler but less rigorous characteristic to use would be elevation and location. *Phacelia fimbriata* is strictly a high elevation endemic found in the mesic mountain forests of east Tennessee, southwest Virginia, Georgia, and western North Carolina while *P. purshii* is widespread throughout the Ohio Valley in both wooded and alluvial habitats, but it is not found at high elevations (Constance 1949). A survey of more than 600 herbarium specimens ranging in

time from 1830-present borrowed from six herbaria showed the two species co-occur in several counties in Tennessee, Virginia, and North Carolina. These specimens were borrowed from: Botanical Research Institute of Texas, Missouri Botanical Gardens, New York Botanical Gardens, Smithsonian US National Herbarium, University of North Carolina Herbarium, and University of Tennessee Knoxville Herbarium (Table 25). Review of the herbarium specimens showed that the minimum elevation for *P. fimbriata* was 640 m (2,100 ft) and maximum elevation was 1,585 m (5,200 ft). *Phacelia purshii*'s range was 305 m (1,000 ft) to 1,173 m (3,850 ft). Based on these elevational ranges, populations of *P. fimbriata* and *P. purshii* may not be completely geographically isolated. While the collection labels indicate that both species are found at overlapping elevations in the same county, there is no indication of sympatric populations.

Table 25 An Overview of the Counties by State and Elevations Where *P. fimbriata* and *P. purshii* Populations Co-Occur Based on a Survey of Herbarium Collections and Field Collections. The elevations listed are based on localities listed on herbarium sheets and represent cases where there is overlap between *P. fimbriata* and *P. purshii* in both county and elevation. In some cases the elevation came from the label, while others are estimations based on the description of the location on the label.

North Carolina		Tennessee		Virginia	
County	Elevation (m)	County	Elevation (m)	County	Elevation (m)
Ashe	853	Sevier	275-393	Smyth	628-640
Haywood	792	Unicoi	586		
Watauga	1,173	Carter	561		

Physiological characteristics for optimal germination and growth differ between *P. fimbriata* and *P. purshii*. These differences are genetically-based because when plants were grown together in the same conditions (the common environment of a growth chamber)

differences were seen in germination frequency as well as growth vigor. Under conditions favorable to *P. purshii*, *P. fimbriata* was difficult to germinate, had a lower frequency of germination, grew slowly, had stunted growth and relatively short inflorescences, and was slower to flower and produce fruits. *Phacelia purshii*, on the other hand, was easy to cultivate under lab conditions where it germinated readily, grew vigorously vegetatively and produced multiple long inflorescences, and when pollinated produced prolific seed crops until senescence. These experiments revealed intrinsic differences in environmental germination cues as well as differences in preferences for daytime/nighttime temperatures during their growing season. Because *P. fimbriata* grows naturally in high elevation woods, it likely would have performed better under the cooler temperatures (perhaps 10^{o} C or below) characteristic of that habitat.

As mentioned above, the two species differ markedly in the extent of their geographic ranges with *P. purshii* extending over much of the Ohio Valley and Appalachian Mountains while *P. fimbriata* is restricted to the higher elevations of the southern Blue Ridge physiographic province. Furthermore, when comparing samples from populations representing a similar geographic area, *P. purshii* is more diverse both morphologically and genetically than *P. fimbriata.* Thus, this pair of species conforms to an often-observed pattern of higher diversity in a widespread species and lower diversity in a closely related endemic. Gitzendanner and Soltis (2000) conducted a literature review to compare genetic diversity between rare and widespread congeneric species. They found a trend of endemic species having a lower percentage of polymorphic loci at the population level, lower mean number of alleles at the population level, and lower level of observed heterozygosity than their widespread congeners. In contrast to this pattern, they found that measures of overall genetic diversity were not significantly different between the endemic and widespread congeners. It is unclear from their analysis whether this

finding is an accurate reflection of trends in genetic diversity or whether it is an artifact of their small sample size; however, they do conclude that when analyzing the diversity of an endemic species it is best to compare it to a close relative to establish the baseline level of diversity within the genus.

In the current study *P. purshii,* the widespread species, clearly had a higher level of diversity when compared to the endemic *P. fimbriata.* The higher level of genetic diversity in *P. purshii* may be a result of an ability to colonize a variety of low-elevation habitats including wooded, riparian, or alluvial habitats. For example, the Rocky Fork population is found in a riparian habitat in a small clearing beside a stream while the Red Hill population is growing along a railroad and adjoining hillside (as well as riparian area) in much more open and sandy soil. In addition to diversity in habitat, *P. purshii* is more morphologically diverse than *P. fimbriata*. In the three *P. purshii* populations involved in this study, there was a wide range of variation in flower color, stem color, and leaf shape. The Red Hill population had stems and leaves tinged with red making these plants easy to distinguish from all other plants in the growth chamber. In comparison, plants from the three *P. fimbriata* populations were indistinguishable from one another in their growth habit, morphology, and flower color. It was also in *P. purshii* that differences in germination between populations were noted in the field germination experiment. The Red Hill population had a lower percentage of germination compared to the Rocky Fork and Paddle Creek populations.

As expected for a species with a higher amount of habitat, morphological, and physiological diversity, *P. purshii* is also more genetically diverse than *P. fimbriata.* One possible explanation for this difference in diversity would be that *P. fimbriata* is a relatively new species that diverged from *P. purshii* and adapted to a new niche at higher elevation over a

relatively small range. The ITS DNA sequence data showed that *P. fimbriata* was more divergent from the other Cosmanthus species, when compared to *P. purshii*. Perhaps *P. fimbriata* became reproductively isolated from *P. purshii* as a result of adapting to a new ecological niche. In a strict cladistic interpretation, *P. purshii* and *P. fimbriata* diverged from a common ancestor and radiated to different habitats that presumably resulted in the development of new traits with the evolution of reproductive isolation as a by-product.

All evidence to date for ecological speciation is based on observations of reduced fitness of hybrids in parental habitats. In the absence of hybrid data, it is a particular challenge to support the hypothesis of ecological speciation of *P. purshii* and *P. fimbriata.* Ecological speciation occurs when a species diverges and adapts to ecologically diverse habitats with associated partial or full reproductive isolation from the progenitor. Ecological speciation is different from allopatric speciation in that it is not a geographic barrier but an ecological barrier separating the incipient species (Stebbins 1950). The primary question regarding ecological speciation is not whether the species have the potential to hybridize, but whether the species hybridize in nature and coexist in the same territory (Stebbins 1950). In the case of *P. purshii* and *P. fimbriata* they are reproductively isolated thus unable to hybridize in nature. Additionally, based on differences in germination and growth when grown under the same conditions, it is unlikely that they could coexist in the same habitat.

Reviewing studies of natural populations Hendry et al. (2007) concluded that ecological speciation could occur in a relatively short period of time according to the contemporary evolution time-scale of a hundred generations or less. One example of rapid ecological differentiation in plants includes *Anthoxanthum odoratum* populations exposed to different fertilizer treatments that resulted in phenotypic changes and the development of reproductive

barriers. Researchers reported reduced survival of seeds planted in their non-native treatment and divergence of flowering times between treatment groups. Divergence in flowering times was greater at treatment group boundaries, an effect that may reduce interbreeding between groups adapted to different fertilizer conditions. These changes were observed between the years of 1856 and the 1960s indicating that adapting to different ecological pressures can quickly lead to reproductive barriers and eventual speciation (Hendry et al. 2007).

Despite the absence of data on hybrid fitness for *P. fimbriata* and *P. purshii*, several observations support ecological speciation. For example, with the exception of a small elevational overlap, these two species may be considered geographically isolated by differences in elevation and range. These two species also diverge in characteristics tied to environmental cues such as germination time. Although hybrids could not be produced and therefore hybrid fitness could not be tested in parental habitats, each species can be grown in heterospecific elevational range and observed for fitness. As previously noted, *P. fimbriata*, is much less fit when grown under controlled laboratory conditions that are favorable to *P. purshii* indicating that they are not equally fit for each other's habitat. This in combination with their designation as closely related sister species, morphological similarity, range overlap, yet complete reproductive isolation provides support for the ecological speciation hypothesis.

In conclusion, *P. fimbriata* and *P. purshii* are closely related sister species that are completely reproductively isolated from each other as a result of a zygotic or early post-zygotic barrier. Although morphologically very similar, *P. fimbriata* can be characterized by a white corolla and sparsely, deflexed stem hairs while *P. purshii* can be characterized by a lavender corolla with strigose, afflexed stem hairs. There is some range overlap both geographically and in elevation; however, *P. fimbriata* is found only in Tennessee, Virginia, Georgia, and North

Carolina, and in those states at elevations ranging from 640-1,585 m while *P. purshii* is widespread throughout the Ohio valley region occurring at elevations ranging from 305-1,173 m. *Phacelia fimbriata* is a species more genetically divergent from other Cosmanthus species when compared to *P. purshii* but less genetically diverse than *P. purshii.*

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APPENDIX

APPENDIX A

Phacelia purshii and *P. fimbriata* Field Germination Data Collected During Fall 2005.

species	population	pot	10/5	10/8	10/12	10/15	10/18	10/23	10/30	11/2	11/9	11/22	12/18	total germinate	% germinated
P. purshii	Red Hill	1a	1	2	0	0	6	8	7	7	7	7	8	9	28.13
		1b	0	0	0	1	3	4	5	4	4	3	3	5	15.63
		1c	0	4	7	8	8	8	6	4	1	2	2	8	25.00
		1d	1	2	5	3	3	4	4	4	5	3	4	7	21.88
	Rocky Fork	2a	0	0	0	0	3	7	9	8	15	7	14	22	68.75
		2b	0	0	0	0	4	10	21	21	22	15	18	25	78.13
		2c	0	0	0	3	2	10	15	15	14	11	12	20	62.50
		2d	0	0	0	5	6	12	13	14	15	11	14	18	56.25
	Paddle Creek	3a	0	0	0	0	1	10	13	10	3	4	4	13	40.63
		3b	0	0	0	0	5	14	16	6	12	7	5	22	68.75
		3c	0	0	3	6	9	15	11	12	12	9	8	16	50.00
		3d	0	0	0	3	9	14	6	13	13	9	9	21	65.63
P. fimbriata	Ripshin	4a	0	0	0	0	0	0	0	0	4	4	5	5	15.63
		4b	0	0	0	0	1	2	4	2	6	4	6	10	31.25
		4c	0	0	0	0	1	4	6	7	17	12	13	18	56.25
		4d	0	0	0	2	2	4	7	14	14	10	9	14	43.75
	Roan	5a	0	0	0	0	1	2	3	4	2	7	9	9	28.13
		5b	0	0	1	1	1	3	10	12	14	11	11	14	43.75
		5c	0	0	0	2	3	5	6	3	4	3	3	7	21.88
		5d	0	0	1	1	6	13	17	17	15	10	12	19	59.38
	Big Bald	6a	0	0	2	1	3	6	9	6	5	7	8	12	37.50
		6b	1	1	0	0	5	3	8	12	4	5	4	15	46.88
		6c	2	0	0	1	1	4	8	8	10	10	9	11	34.38
		6d	0	0	2	4	4	8	8	10	7	6	5	10	31.25

APPENDIX B

Phacelia purshii and *P. fimbriata* Laboratory Germination Data Collected During Fall 2005.

Species	Population	9/19	9/20	9/26	9/27	9/29	9/30	10/3	10/4	10/6	10/10	10/12
P. purshii	Red Hill-1	0	1	10	10	11	11	11	11	11	11	11
	Red Hill-2	1	2	10	13	11	13	13	13	13	13	13
	Red-Hill Mega-1	1	0	7	6	10	11	13	13	13	13	14
	Red-Hill Mega-2	0	2	13	13	13	13	13	12	13	13	13
	Paddle Creek-1	0	0	2	3	4	4	4	4	8	10	12
	Paddle Creek-2	0	3	4	6	5	6	6	7	7	7	7
	Rocky Fork-1	0	0	1	2	2	1	3	3	3	3	5
	Rocky Fork-2	0	0	0	0	0	0	1	1	2	5	7
P. fimbriata	Roan Mountain-1	0	0	0	0	0	1	1	1	1	2	2
	Roan Mountain-2	0	0	0	0	0	0	0	0	0	0	1
	Big Bald-1	0	0	0	0	0	1	1	1	1	1	1
	Big Bald-2	0	0	0	0	0	0	0	0	0	0	0
	Ripshin-1	0	0	0	0	0	0	0	0	0	0	0
	Ripshin-2	0	0	0	0	0	0	0	0	0	0	0

Species	Population	10/13	10/20	10/24	10/25	10/27	11/1	11/3	11/7	11/8	11/12	11/15
P. purshii	Red Hill-1	12	13	12	12	12	12	12	12	12	12	11
	Red Hill-2	14	14	14	14	14	14	14	14	14	13	13
	Red-Hill Mega-1	14	14	15	14	14	14	14	13	13	15	14
	Red-Hill Mega-2	13	13	13	13	13	13	15	15	13	13	12
	Paddle Creek-1	12	12	12	12	13	12	14	11	11	11	10
	Paddle Creek-2	8	11	10	11	11	10	10	10	11	11	13
	Rocky Fork-1	6	7	7	7	7	8	8	8	6	7	7
	Rocky Fork-2	10	12	12	12	13	14	14	14	14	14	14
P. fimbriata	Roan Mountain-1	2	5	7	6	6	6	6	6	6	6	7
	Roan Mountain-2	2	2	4	4	5	5	6	7	7	7	7
	Big Bald-1	2	2	4	2	3	3	4	4	4	4	4
	Big Bald-2	0	1	1	1	2	2	2	2	3	3	3
	Ripshin-1	0	0	0	0	0	0	0	1	2	3	3
	Ripshin-2	0	0	0	0	0	0	0	2	2	2	1

Species	Population	11/17	11/19	11/21	11/22	11/28	12/1	12/6	12/8	12/13	Percent Germinated
P. purshii	Red Hill-1	11	11	11	11	11	10	11	9	10	71.43%
	Red Hill-2	14	13	13	13	13	13	12	13	12	80.00%
	Red-Hill Mega-1	14	14	13	13	13	13	13	12	13	86.67%
	Red-Hill Mega-2	13	13	13	13	14	12	13	12	13	92.86%
	Paddle Creek-1	12	12	12	12	12	12	12	11	12	75.00%
	Paddle Creek-2	10	11	11	10	11	11	11	12	11	73.33%
	Rocky Fork-1	7	8	7	6	6	6	6	5	6	40.00%
	Rocky Fork-2	13	14	13	14	14	14	14		14	100.00%
P. fimbriata	Roan Mountain-1	9	8	8	8	8	7	8	8	8	53.33%
	Roan Mountain-2	7	7	7	7	7	7	7	7	7	46.67%
	Big Bald-1	4	4	4	4	5	4	5	5	5	35.71%
	Big Bald-2	4	4	4	4	4	4	4	4	4	26.67%
	Ripshin-1	3	3	3	4	4	4	5	5	4	26.67%
	Ripshin-2	3	4	4	4	5	5	5	5	5	33.33%

APPENDIX C

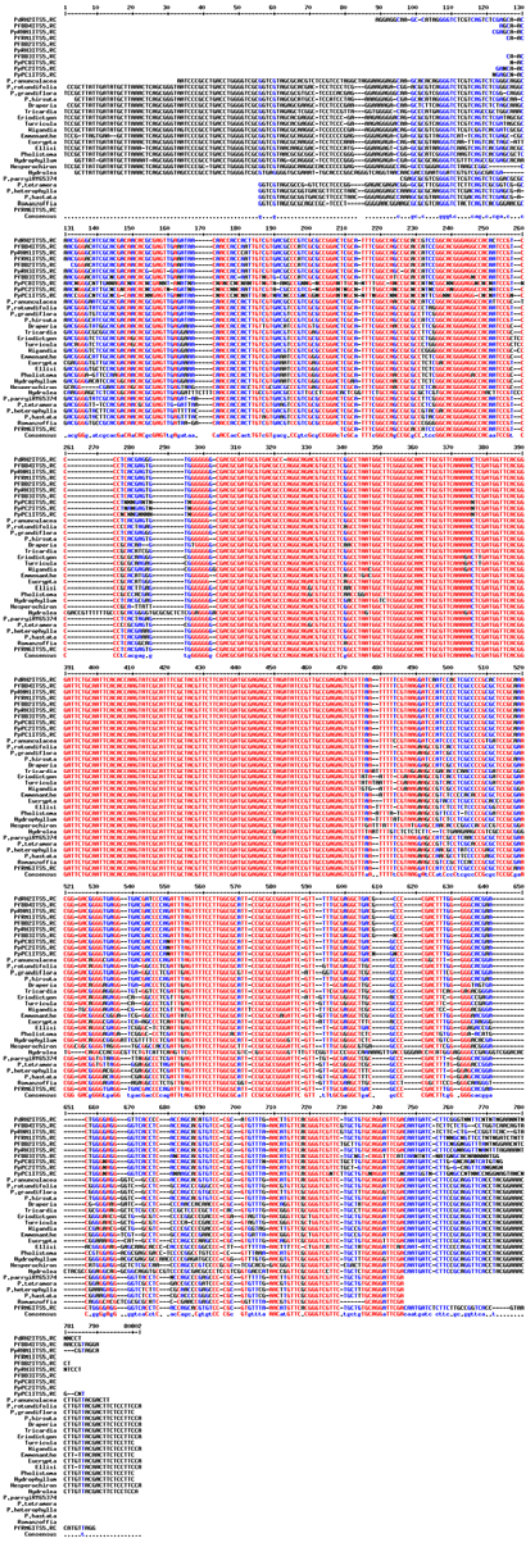
Phacelia fimbriata and *P. purshii* Pollen Tube Data.

					Pollen Tubes				
Type	Hour	Cross	Slide #	Pollen grains	Stigma	Stigma to fork	Fork to ovary	Ovary	Fertilization
Pf x Pf	4	Pf2 x Pf6	5	0	0	0	0	0	N
		Pf1 x Pf3	16	0	0	2	0	0	N
		Pf2 x Pf6	23	2	1	1	0	0	N
		Pf1 x Pf3	32	0	0	0	Picture	0	N
		Pf1 x Pf6	36	1	2	1	3	0	Y-picture
		Pf2 x Pf3	37	0	3	2	0	0	N
	24	Pf5 x Pf6	4	7/4	1	10	3	0	Y
		Pf1 x Pf6	9	0	2/1	6/2	2	2	N
		Pf1 x Pf6	15	2/4	2	2	2	2	Y
		Pf1 x Pf3	24	0	0	2	2	5	Y
		Pf1 x Pf6	30	0	2	0	0	0	N
		Pf1 x Pf6	33	0	4	4	5	0	N
		Pf1 x Pf3	38	0	0	0	0	0	N
		Pf5 x Pf3	46	6/0	4/3	3	3	1	N
Pf x Pp	4	Pf1 x PpRH3xRF1-1	1	0	0	0	0	0	N
		Pf2 x PpRH3Xrf1-4	27	0	0	5	2	5	Y
		Pf2 x PpPC6xRF1-5	43	3/5	3/7	0	0	0	N
		Pf1 x PpPCxRF1-5	44	0	0	0	0	0	N
		Pf5 x PpPCxRF1-5	45	0	4/3	3	3	1	N
		Pf1 x PpPC6xRF1-5	50	0	0	6	0	0	N
	24	Pf1 x PpRH3xRF1-4	10	10/0	1	1	4	0	Y
		Pf1 x PpPC6xRF1-5	24	0	0	2	2	5	Y
		Pf1 x PpPC6xRF1-5	18	0	6/7	10	20	8	Y
		Pf5 x PpPC6xRF1-5	25	Flower broke apart during staining					
		Pf2 x PpRH3xRF1-4	28	16/2	3/3	5/3	6	2	Y
		Pf1 x PpPC6xRF1-5	29	0	3	4	3	0	Y

		Pf2 x PpPC6xRF1-5	35	Flower broke apart during staining					
		Pf5 x PpRH3xRF1-4	52	0	0	6	2	2	Y
Pp x Pf	4	PpPC6xRF1-5 x Pf4-1	2	0	0	0	0	0	N
		PpPC6 x Pf4b	6	0	0	0	0	0	N
		PpPC6xRF1-3 x Pf4a	41	0	0	4	0	0	N
		PpPC6xRF1-3 x Pf4b	42	0	0	0	0	0	N
	24	PpPC6xRF1-9 x Pf3	8	1/0	0	0	0	0	N
		PpPC6xRF1-9 x Pf6	26	3	0	1	2	9	Y
		PpPC6xRF1-3 x Pf4a	31	7	1	4	4	5	Y
Pp x Pp	4	PpPC6xRF1-3 x PpRH3xRF1-4	7	1/0	1	1	0	0	N
		PpPC6xRF1-9 x PpPC6RF1-5	14a	5/0	4	5	0	0	N
		PpPC6xRF1-9 x PpPC6RF1-5	14b	0	0	0	0	0	N
		PpPC6xRF1-3 x PpRH3xRF1-4	39	6/3	0	3/3	0	0	N
		PpPC6xRF1-3 x PpRH3xRF1-4	40	2/2	0	1/1	0	0	N
		PpPC6xRF1-3 x PpPC6xRF1-5	48	0	0	0	0	0	N
		PpPC6xRF1-3 x PPPC6xRF1-5	49	0	0	0	0	0	N
	24	PpPC6xRF1-3 x PpPC6xRF1-5	3	0			6	0	
		PpPC6xRF1-9 x PpRH3xRF4	11	0	0	0	0	0	N
		PpPC6xRF1-9 x PpRH3xRF1-5	47	0	0	1	2	2	Y
		PpPC6xRF1-3 x PpPC6xRF1-5	51	16	10	10/3	6	3	Y

APPENDIX D

DNA Sequences Aligned Using Multalin (Corpet 1988). Red = High Consensus (90%); Blue = Low Consensus (50%); Black = Neutral



VITA

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	Magna Cum Laude
	Dean's List
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