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Hiding in Plain Sight, a New Species of *Phacelia* for the Southern Appalachian Mountains

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ABSTRACT

We examined the genetic basis for the spatial segregation of two different morphotypes within populations of *Phacelia bipinnatifida*, a biennial plant species associated with cove forest communities of the southern Cumberland Plateau and determined that these morphotypes were actually two distinct species. Given that there are no reported subspecies or varieties in the literature that would conform to these morphotypes, we believe we are the first to describe these morphological and genetic differences. We describe and name *Phacelia sewaneensis* as a new species that is differentiated from *Phacelia bipinnatifida* by having purple corollas, longer stamens, basal leaves without gray blotches, and more highly dissected basal leaves. Genetic analyses of individuals from sympatric populations found in three geographically distant locations on the southern Cumberland Plateau revealed that *Phacelia sewaneensis* (purple morphotype) was more similar genetically to purple morphotypes at the other locations than to sympatric individuals of *Phacelia bipinnatifida* (blue morphotype). We found that at a given site, the two species occur in large, non-overlapping, yet adjacent patches within a cove, and these patches remain homogeneous (with respect to species) from year to year. *Phacelia sewaneensis* prefers rocky soils and is found in higher density populations than *Phacelia bipinnatifida*. We establish a new epitype for *Phacelia bipinnatifida* and holotype and isotypes for the newly described *Phacelia sewaneensis*. We discuss why this new species of *Phacelia* has been missed by botanists, despite its multiple distinguishing features.

Key words: Cumberland Plateau, epitypification, microsatellites, *Phacelia bipinnatifida*, population genetics

INTRODUCTION

There has been a resurgence in taxonomic studies in recent years, such that as many as 422 new plant species have been described in the southeastern United States alone (unpublished data, Weakley, pers. comm.). Recently named plant species from this region are generally associated with one of three scenarios: 1) taxonomic revisions where already recognized subspecies or varieties with defined morphological differences become elevated to species-level status (Weakley et al. 2017); 2) discovery of rare plants with restricted distributions (Estes and Beck 2011; Estes et al. 2015; McClelland et al. 2023; Ungberg et al. 2024; Weakley et al. 2024); or 3) taxa that vary genetically, but are morphologically very similar (i.e., cryptic species, Carstens and Satler 2013; Ciafre and Naczi 2022, Edwards et al. 2021, Pace et al. 2017, Pantinople et al. 2024). An unlikely scenario would be two relatively common, morphologically distinct species with sympatric distributions that have been mistakenly lumped together as one species. This paper describes an unusual example of such a case involving *Phacelia bipinnatifida* Michx. (Hydrophyllaceae), a biennial forb found in mesic forest communities throughout the Southern Appalachian Mountains. Populations of two distinct morphotypes, characterized by multiple differences in both vegetative and reproductive traits,

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were found to have sympatric distributions within the southern range of *P. bipinnatifida*. Indeed, both morphotypes can be commonly observed growing together in some of the most frequently visited wildflower locations in the Southern Appalachians, yet no taxonomic treatment has recognized this variation at any level.

In spring 2021, there was a major flowering event of *Phacelia bipinnatifida* in Shakerag Hollow, an old-growth, mixed mesophytic cove on the campus of the University of the South, Sewanee, in Franklin County, Tennessee. Large areas of the north-facing slope within this cove were covered with a high density of flowering individuals (Figure 1a). Since this species is biennial, these plants had germinated in 2020. What was striking about these populations was that there appeared to be two distinct flower morphotypes (purple and blue—see Figure 1b–d) and these two morphotypes were distributed in discrete, non-overlapping patches spread across the slope. This observation served as the basis for a study to determine how it was possible for these distinct patch structures to be maintained despite observed pollinator movement among patches. Consequently, the objective of this study was to assess variation in morphology, ecology, and genetic structure between the two morphotypes across multiple sampling locations to determine if taxonomic recognition was warranted.

Taxonomic history of *Phacelia*

Phacelia Juss. (1789), commonly referred to as scorpion weed, is the largest genus within the Hydrophyllaceae (~200 sp.) and is distributed throughout diverse habitats in North and South America (Hoffmann et al. 2016; Luebert et al. 2016). While some researchers have nested the traditionally recognized Hydrophyllaceae within the Boraginaceae, the Boraginales Working Group recognizes a monophyletic Hydrophyllaceae after splitting out representatives of what is now Namaceae (Luebert et al. 2016). Botanists have debated and revised the placement of taxa within the *Phacelia* genus since its initial description, with numerous subgenera, sections, subsections, and other informal groupings proposed (see Constance 1949 and Walden and Patterson 2012 for an in-depth review). Constance (1949, 1963) considered *Phacelia* subg. *Cosmanthus* (Nolte ex A.DC.) A.Gray, as distinct from two other subgenera (*Howellanthus* Constance and *Phacelia*) due to the lack of corolla scales and presence of glands or nectaries at the base of the corolla tube (Constance 1949), as well as base chromosome number (5, 6, 8, 9, or 14, but not 11—unlike other members of the genus; Constance 1963). Constance (1949, 1963), included *Phacelia bipinnatifida* in this group along with all other eastern North American taxa. Morphological and phylogenetic studies since Constance have attempted to address confusion at the subgeneric level within *Phacelia*, including Ferguson (1999), Walden and Patterson (2012), and Vasile et al. (2020), among others. Ferguson (1999) noted, "*Phacelia* is in a state of taxonomic flux," and recent treatments did not include all recognized members of the genus. Ferguson, however, did not propose a formal taxonomic revision, nor include *P. bipinnatifida* in their phylogenetic analysis.

Walden and Patterson (2012) largely followed Ferguson (1999) in their circumscription, defining three subgenera (*Pulchellae* (Rydb.) Walden & R.Patt., *Microgenetes* (A.DC.) A.Gray, and *Phacelia*), and approximately 11 sections and 12 subsections, although the relationships among these groupings was unclear. They resurrected *Phacelia* Juss. subsect. *Bipinnatifidae* (Small) Walden & R. Patt. as a monotypic subsection containing *P. bipinnatifida*. Gilbert et al. (2005), Walden et al. (2014), and more recently Vasile et al. (2020) all inferred phylogenetic structure among members of *Phacelia*, finding support for monophyly of many (but not all) of the subgeneric groupings of Walden and Patterson (2012). These studies uncovered limited genetic divergence among taxa within *Phacelia*, suggesting a possible recent diversification among extant taxa. However, to our knowledge, *P. bipinnatifida* has not been included in any published phylogenetic treatment of the genus. As a result, its placement within the genus remains unclear.

Weakley et al. (2024) recognize 16 species and 12 infraspecific taxa in the southeastern United States, including *Phacelia bipinnatifida*. Constance (1949) noted several synonyms for *P. bipinnatifida*, including *P. brevistylis* Buckl., *P. bipinnatifida* var. *plummeri* Wood, and *P. bipinnatifida* var. *brevistylis,* all of which are treated as synonyms by Weakley et al. (2024). They indicate that these

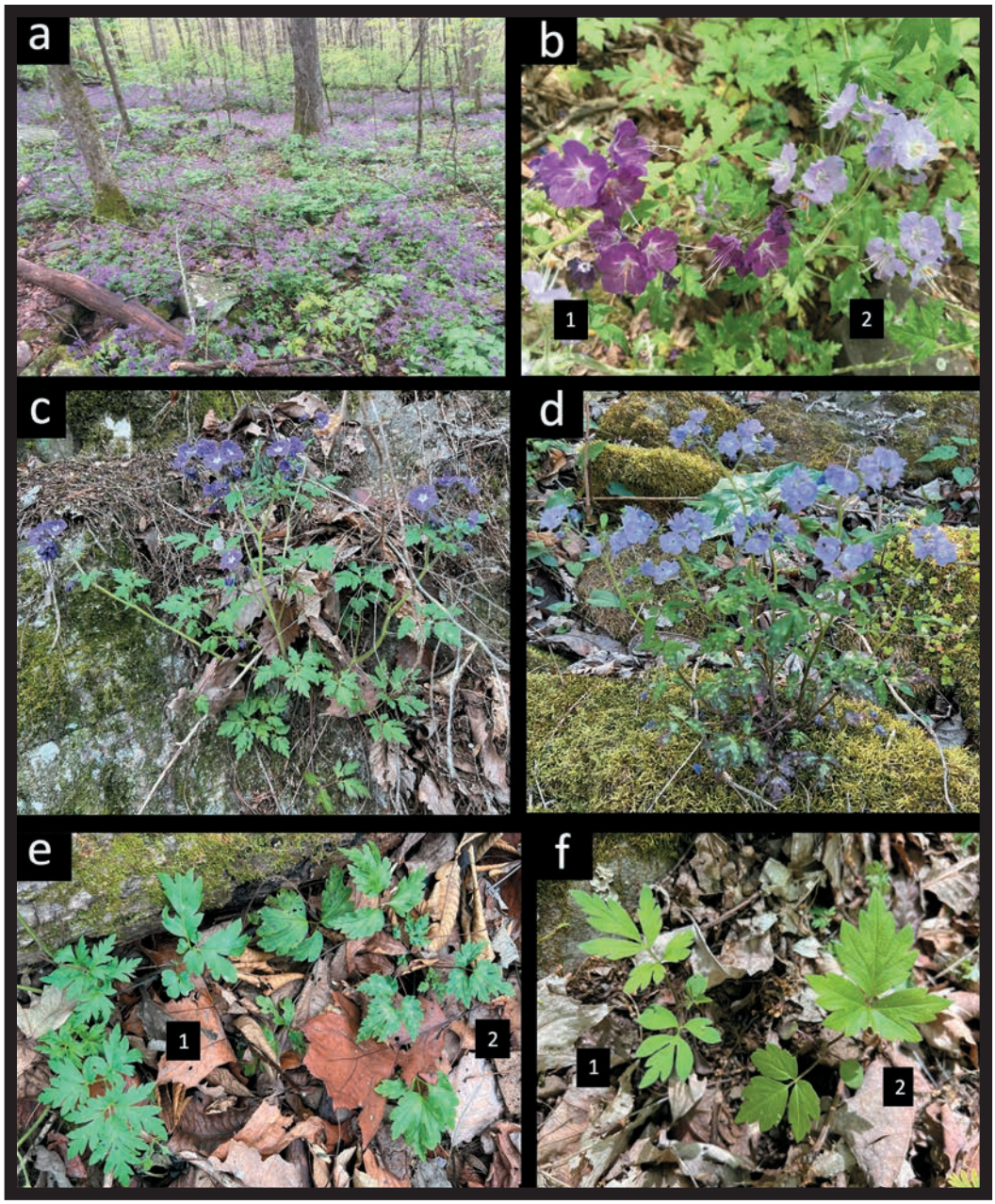

Figure 1. Comparative photographs from *Phacelia* morphotype populations: a) Flowering population of purple morphotype on north-facing, upper slope of Shakerag Hollow, Tennessee (10 April 21); b) Flowers: 1-purple morphotype and 2-blue morphotype (Shakerag Hollow, Tennessee, 18 April 2021); c) Second-year flowering individua—purple morphotype (Savage Gulf State Park, Tennessee, 24 March 2024) d) Second-year flowering individual—blue morphotype (Shirley Miller Wildflower Trail, Georgia, 30 March 2024); e) First year overwintering individuals: 1-purple morphotype, 2-blue morphotype (Shakerag Hollow, Tennessee, 8 December 2023); f) Seedlings 1-purple morphotype, 2-blue morphotype (Shakerag Hollow, Tennessee, 17 April 2023). The purple morphotype is described as a new species, *Phacelia sewaneensis*, that is differentiated from *Phacelia bipinnatifida* (the blue morphotype).

taxa were distinguished by "variation with sparser pubescence, larger and less divided leaf segments, smaller flowers, and sub-included stamens and style" and go on to say that "these variations are not concomitant, and the distribution of forms showing a complete or partial combination of them is sporadic." They do, however, acknowledge the presence of heterostyly in the species. As Weakley et al. (2024) noted, more clarification of the taxonomic status of this group is still needed. Population genetic work has been published for some of the eastern taxa, with a strong emphasis on the *P. dubia* (L.) Trel. & Small species complex (Levy 1991; del Castillo 1994; Levy et al. 1996; Levy and Neal 1999; Levy and Malone 2001; Glass and Levy 2011). No such work has yet been published for *P. bipinnatifida*.

MATERIALS AND METHODS

Population surveys

Shakerag Hollow, Franklin County, Tennessee

Two distinct morphotypes of *Phacelia bipinnatifida* were first identified in 2021 in Shakerag Hollow on the campus of the University of the South, Sewanee, Franklin County, Tennessee. Purple and blue morphotypes of *Phacelia bipinnatifida* form distinct patches across the north-facing upper slope of this cove. There are no other species of *Phacelia* known from Shakerag Hollow (Evans et al. 2016). The study site is an old-growth, "mixed mesophytic" forest habitat (Duffy and Meier 1992) and is characterized by a diverse assemblage of canopy species including *Acer saccharum* Marshall, *Aesculus flava* Sol., *Carya ovata* (Mill.) K.Koch, *Fraxinus biltmoreana* Beadle, *Juglans nigra* L., *Quercus rubra* L., and *Tilia americana* L. (Evans et al. 2016).

Understory trees and shrubs include *Asimina triloba* (L.) Dunal, *Cercis canadensis* L., *Lindera benzoin* (L.) Blume, and *Staphylea trifolia* L.. There is a diverse vernal herbaceous flora, that in addition to *Phacelia bipinnatifida*, includes over 50 other species including *Arisaema triphyllum* (L.) Schott, *Caulophyllum thalictroides* (L.) Michx., *Claytonia virginica* L., *Delphinium tricorne* Michx., *Dicentra cucullaria* Bernh., *Hydrophyllum canadense* L., *Podophyllum peltatum* L., *Polymnia laevigata* Beadle, *Phlox divaricata* L., *Sanguinaria canadensis* L., *Tradescantia subaspera* Ker Gawl, *Trillium spp.* L., and *Viola spp.* L. (Evans et al. 2016). Soil depth varies across the slope as a function of the distribution of sandstone colluvium formerly part of the Plateau surface, which can form extensive expanses of loose surface rock in places.

Three 200 m × 2 m belt transects were established perpendicular to the slope to capture several transition zones between patches of purple and blue morphotypes. In late April 2021, all adult individuals of each morphotype were counted within consecutive 2 m × 2 m subplots along each transect. In 2023, six purple morphotype individuals and seven blue morphotype individuals were collected outside of Transect 3 as vouchers, along with corresponding photographs to capture characters that do not preserve well in herbarium specimens (Parnell et al. 2013). We uploaded our field photographs to iNaturalist and grouped them into a new Project, "*Phacelia bipinnatifida* Integrative Taxonomy." We followed the protocol outlined by Heberling and Isaac (2018) to create QR code references to attach to our herbarium specimen labels that link to each specimen's field photos on iNaturalist. In April 2023 and April 2024, all transects were re-censused for adult individuals as well as for seedlings of both morphotypes; seedlings could be distinguished based on the number of basal leaf segments (see Figure 1f).

To determine if the two morphotypes occurred in different microhabitats, we also characterized the surface rock cover of each 2 m × 2 m subplot along all transects in 2024. Subplots were assigned one of three types based on the visible rock cover across the surface: <30%, 30–60%, >60%. The relationship between morphotype presence and rock cover in the Shakerag Hollow transects was statistically assessed using a likelihood ratio chi-square test.

Surveys in Georgia and Alabama

Based on the Shakerag populations, we found that, in addition to corolla color, three other traits could reliably be used to distinguish between the two morphotypes: stamen length, gray blotches

on basal leaves, and the number of basal leaf segments (see character analysis below). These differences are often discernible in photographs, so in December 2023, we downloaded all available observations of *Phacelia bipinnatifida* from iNaturalist (2023) to locate two additional populations where the purple and blue morphotypes could be found growing in sympatry in north-facing cove communities: Shirley Miller Wildflower Trail in the Crockford-Pigeon Mountain Wildlife Management, Walker County, Georgia; and Monte Sano State Park, Madison County, Alabama. Both locations have a similar flora and rock cover to Shakerag Hollow. In April 2024, we established a 100 m belt transect at each of the two sites and, using the same methods described above, counted the number of mature adults and seedlings of the two morphotypes in each 2 m × 2 m subplot. Rock cover of subplots was categorized as described above. For each site, the relationship between morphotype and rock cover was statistically assessed using a likelihood ratio chi-square test. Additionally, data were combined across sites (all three transects at Shakerag Hollow, one transect each at Shirley Miller Wildflower Trail and Monte Sano State Park) and assessed using a l likelihood ratio chi-square test (morphotype [purple or blue] by visible rock cover [<30%, 30–60%, >60%]).

Genetic assessment

Population sampling

Leaf material was collected from equal numbers of each morphotype evenly along the length of 100 m transects at each of the three locations above (Shakerag Hollow, Tennessee; Shirley Miller Wildflower Trail, Georgia; and Monte Sano State Park, Alabama). In order to create genetic reference data from the broader range of *Phacelia bipinnatifida*, leaf material was obtained from several additional sites that appear to be characterized by only one morphotype: Cove Spring Park, Franklin County, Kentucky; Ferne Clyffe State Park, Johnson County, Illinois; Savage Gulf State Natural Area, Grundy County, Tennessee; St. Francis National Forest, Phillips County, Arkansas; Mulberry Fork River, Blount County, Alabama; and North Saluda Reservoir, Greenville County, South Carolina (see Table 1).

Microsatellite development and genotyping

Total genomic DNA was extracted from silica dried leaf material using the Qiagen DNeasy Plant Pro Kit (Qiagen Sciences, Germantown, Maryland USA) following manufacturer's instructions with modifications for problematic samples. DNA of a single individual of *Phacelia bipinnatifida* (blue morphotype) from Shakerag Hollow was extracted and submitted to Steve Bogdanowicz (Evolutionary Core Genetics Facility [ECGF], Cornell University, Ithaca, New York) for microsatellite locus development. A genomic library was constructed and enriched for tetrameric repeats; fragments were then PCR-amplified, barcoded, and sequenced on an Illumina MiSeq platform. We received files containing all resulting data, including primer pair sequences to be tested for potential utility in the study system.

Approximately 100 loci were screened through a combination of direct sequencing (protocol described below) and fragment-based analyses (methods following Morris et al. 2016).

Nextera-tagged primers for 65 loci and DNA for 12 individuals of *P. bipinnatifida* (six purple/six blue morphotypes) from Shakerag Hollow were submitted to Cornell University for the genotype-by-sequencing (GBS) pilot. Methods generally followed D'Aloia et al. (2017) and are described here in brief. Loci were amplified in multiplex reactions containing a maximum of 25 loci, with successful amplification verified by gel electrophoresis. PCR products were barcoded with dual llumina Nextera barcodes and pooled into a single library, which was size selected and quantified prior to sequencing. Products were sequenced on an Illumina Miseq (paired-end reads, 2 x 150), and haplotypes (i.e., alleles) were called using custom Python scripts written by Qi Sun (**https://bitbucket.org/cornell_bioinformatics/amplicon/src/master/**). Due to an unexpectedly low amplification success rate (22 out of 65 loci, 33.8%), an additional 35 loci were screened using a fragment-based approach (see Morris et al. 2016). Ultimately, only 18 loci amplified consistently and cleanly such that those were the loci chosen for the full GBS project. Such a low success rate (19%) for primer development is surprising given our experience with other taxa (*Sagittaria fasciculata* E.O.Beal 41%; *Shortia*

Table 1. Sampling locations for *Phacelia bipinnatifida* included in the present study.

Site Name	Co., State	Site Code	Morph obs.[1]	Collector(s)
Cove Spring Park	Franklin Co., KY	KY	ambiguous	J.T. Michel
Ferne Clyffe State Park	Johnson Co., IL	IL	blue	J.T. Michel
Savage Gulf State Natural Area	Grundy Co., TN	TN1	purple	Jon Evans
Shakerag Hollow, Sewanee	Franklin Co., TN	TN2	blue & purple	Jon Evans, J.T. Michel, Skyler J. Fox
St. Francis National Forest	Phillips Co., AR	AR1	unknown	Brendan Kosnik
St. Francis National Forest	Phillips Co., AR	AR2	unknown	Brendan Kosnik
Mulberry Fork	Blount Co., AL	AL1	blue	Wayne Barger, Priscilla Barger
Monte Sano State Park	Madison Co., AL	AL2	blue & purple	Jon Evans, J.T. Michel
Shirley Miller Wildflower Trail	Walker Co., GA	GA	blue & purple	Jon Evans, J.T. Michel
Greenville Water Property	Greenville Co., SC	SC1	ambiguous	Ashley Morris, McKenzie Boyd, Maura Champley
Greenville Water Property	Greenville Co., SC	SC2	unknown	McKenzie Boyd, Maura Champley

[1]Morph obs. is defined by a suite of characters described herein. Each site was designated as having either the blue or purple morph where characters clearly aligned with those morphotypes; two sites were ambiguous in their characters, sharing some of each morphotype, while individuals at three other sites were not characterized in the field and are therefore scored as unknown.

galacifolia Torr. & A.Gray 41%; *Sarracenia oreophila* Wherry 61%; *Sassafras albidum* (Nutt.) Nees 48%, unpublished data, Morris et al. in prep). It is unclear at this time what factors could be driving this issue.

Eighteen loci (Table 2) were used to genotype 325 individuals across 11 locations (Table 1), plus five additional samples of *P. brevistyla* from Cherokee County, Georgia, as identified by Tom Diggs (Department of Biology, University of North Georgia). Methods followed those described above. Upon receipt of the data, all haplotype (i.e., allele) calls were cross referenced with depth of coverage; any genotype with one or more haplotypes with less than 10 reads was marked as missing data. This was done to avoid any erroneous genotype calls from poor quality reads.

Genetic analysis

Summary statistics were calculated using the package GenAlEx 6.5 (Peakall and Smouse 2006; Smouse and Peakall 2012). Populations were characterized by percent polymorphic loci (%*P*), mean number of alleles (N_a), mean number of effective alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), unbiased expected heterozygosity (uH_e), the fixation index (F), and pairwise population F_{ST} values. To further explore population structure, a Principal Coordinates Analysis (PCoA) was performed. The R package 'poppr' was used to construct a UPGMA dendrogram based on Nei's genetic distance, with bootstrap support determined by 1,000 replicates using code from Kamvar et al. (2014).

Character analysis

To distinguish between the two morphotypes, we initially intended to use the data obtained from our own field observations of the individuals sampled from Shakerag Hollow, Shirley Miller Wildflower Trail, and Monte Sano State Park, as well as data obtained from SERNEC images (SERNEC Data Portal 2024). SERNEC specimen records were accessed from the counties in which our three research sites were located (Franklin County, Tennessee; Walker County, Georgia; and Madison County, Alabama) since these were counties where both morphotypes were known to co-occur. Forty-two digitized images of herbarium specimens were assessed for the traits we used to identify morphotypes in the field: corolla color, stamen length, gray blotches on basal leaves, and the number of basal leaf segments. However, we ultimately chose to remove all SERNEC records

Table 2. Characterization of 18 nuclear microsatellite loci developed for *Phacelia bipinnatifida* in the present study.

Locus	Primers (5´–3´)	Product Size	N_a^2
Phab84	F-GAAGTTTGGTTTCAGCTGAGC		4.6000
	R-TCCCACATCCCTTTACTCTTTG	207	(0.888)
Phab101	F-ACCGAACCTTATCCTCCATTG		3.867
	R-CTCTCTCATCGCCAAAGACAG	208	(0.768)
Phab202	F-CTCAGCCCATTCAATCACCAC		5.000
	R-ACAAGAAGGTGTCCAATGGG	200	(1.313)
Phab260	F-ACTCGTTTACAACCTGATTCACAC		4.867
	R-AACGGTTAGAGCACATCCTTG	209	(1.014)
Phab317	F-ACATACTTTCCAGGTTCACACG		3.133
	R-TGCTGTGTCCCATATTTGTGC	206	(0.668)
Phab347	F-TTTGTGGGTTGGTTTAGCTGAAG		1.333*
	R-TCCCTCTCCTGACTATTATCACATC	190	(0.187)
Phab349	F-TGATGAAGTTTGTGATGCCCTC		3.133
	R-TCTTCTGTTGTTGGTGCCATC	190	(0.487)
Phab504	F-CATCATCACCTCTCCTGACCC		4.800
	R-TCTCACATAACTTCCAGGCAC	192	(1.239)
Phab1016	F-TGTTGGGTGATCATGTTATGCTG		2.467*
	R-GCTATTATATAAGCATTGCAAAGCC	197	(0.608)
Phab1025	F-GATGTGTAAATTTCTTCGTTGGTTC		3.267
	R-GCACACGTGGATGCTCAAG	201	(1.016)
Phab1155	F-TCTTCTGCCTCAACACCTAAC		4.333
	R-CGTATCTTGGTCACACTCTTGG	210	(0.95)
Phab1505	F-GGTGATACCCTTGTTGTTCATG		2.400
	R-TGCCTGCAATATCAATGAATAACG	207	(0.689)
Phab1513	F-TTCGATGAGTACCGTTTCTACC		0.933
	R-CTCAGGGAGATGTACTATGCATG	190	(0.452)
Phab1820	F-TGTCCTAACATGTGATTGGTCC		3.600*
	R-GGACCAACTGTAACTCCATGG	209	(1.036)
Phab4089	F-CTAATCTCCCTCGGATCAGCG		3.600
	R-GCAAGTGAGGAATTTCAAACCC	192	(1.036)
Phab4771	F-GTGAATTAGCTAGTTGATTGGAAGC		5.933
	R-GTGTGATGTGAAGATGGAATGC	200	(0.1634)
Phab5565	F-CATCAGCTTTGTCTTTCGATCAC		3.067
	R-AACAATGAACACCCAGAAGCG	190	(1.030)
Phab6056	F-GTTAAGACACTGCCACCGC		4.267
	R-GTATGTGGAAAGAGGAAAGTAATGG	208	(1.193)

[1]Product size is reported PCR product size at time of locus development

[2]N_a = mean number of haplotypes (i.e., alleles) observed over all populations sampled in the study, with standard error (s.e.) in parentheses

*Indicates a locus that consistently amplified poorly in *P. sewaneensis*, resulting in scores of 'missing data'

from our analysis of character traits due to the absence or ambiguity of one or more of those traits from each imaged specimen. For example, corolla color and filament color are often not preserved or change over time depending on specimen preparation, and basal leaves are often not included on specimens. As a result, we focused our character analysis on field observations from Tennessee, Georgia, and Alabama. In Georgia and Alabama, morphological data were collected in 2024 for each of the genetically sampled individuals, including stamen length, filament color, presence of gray blotches on basal leaves, and the number of basal leaf segments. We took corresponding photographs of flowers and basal leaves for each sampled individual. In Tennessee, morphological data were collected in 2021 prior to genetic sampling in 2023.

Box plots were constructed for each of four characters by morph (stamen length, filament color, gray blotches on basal leaves, and the number of basal leaf segments), and Welch's t-test was used to determine significance of observed differences between morphs.

RESULTS

Population surveys

At all three sites, the distribution of morphotypes along transects revealed a distinct, and mostly non-overlapping patch structure (Figures 2 and 3). Morphotypes were distinctly segregated from each other. Seedling distribution mirrored adult distribution at all transects. Data from 2021 to 2024 in Shakerag Hollow, indicate that morphotype patch size and position remained constant between years (Figure 2). Seedlings were repeatedly established within the same patches that included their parents and second-year individuals of the same morphotype. This reflects the limited seed dispersal (gravity) within these patches. Adult abundance and seedling recruitment varied between morphotypes. The adult density for the purple morphotype was higher than the blue morphotype in 2022 and 2024 but lower in 2023. In both 2023 and 2024, the purple morphotype consistently produced more offspring that germinated and survived in their first year as compared to the blue morphotype. Adult density within patches for both morphotypes was highly variable between years. Adult abundance was relatively low in 2023 compared to both 2021 and 2024 (Figure 2).

Chi-square analyses to assess the relationship between morphotype and visible rock cover were significant at each of the three locations: Shakerag Hollow (χ^2=123.860, df=5, N=600, p<0.001), Shirley Miller Wildflower Trail (χ^2=98.09, df=5, N=100, p<0.01), and Monte Sano State Park (χ^2=14.981, df=5, N=100, p=0.01). Additionally, the chi-square analysis across sites was significant (χ^2=193.59, df=5, N=800, p<0.001). Row percentages for the chi-square analysis indicate that the blue morphotype was more often associated with plots scored as '<30% rock cover' or '30–60% rock cover' (82.47% and 72.92% of those plots, respectively), and less often associated with plots scored as '>60% rock cover' (35.86% of those plots). In contrast, the purple morphotype was more often associated with plots scored as '>60% rock cover' or '30–60% rock cover' (51.52% and 41.67% of those plots, respectively), and less often associated with plots scored as '<30% rock cover' (12.34% of those plots).

Genetic analysis

Three of the 18 loci (*Phabip347, Phabip1016,* and *Phabip1025*) used in the present study consistently failed to amplify in the purple morphotype or only amplified in a few individuals. In contrast, there were no loci that consistently failed to amplify in the blue morphotype, but there were loci that differentially amplified in certain geographic areas. Summary statistics for the full data set are shown in Table 3. Mean number of alleles averaged across loci for each population (N_a) ranged from 1.000 ± 0.000 (AR2) to 11.278 ± 1.328 (TN2 blue). Mean number of effective alleles (N_e) ranged from 0.985 ± 0.181 (SC2) to 4.760 ± 0.694 (TN2 blue). Observed heterozygosity (H_o) ranged from 0.000 ± 0.000 (AR2) to 0.589 ± 0.043 (TN2 blue). Notably, H_o was consistently lower in purple sympatric sites (0.237 ± 0.055 to 0.323 ± 0.071) than in blue sympatric sites (0.429 ± 0.069 to 0.589 ± 0.043). Unbiased expected heterozygosity (uH_e) ranged from 0.000 ± 0.000 (AR2) to 0.716 ± 0.042 (TN2 blue). Again, values for uH_e were consistently lower in purple sympatric sites (0.376 ± 0.072 to 0.479 ± 0.085) than in blue sympatric sites (0.531 ± 0.066 to 0.716 ± 0.042). All sites except two (AR2 and AL1) had positive fixation indices (F), ranging from 0.119 ± 0.055 (IL) to 0.781 ± 0.106 (*P. brevistyla*). Fixation indices were not calculated for AR2 and AL1. Pairwise population F_{ST} values show greatest similarity between GA blue and TN2 blue (0.092) and greatest difference between SC2 and AR2 (0.875) (Table 4). In the PCoA, the percentage of variation explained by the first and second axes combined was 29.58% (Figure 4). All individuals scored as the purple morphotype formed a relatively tight cluster. Most individuals scored as the blue morphotype clustered together as well, with ambiguous or unknown individuals falling out separately. The dendrogram strongly supported two clusters (100% b.s.), with individuals scored as purple in one cluster and all other individuals in the second cluster (Figure 5).

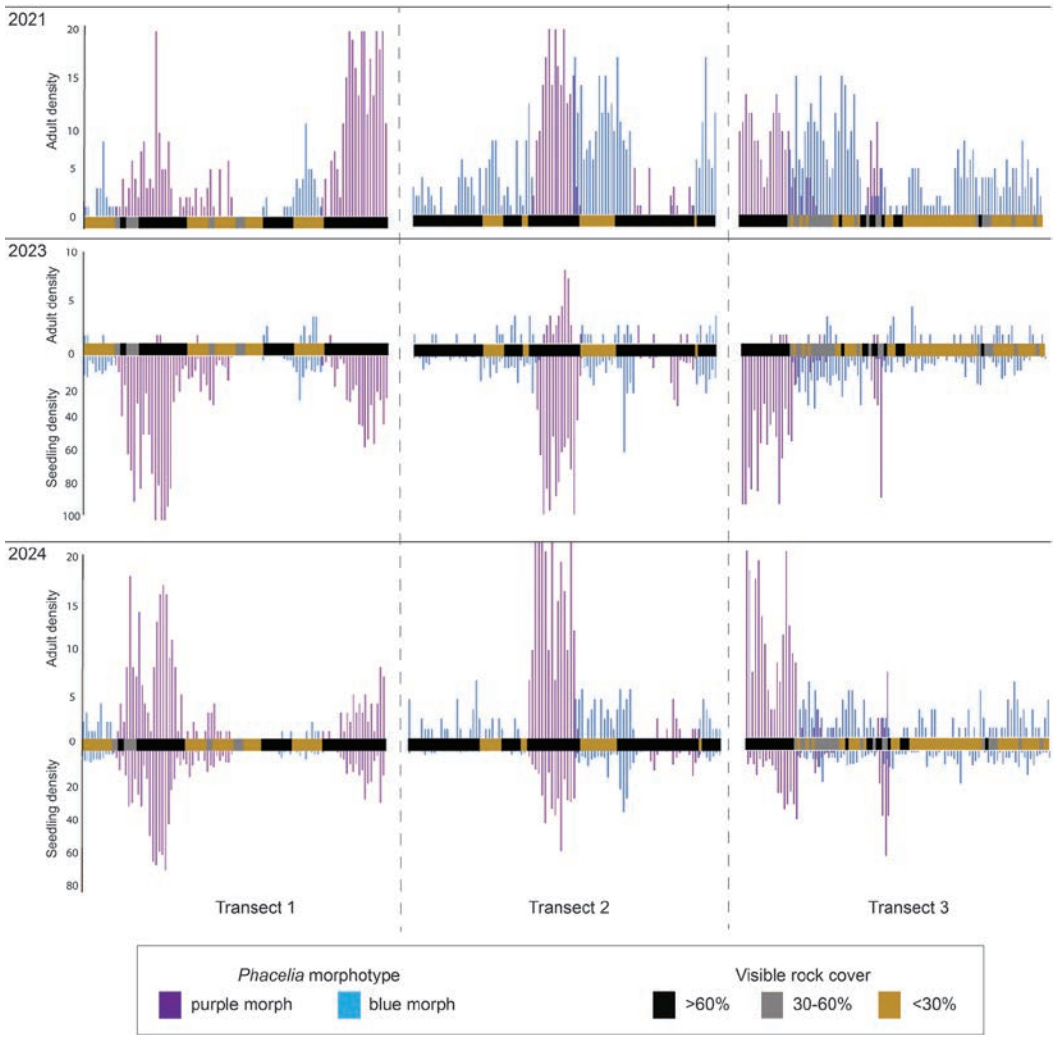

Figure 2. Patch structure of the purple and blue morphotypes of *Phacelia* observed in Shakerag Hollow, Sewanee, Tennessee. Observations for the three transects recorded three years (2021, 2023, and 2024) are graphically illustrated, with transects separated by dotted lines and years separated by solid black lines. For each transect, the y-axis shows adult and/or seedling density, with purple morph indicated by a purple bar and blue morph indicated by a blue bar. The x-axis shows position along the transect and notes percent visible rock cover: black=<60%; grey=30-60%, and yellow=30%.

Character analysis

For the four characters (corolla color, stamen length, gray blotches on basal leaves, and the number of basal leaf segments), the two morphotypes were consistently distinct when observed in sympatry (Table 5; Figure 1b, e–f). Filament color, gray blotches on basal leaves, and the number of basal leaf segments were always true to morphotype, with the blue morphotype having white filaments, gray blotches on basal leaves, and three leaf segments, while the purple morphotype had purple filaments, no blotches on basal leaves, and five basal leaf segments. Using a Welch's t-test, we determined that the continuous variable of stamen length varied significantly between the two morphotypes ($t=-16.44$, $df=43.34$, $p<0.0001$), with the blue morphotype having stamens ranging from 0.5–1.3 cm in length, and purple having stamens ranging from 1.2–1.6 cm in length (Table 5).

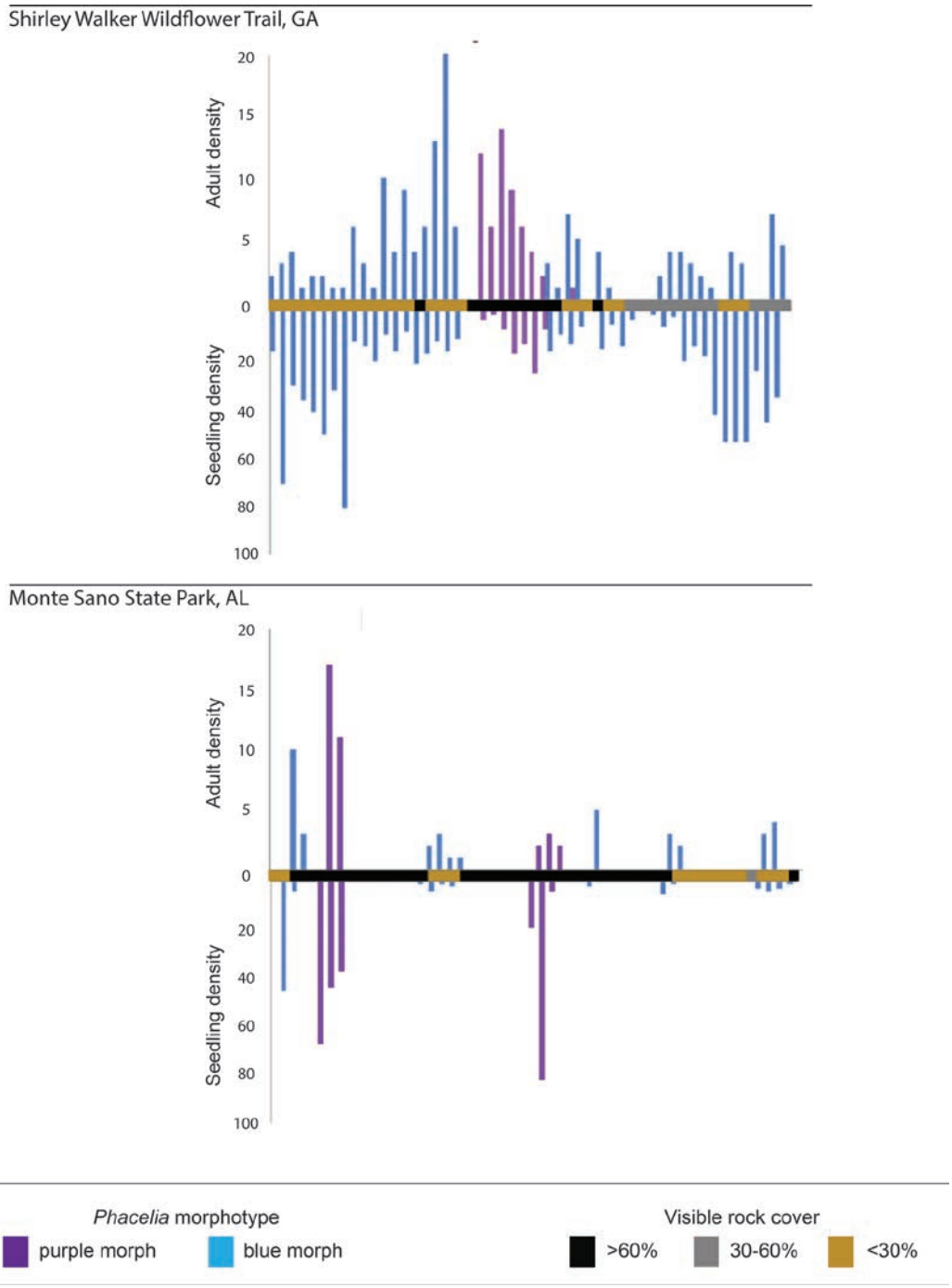

Figure 3. Patch structure of the purple and blue morphotypes of *Phacelia* observed at the Shirley Miller Wildflower Trail, Georgia and Monte Sano State Park, Alabama. Observations for a transect recorded in 2024 at each site are graphically illustrated. The y-axis shows adult and/or seedling density, with purple morph indicated by a purple bar and blue morph indicated by a blue bar. The x-axis shows position along the transect and notes percent visible rock cover: black=<60%; grey=30-60%, and yellow=30%.

Table 3. Summary statistics averaged over 18 loci for populations of *Phacelia* sampled in the present study.

Site code and morph	N	N_a	N_e	H_o	H_e	uH_e	F
KY (ambiguous)	22.000	3.889	1.998	0.275	0.391	0.399	0.265
	(1.36)	(0.690)	(0.314)	(0.047)	(0.058)	(0.06)	(0.065)
IL (blue)	13.444	4.667	3.277	0.516	0.593	0.617	0.119
	(1.261)	(0.464)	(0.351)	(0.064)	(0.063)	(0.066)	(0.055)
TN1 (purple)	7.389	3.667	2.144	0.295	0.363	0.380	0.181
	(1.334)	(0.792)	(0.475)	(0.07)	(0.081)	(0.085)	(0.048)
TN2 (blue)	59.556	11.278	4.760	0.589	0.710	0.716	0.162
	(0.879)	(1.328)	(0.694)	(0.043)	(0.041)	(0.042)	(0.037)
TN2 (purple)	33.778	6.500	2.991	0.323	0.465	0.479	0.319
	(4.776)	(1.178)	(0.547)	(0.071)	(0.083)	(0.085)	(0.074)
AR1 (unknown)	11.000	1.167	1.083	0.005	0.049	0.051	0.651
	(0.000)	(0.09)	(0.058)	(0.005)	(0.031)	(0.033)	(0.143)
AR2 (unknown)	23.000	1.000	1.000	0.000	0.000	0.000	–
	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	–
AL1 (blue)	24.000	1.389	1.143	0.058	0.075	0.076	0.468
	(0.000)	(0.200)	(0.091)	(0.048)	(0.039)	(0.039)	(0.141)
AL2 (blue)	11.944	3.500	2.463	0.429	0.509	0.531	0.180
	(1.116)	(0.473)	(0.321)	(0.069)	(0.063)	(0.066)	(0.084)
AL2 (purple)	7.889	2.222	1.544	0.237	0.344	0.376	0.303
	(1.549)	(0.401)	(0.27)	(0.055)	(0.065)	(0.072)	(0.08)
GA (blue)	12.222	6.167	3.343	0.531	0.630	0.668	0.145
	(0.85)	(0.673)	(0.407)	(0.059)	(0.044)	(0.046)	(0.069)
GA (purple)	7.778	3.611	2.203	0.293	0.370	0.391	0.176
	(1.19)	(0.829)	(0.508)	(0.064)	(0.078)	(0.082)	(0.061)
SC1 (ambiguous)	18.444	1.833	1.245	0.186	0.217	0.222	0.190
	(2.394)	(0.406)	(0.212)	(0.053)	(0.06)	(0.061)	(0.09)
SC2 (unknown)	16.833	1.111	0.985	0.120	0.136	0.139	0.157
	(2.57)	(0.212)	(0.181)	(0.054)	(0.05)	(0.051)	(0.087)
P. brevistyla	4.222	1.889	1.543	0.067	0.269	0.308	0.781
	(0.367)	(0.254)	(0.188)	(0.044)	(0.063)	(0.072)	(0.106)

DISCUSSION

In this study, we used an integrative taxonomy approach, to reach the conclusion that *Phacelia bipinnatifida sensu* Weakley et al. (2024) should be split into two separate species. Using three lines of evidence: morphology, genetics, and ecology, we distinguish between *P. bipinnatifida* (i.e., the blue morphotype) and a new species (i.e., the purple morphotype) that we are naming *P. sewaneensis*.

While there were several key diagnostic traits that differentiate the two taxa, *P. sewaneensis* is best distinguished from *P. bipinnatifida* in its acaulescent first-year growth form. In this stage, *P. sewaneensis* lacks the gray blotches on basal leaves that are always associated with *P. bipinnatifida*, and it has leaves that are distinctly more dissected (Figure 1e). In sexually mature second-year plants, corolla color and stamen length are most diagnostic. Corolla color in *P. sewaneensis* is purple, while *P. bipinnatifida* corollas vary from light blue to lavender (Figure 1b–d). Stamen length is consistently longer in *P. sewaneensis*.

Phacelia sewaneensis and *P. binnatifida* both maintain populations in cove habitats on the southern Cumberland Plateau. Patches of the two species juxtapose one another but rarely overlap. *Phacelia sewaneensis* maintains patches of much higher density, and this patch structure appears to reflect surface rock conditions (Figures 2 and 3). On a given forested slope, *P. sewaneensis* tends to grow on loose rock and boulders, whereas *P. bipinnatifida* is generally found where greater exposed soil is present. Genetic analyses of individuals from sympatric populations found

Table 4. Population pairwise F_{ST} values for sampled sites separated by color morph.

	KY	IL	TN1	TN2-b	TN2-p	AR1	AR2	AL1	AL2-b	AL2-p	GA-b	GA-p	SC1	SC2	*P. brev*
KY (ambiguous)	0.000														
IL (blue)	0.313	0.000													
TN1 (purple)	0.496	0.422	0.000												
TN2 (blue)	0.270	0.130	0.353	0.000											
TN2 (purple)	0.407	0.306	0.376	0.251	0.000										
AR1 (unknown)	0.576	0.450	0.698	0.335	0.608	0.000									
AR2 (unknown)	0.603	0.477	0.726	0.381	0.636	0.337	0.000								
AL1 (blue)	0.579	0.444	0.689	0.327	0.589	0.754	0.860	0.000							
AL2 (blue)	0.379	0.249	0.457	0.167	0.358	0.444	0.484	0.449	0.000						
AL2 (purple)	0.492	0.401	0.474	0.361	0.396	0.700	0.729	0.586	0.454	0.000					
GA (blue)	0.299	0.117	0.393	0.092	0.281	0.394	0.419	0.376	0.191	0.381	0.000				
GA (purple)	0.473	0.404	0.417	0.349	0.327	0.692	0.719	0.674	0.444	0.435	0.285	0.000			
SC1 (ambiguous)	0.509	0.431	0.603	0.370	0.548	0.776	0.815	0.771	0.497	0.613	0.422	0.603	0.000		
SC2 (unknown)	0.592	0.475	0.659	0.404	0.568	0.835	0.875	0.829	0.526	0.659	0.440	0.642	0.712	0.000	
P. brevistyla	0.536	0.355	0.573	0.229	0.465	0.595	0.671	0.608	0.316	0.578	0.291	0.558	0.630	0.708	0.000

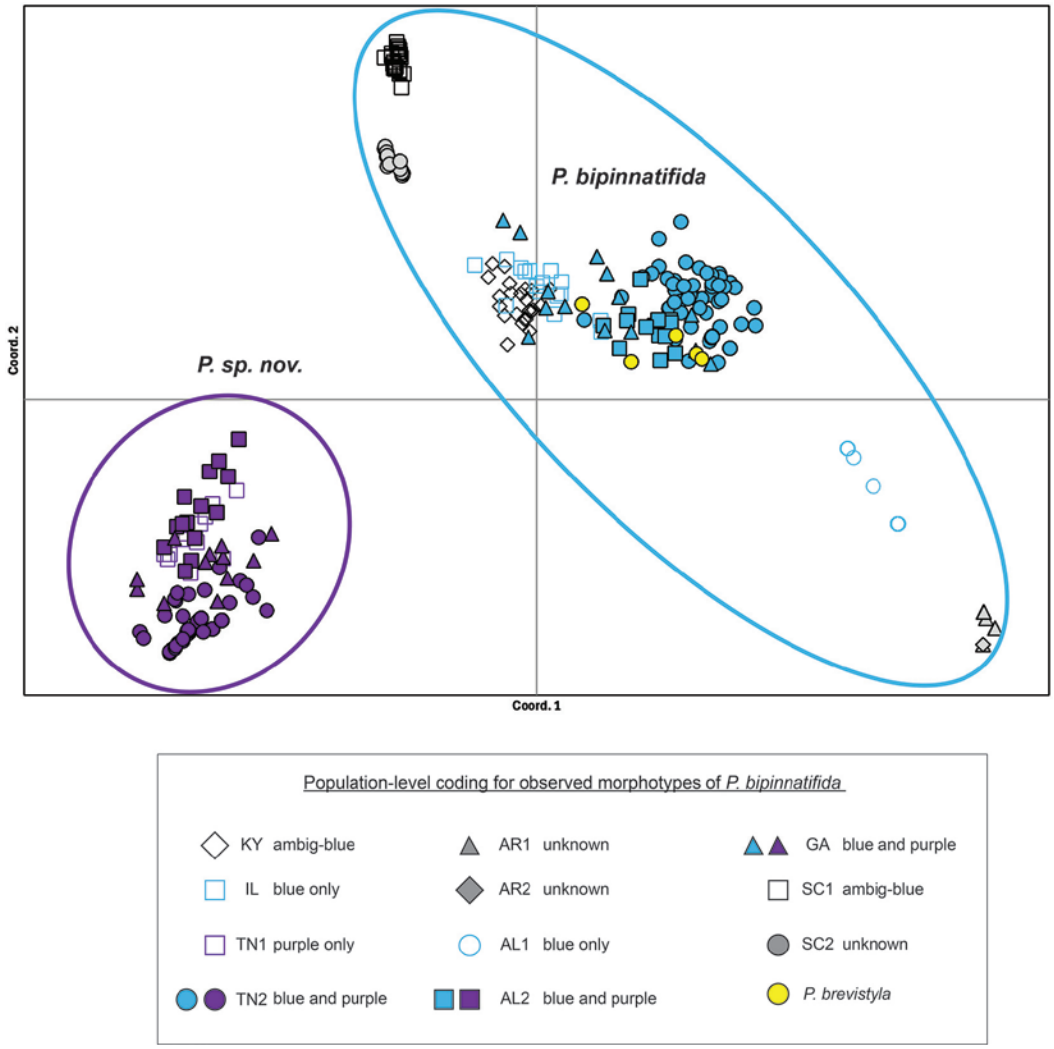

Figure 4. Principal Coordinates Analysis of sampled *Phacelia bipinnatifida* based on 18 GBS microsatellite loci. Population codes follow those provided in Table 1; color coding indicates observed morphotypes at each site as detailed in the text. Ellipses (blue and purple) are consistent with clusters in the dendrogram presented in Figure 5; 100% bs). Additional samples identified as *P. brevistyla* are coded in yellow and cluster within the "blue" morph, which we are recognizing as *P. bipinnatifida*.

in three geographically distant locations on the southern Cumberland Plateau revealed that *P. sewaneensis* was more similar genetically to *P. sewaneensis* at the other locations rather than to *P. bipinnatifida* found in sympatry (Table 4, Figures 4 and 5) and that observed and expected heterozygosities were lower among *P. sewaneensis* populations than *P. bipinnatifida* populations. All individuals scored as *P. sewaneensis* based on morphology consistently segregated as a genetically distinct cluster in our analyses (Figure 4). Other sites included for broader context all fell within the larger cluster of sites identified as *P. bipinnatifida* (Figure 4). However, we noted that several sites for which morphology either was not documented (AR1, AR2, and SC2) or was ambiguous (KY and SC1) do not cluster tightly with the other members of the *P. bipinnatifida* clade (Figure 5), indicating the need for additional investigation.

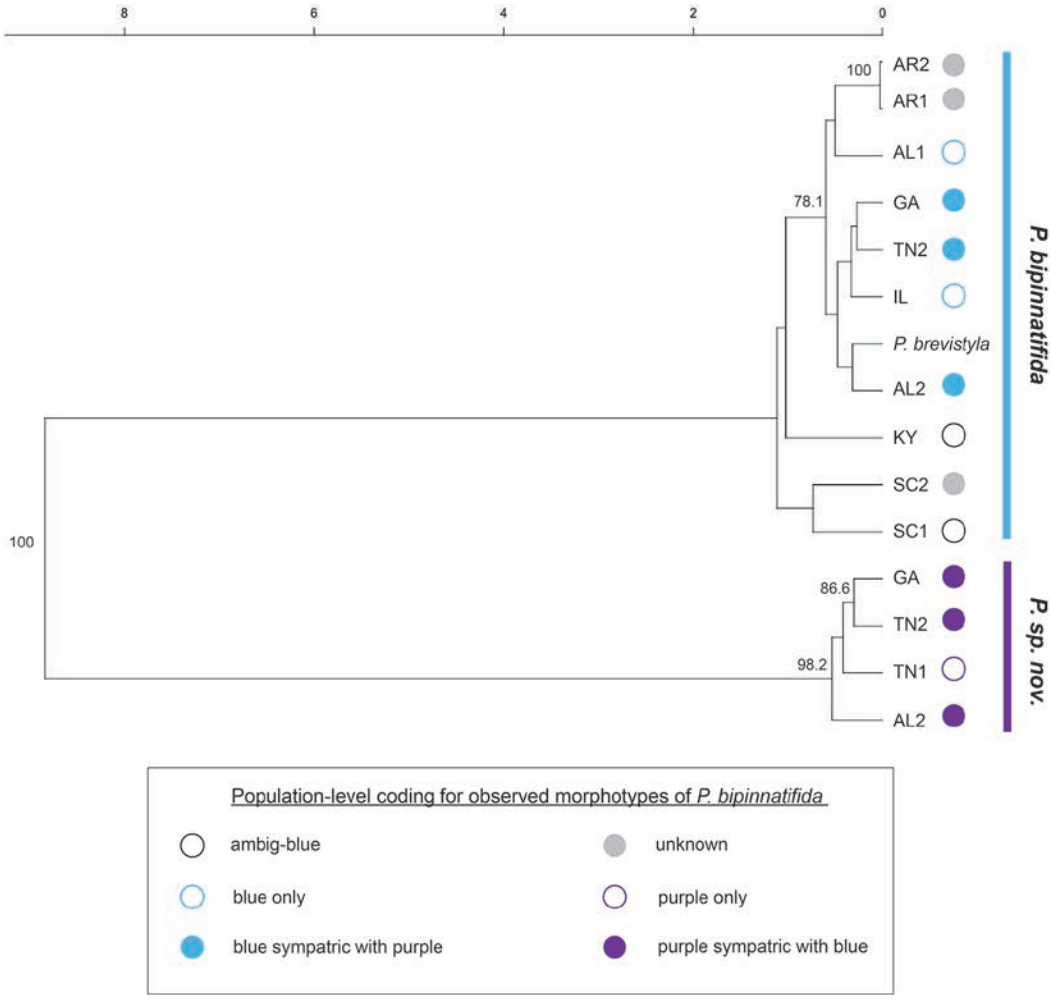

Figure 5. Dendrogram of sampled *Phacelia bipinnatifida* based on Nei's genetic distance of 18 GBS microsatellite loci. Population codes follow those given in Table 1; color coding indicates observed morphotypes at each site as detailed in the text. Numerical values above branches represent bootstrap support greater than or equal to 70%.

Furthermore, our observations regarding failure rates in marker development, and differential amplification of selected loci in geographic space may be indicative of other as yet unidentified taxa hiding within this group. Additionally, the samples identified as *P. brevistyla* fall well within the cluster that is *P. bipinnatifida* (Figures 4 and 5), suggesting that *P. brevistyla* is not supported as being a distinct species based on the genetic data. This is in keeping with current taxonomy under Weakley et al. (2024), who treat *P. brevistyla* as a synonym of *P. bipinnatifida*.

Several mechanisms commonly observed across *Phacelia* may be responsible for reproductive incompatibility and species-level differentiation between *Phacelia sewaneensis* and *Phacelia bipinnatifida*. Polyploidy, aneuploidy, postzygotic incompatibility, mating system shifts, and ecological specialization have all been suggested as mechanisms that allow *Phacelia* species to maintain distinct evolutionary identities despite overlapping ranges and shared pollinators (Glass and Levy 2011; Walden et al. 2014). In *Phacelia dubia*, crosses between varieties result in hybrid sterility driven by nuclear-cytoplasmic incompatibilities and partial gametic sterility (Levy 1991). Similar postzygotic barriers occur between *Phacelia fimbriata* and *Phacelia purshii*, where hybrid seeds

Table 5. Trait differences in sympatric population. A total of 66 plants were sampled, and flower color was used to categorize morphotype.

Population	Stamen Length (cm)		Filament Color		Leaf Spots		Basal Leaf Segments	
	Purple	Blue	Purple	Blue	Purple	Blue	Purple	Blue
Shakerag Hollow, Sewanee	1.44 ±0.20	1.07 ±0.04	purple	white	n	y	5	3
Monte Sano State Park	1.34 ±0.11	0.92 ±0.14	purple	white	n	y	5	3
Shirley Miller Wildflower Trail	1.46 ±0.09	1.04 ±0.11	purple	white	n	y		

fail due to embryo-endosperm incompatibility (Glass and Levy 2011). Chromosomal shifts have also played a major role, particularly in species within subgenus *Cosmanthus*, where descending dysploidy—from n = 11 to n = 9 or 5—creates immediate genetic barriers to hybridization (Gillett 1968; Walden et al. 2014). These shifts could potentially have been facilitated by structural genomic changes mediated by transposable element (TE) insertions or transposable element-induced recombination. This type of TE-mediated chromosomal rearrangement has been documented in other plant genera with similar karyotypic variation (Bennetzen and Wang 2014). These chromosomal changes often accompany shifts from self-incompatibility to self-compatibility, further reinforcing isolation and allowing new polyploids to establish (Mable 2004).

Given the clear differences between *Phacelia bipinnatifida* and *P. sewaneensis* along multiple lines of evidence, how was this species missed? Historically, taxonomic revisions and species treatments have relied heavily on data generated from the analysis of herbarium specimens, which has traditionally been the case with revisions of the genus *Phacelia* (Constance 1949, 1963; Walden and Patterson 2012). This can be problematic if herbarium specimens do not accurately capture distinguishing characteristics (Parnell et al. 2013; Botes et al. 2020). In our case, the most defining features of *P. sewaneensis* are not associated with herbarium specimens for various reasons. Basal leaf characteristics of first year plants represent the most visibly striking differences between *P. sewaneensis* and *P. bipinnatifida*. These characters cannot be discerned from the herbarium record, since all collected specimens of this species that we examined (including the original holotype for *P. bipinnatifida*) were of second year plants that were flowering or in fruit. Most herbarium specimens no longer had first year basal leaves attached, so these characters were missing. The difference in corolla color (blue versus purple), while striking when comparing live plants in the field (Figure 1), is also not as apparent on herbarium specimens, becoming less discernible as specimens age. The small but significant difference in stamen length is also very difficult to measure consistently on pressed specimens. For our analyses we found the photographic archive of iNaturalist to be invaluable, as we were able to examine over 5000 observations from populations across the entire range of the two taxa. Within highly visited populations such as at our three study sites, we were able to determine exactly where the two species were growing together. Photographs of plants in these populations taken over the course of several years represented plants in various life history stages. Photographs of flowers were truer to color and often included close-up images that allowed us to examine stamen exertion. We believe photo documentation such as iNaturalist records should be used to supplement herbarium collections (Heberling and Isaac 2018) of these two species in the future.

Epitypification of *Phacelia bipinnatifida*

Michaux (1803) first described *Phacelia bipinnatifida* in his *Flora Boreali-Americana* and the Muséum National D'Histoire Naturelle Paris (PCU), holds both holotype (no accession number provided; **Supplemental Figure 1**) and isotype (MNHN-P-P00640081; **Supplemental Figure 2**)

specimens for *P. bipinnatifida*. A translation of Michaux's original description is as follows: "with pinnatifid leaves, incised and lobed; spikes generally bifid, oblong, multi-flowered; corolla with blue lobes with a simple margin. The habitat is described as in the western mountains of the Alleghenies and Kentucky." We translated the undated label for the holotype from French to English as "... down very wet and rich ... at the foot of mountains after ... turned right hand ... with T. Yong dear DavinPort." The specimen appears to have both fruits and flowers. The isotype label simply translates to "in the high mountains west of the Allegany," and that specimen also appears to have both fruits and flowers.

Based on information gathered from the original description, holotype, isotype, and from translated journals and letters of Michaux himself, we hypothesize that *P. bipinnatifida* was originally collected and described from the area between Roan Mountain, Tennessee, and Linville Gorge, North Carolina. Williams et al. (2020) reported that Martin Davinport was a mountain guide who lived northwest of Linville Gorge and accompanied Michaux on the following dates: 17 Aug–1 September 1794; 5 May–13 May 1795; and 23 March–29 March 1796. Additionally, they reported a Thomas Young who lived less than 10 miles from Davinport. It is not always clear on which dates Young was traveling with Michaux and Davinport. However, given the travel routes described in the journals on the dates above and the apparent presence of fruits on Michaux's holotype and isotype, we hypothesize that the specimens were collected during the May 1795 trip. During that trip, Michaux described botanizing in the mountains "around the home of Davinport" as well as traveling to Roan Mountain and Yellow Mountain. Beyond these details, it is not possible to determine exactly what, when, or where Michaux collected as *P. bipinnatifida*.

According to the International Code of Nomenclature for Algae, Fungi, and Plants, an epitype can be established when "the holotype, lectotype, or previously designated neotype, or all original material associated with a validly published name, is demonstrably ambiguous and cannot be critically identified for purposes of the precise application of the name to a taxon" (Ch II, Sec. 2, Art. 9.9; Turland et al. 2018). As noted in the current work, it is not possible to identify the two taxa we describe herein using these specimens due to loss of important diagnostic characters during preservation as well as the lack of detail surrounding geographic range. Specifically, Michaux's type specimen does not have observable stamens, such that the key feature of stamen length is missing. Flower color is not retained in the specimen, nor are basal leaves (see Supplemental Figures 1 and 2).

Additionally, as noted from the genetic data, there are still some uncertainties regarding the status of material in the Carolinas that require further study. For clarification, we establish a new epitype for *P. bipinnatifida* (*Michel and Evans 9358*: UOS); Figure 6) in support of the holotype (Figure 7). The holotype and isotypes for the newly described *P. sewaneensis* are given below. All specimens are collected from the same location (Shakerag Hollow) and are supplemented with iNaturalist observations to preserve diagnostic characters that are typically not preserved in herbarium specimens.

TAXONOMIC TREATMENT

Phacelia sewaneensis J.P. Evans, J.T. Michel, S.J. Fox, and A.B. Morris, sp. nov-TYPE: **Tennessee. Franklin County:** Shakerag Hollow, Domain of the University of the South, 17 Apr 2023, *J.T. Michel and Jon Evans* (HOLOTYPE: UOS; ISOTYPES: UOS, FUGR, UCHT). Figure 7.

Description

Biennial; stems branching above the base, branches ascending to erect, densely hirsute with spreading or deflexed stiff hairs, more densely so at base; basal leaves petiolate, triangular-ovate, 3–12 cm. long and broad, segments 5–7, terminal segment tripartite incised nearly to the midvein, lacking waterspots; leaflets pinnatifid, deeply lobed to highly dissected, leaf glands strongly odiferous; the inflorescence densely spreading-hirsutulous or -hirsute and glandular-villous with small slender-stalked glands, terminal, cymes paired or clustered, 5–50-flowered, mature pedicels 6-15 mm. long, arcuately recurved,; calyx lobes linear, 4-8 mm × 0.5-1.5 mm. broad, subequal, acute, surfaces glandular-

Figure 6. Epitype of *Phacelia bipinnatifida*.

Figure 7. Holotype of *Phacelia sewaneensis*

villosulous; corolla purple-indigo, broadly campanulate, 10–15 mm. broad, the lobes obovate, length × width, minutely crenulate, abaxial surface hispidulous; gland flaps wholly adnate, puberulent, corolla tube conspicuously distended by the apparently functional glands; stamens 12–16 mm. long, exserted, anthers oblong, 1–1.5 mm. long, filaments villous on their lower ⅔ and often purple in color; style 8–15 mm. long when mature, exserted in flower, cleft ⅓ to ⅔, hirsutulous at base, ovary summit hirsute; mature capsule subglobose, 4–6mm. in diameter; ovules 2 to each placenta; seeds usually 4, dark brown, ovoid-angled, 3 mm. long, areolate and finely alveolate.

Diagnosis

The following combination of characteristics distinguishes *Phacelia sewaneensis* from *P. bipinnatifida*: corolla purple–indigo (vs. blue–lavender), longer stamens 12–16mm (vs. 5–13mm), filaments purple (vs. white), gray blotches on basal leaves absent (vs. present), and five basal leaf segments (vs. three; Table 5).

Habitat and Distribution

Rocky, north-facing upper slopes of the Cumberland Plateau. Locally abundant at the base of the bluff in association with sandstone boulder fields and areas of loose surface rocks.

Etymology

The specific epithet, *sewaneensis*, refers to the type locality and center of the known range based on our current sampling. In an article in the Sewanee Mountain Messenger from 23 August 1985, Elizabeth N. Chitty states that Sewanee is a Shawnee word for 'southern' and was often used by Native people west of the Smokies to describe the Cumberland Plateau. Another article on the university website indicates that the word is a Native American word meaning 'lost.' Given these comments without substantial reference to support them, we felt it was important to carefully document the role of Traditional Knowledge in recognition of this new species. We contacted Scott Miller, Language Coordinator of the Language Department of the Absentee Shawnee Tribe of Indians of Oklahoma for clarification. Miller communicated with tribal elders, and he indicated that they could not find a direct translation for the word 'Sewanee' in their lexicon. He noted that their word for south is *Ya li wi k'wa ke* (yay lah wah k'way key), and their word for land is *Hi se s'ke* (hah see s'key), neither of which is close to 'Sewanee.' He did, however, tell us that they found the word *wi ne* (wah nee), which means lost, and they thought that perhaps the word Sewanee may be derived from *Ne ta se wi ne* (nee tay see wah nee), meaning 'I'm lost.' He also told us that the area where Sewanee exists today was most likely inhabited by a Shawnee band spanning eastern Tennessee, western North Carolina, and Alabama.

Range

Phacelia sewaneensis is currently known from four sites: two in Tennessee and one each in Alabama and Georgia. The inability to distinguish *P. sewaneensis* from *P. bipinnatisecta* in herbaria collections is cause for this initial restricted range. More field surveys along the Cumberland Plateau and in Appalachia will certainly result in the discovery of additional populations.

Conservation

Based on current sampling, *P. sewaneensis* is only known from two counties in Tennessee, and one county in each of Georgia and Alabama. Additionally, genetic data indicate much lower observed and expected heterozygosities for *P. sewaneensis* than for *P. bipinnatifida*. Given this limited distribution and genetic variation, we propose that the species be considered for special conservation concern at this time. We anticipate that further survey work will likely expand the documented range, at which point, a formal conservation assessment will be needed.

KEY TO *PHACELIA BIPINNATIFIDA* AND *P. SEWANEENSIS*

1a. Corollas blue-lavender, stamen length 5-13 mm., basal leaves mottled with gray blotches, and pinnate with 3 segments . . . . . *P. bipinnatifida*

1b. Corollas purple-indigo, stamen length 12-16mm., basal leaves lacking gray blotches, and pinnate with 5-7 segments . . . . . *P. sewaneensis*

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