Title: Phylogenomic patterns of divergence and gene flow detail the evolution of reinforcement and hybrid speciation in *Phlox* wildflowers

Authors: Austin G. Garner [1,2*], Benjamin E. Goulet-Scott [1,2], and Robin Hopkins [1,2*]

Affiliations:

[1] *Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 021382, USA*

[2] *The Arnold Arboretum, Harvard University, Boston, MA 02131, USA*

[*] Authors to whom correspondence should be addressed:

Austin G. Garner; email: aggarner@g.harvard.edu, phone: 617-496-9613

Robin Hopkins; email: rhopkins@fas.harvard.edu, phone: 617-496-9613

SUMMARY

- The tree of life is riddled with reticulate evolutionary histories, and some clades, such as the eastern standing *Phlox*, appear to be hotspots of hybridization. In this group there are two cases of reinforcement and nine hypothesized hybrid species. Given the historical importance of this group for our understanding of plant speciation, the relationships of these taxa and the role of hybridization and gene flow in their diversification require genomic validation.
- Using phylogenomic analyses, we resolve the evolutionary relationships of the eastern standing *Phlox* and test hypotheses about if and how hybridization and gene flow were creative forces in their diversification.
- Our results provide novel resolution of the phylogenetic relationships in this group, including well-supported paraphyly across some species. We identify gene flow during one of two cases of reinforcement and find support for one of the five hypothesized homoploid hybrid speciation events. Additionally, we infer the ancestries of four allotetraploid hybrid species.
- Hybridization has contributed to diverse evolutionary outcomes within this *Phlox* group; although, not as extensively as previously hypothesized. This study demonstrates the importance of phylogenomics in confirming hypothesized histories of non-model systems

and adds to the growing evidence of interspecific genetic exchange in the generation of biodiversity.

KEYWORDS: gene flow; hybridization; hybrid species; phylogeny; polyploid; RADseq; reinforcement; speciation

INTRODUCTION

Hybridization and gene flow can play important and diverse roles during the formation and divergence of species (Taylor & Larson, 2019). While these processes are often viewed as homogenizing forces, they can also generate novel variation. For example, gene flow can create stable hybrid species and/or fuel the diversification of adaptive radiations (Abbott *et al.*, 2013; Schumer *et al.*, 2014; Marques *et al.*, 2019). The act of hybridization itself can also create selective pressures favoring the evolution of reproductive trait divergence to reduce mating between species, i.e. reinforcement (Howard, 1993; Garner *et al.*, 2018). The effects of hybridization and gene flow on speciation have been investigated across the tree of life, yet we lack a broader understanding of the frequency and extent to which these forces impact speciation across clades of organisms (but see Eaton *et al.*, 2015; Pease *et al.*, 2016**)**. Characterizing the context and consequences of hybridization and gene flow across groups of species is necessary for discerning how these forces influence the generation and maintenance of biodiversity.

Much of the foundational work on hybridization as a creative force in speciation comes from historic research on non-model plant systems (Anderson, 1949; Stebbins, 1959; Grant, 1981). Patterns of morphological, physiological, and ecological trait variation across and within plant species created taxonomic conflict, inspiring biologists to propose some species arose from interspecific mating (Goulet *et al.*, 2017). In particular, observations of populations with combinations of intermediate, recombinant, and novel traits to those of nearby geographically overlapping species inspired hypotheses of polyploid and homoploid hybrid speciation (Gottlieb, 1972; Soltis & Soltis, 2009; Yakimowski & Rieseberg, 2014; Barker *et al.*, 2016). It is important to return to these known and hypothesized cases of reticulate evolution with genomic tests for gene flow to confirm these evolutionary processes and further understand the context of their evolution (Goulet *et al.*, 2017).

Similarly, patterns of trait divergence in sympatry, where species coexist, but not in allopatry, motivated hypotheses of hybridization causing reproductive trait divergence by reinforcement (Hopkins, 2013). Yet, only a few empirical studies have evaluated the presence and extent of gene flow during the evolution of reinforcement in nature (Kulathinal *et al.*, 2009; Roda *et al.*, 2017; Turissini & Matute, 2017; Lemmon & Juenger, 2017; Dyer *et al.*, 2018)**.** Maladaptive hybridization can drive selection for reinforcement and the divergence of plant reproductive traits such as flower color, flowering time, and cross-compatibility (McNeilly &

Antonovics, 1968; Levin, 1985; Fishman & Wyatt, 1999). However, gene flow and recombination can also impede divergence by reinforcement (Felsenstein, 1981). Much debate and theory has focused on if and how reinforcement can evolve if hybridization also causes interspecific gene flow (Servedio & Noor, 2003), but further genomic study of cases of reinforcement are needed to understand if and how this reproductive trait divergence has evolved with gene flow.

Hybridization and gene flow result in the mixing of genetic variation between species. If these processes coincide with the formation of novel diverging lineages, admixture of parental species' genetic variation will be present in the new lineage. Traditional phylogenetic methods infer evolution along bifurcating trees. However, modern phylogenomic analyses can use discordance in allele patterns under these trees (Green *et al.*, 2010; Durand *et al.*, 2011) or model allele frequencies at the tips (Gutenkunst *et al.*, 2009; Pickrell & Pritchard, 2012; Excoffier *et al.*, 2013) to identify a history of gene flow between taxa (reviewed in Hibbins & Hahn, 2021). Contemporary genome-wide sequencing methods, such as restriction site-associated DNA (RAD) sequencing (Andrews *et al.*, 2016)**,** allow cost-efficient application of these methods to infer the evolutionary histories of genetic exchange in non-model systems (Eaton & Ree, 2013; Eaton *et al.*, 2015; Léveillé-Bourret *et al.*, 2020; Bombonato *et al.*, 2020; Guo *et al.*, 2021; Suissa *et al.*, 2022).

The eastern standing *Phlox* (Polemoniaceae) wildflowers are an ideal system for exploring the role of hybridization and gene flow in speciation. They are a monophyletic group (Landis *et al.*, 2018) distinct from the rest of *Phlox* by their upright growth habit and natural occurrence across mid to eastern North America. Their natural ranges overlap and intertwine, generating numerous zones of species contact (Fig. 1), and reproductive barriers between these taxa are highly permissive (Levin, 1966b)**.** Decades of morphological, ecological, and biochemical evidence have suggested hybridization and gene flow between taxa**,** leading to hypotheses of their diversification by hybrid speciation and reinforcement (Anderson & Gage, 1952; Erbe & Turner, 1962; Levin, 1966a; Levin & Smith, 1966; Levin & Kerster, 1967; Levin & Schaal, 1970b; Levin, 1985).

There are five hypothesized cases of homoploid hybrid species (*P. argillacea*, *P. maculata* subsp. *pyramidalis*, *P. pilosa* subsp. *deamii*, *P. pilosa* subsp. *detonsa*, and *P. amoena* subsp. *lighthipei*) and at least four cases of allotetraploid hybrid species (*P. villosissima* subsp.

98 *villosissima, P. villosissima* subsp. *latisepala, P. floridana, P. buckleyi*) (Table 1) within the
99 eastern standing *Phlox*. Support for these hypotheses were originally based on observations that
100 putative hybrid lineages had reproductive, morphological, and ecophysiological trait values that
101 appear to be intermediate, recombined, or transgressive with putative parental taxa, making them
102 potentially reproductively and ecologically distinct from their parental lineages (Levin, 1963,
103 1969; Levin & Smith, 1966; Hadley & Levin, 1969)**.** Further analysis of karyology (Smith &
104 Levin, 1967; Levin, 1968), cross compatibility (Levin, 1966b), biochemical profiles (Levin &
105 Schaal, 1970b, 1972; Levy & Levin, 1974, 1975), and microsatellite markers (Fehlberg *et al.*,
106 2014) built support for these hypotheses. Finally, natural hybrid zones and synthetic crosses
107 between pairs of putative parental taxa generated hybrids with phenotypes similar to the putative
108 stable hybrid lineages in nature (Levin, 1963, 1966a; Levin & Smith, 1966). However, other
109 treatments of these putative hybrids species have assumed them to be diverging varieties without
110 hybridization defining their formation (Wherry, 1956) or found no support for hybrid ancestry
111 with genome-wide markers (Goulet-Scott *et al.*, 2021).
112 This clade of *Phlox* also has two cases of reinforcement. During the speciation of both *P.*
113 *drummondii* and *P. pilosa* subsp. *pilosa*, flower color divergence within sympatric populations
114 decreases hybridization with closely related species (reviewed in Hopkins, 2013; Fig. 3). *P.*
115 *drummondii* has evolved from having light-blue to dark-red flower color to prevent hybridization
116 with sympatric light-blue colored *P. cuspidata* (Levin, 1985; Hopkins & Rausher, 2012), and *P.*
117 *pilosa* subsp. *pilosa* have evolved from pink to white flower color to prevent hybridization where
118 it cooccurs with pink colored *P. glaberrima* subsp. *interior* (Levin & Kerster, 1967).
119 Biochemical evidence suggests gene flow occurred between sympatric *P. pilosa* subsp. *pilosa*
120 and *P. glaberrima* subsp. *interior* (Levin & Schaal, 1972), and genomic analyses have inferred a
121 history of gene flow between sympatric *P. drummondii* and *P. cuspidata,* but analyses were
122 limited to single individuals (Roda *et al.*, 2017).
123 *Phlox* has been a taxonomically and phylogenetically difficult genus. Traditional
124 delineations of *Phlox* species relied on morphological, ecological, and geographic characteristics,
125 resulting in a long history of describing, promoting, and demoting species, subspecies, and
126 varieties (Whitehouse, 1945; Wherry, 1955, 1965; Erbe & Turner, 1962; Turner, 1998; Locklear,
127 2011a,b). Conspicuous variation in pistil style length has been used to divide the eastern standing
128 *Phlox* into long-styled (10-26mm) and short-styled (1.5-4mm) species groups (Wherry, 1930,

129 1931, 1932, 1955; Ferguson *et al.*, 1999). However, other morphological traits vary widely
130 within and between species, and species-level relationships within the group remain either poorly
131 supported or incongruent across phylogenetic studies (Ferguson *et al.*, 1999; Ferguson & Jansen,
132 2002; Landis *et al.*, 2018; Goulet-Scott *et al.*, 2021). Resolving the phylogenetic relationship of
133 the eastern standing *Phlox* with modern genomic methods will provide a powerful framework for
134 returning to evolutionary hypotheses about their speciation and understanding the dynamics by
135 which their diversification has occurred.

136 Here, we apply double-digest RAD (ddRAD) sequencing with phylogenomic approaches
137 to resolve the evolutionary relationships of the eastern standing *Phlox*. We leverage this
138 phylogenetic framework to examine the contribution of hybridization and gene flow in the
139 diversification of this system. Specifically, we test if gene flow has occurred alongside the
140 evolution of two cases of reinforcement and investigate patterns of genetic composition and gene
141 flow in multiple hypothesized cases of homoploid and allotetraploid hybrid speciation.
142

143 **MATERIALS AND METHODS**

144 *Sampling, sequencing, and data processing*

145 We collected wild plants from across the native ranges of the eastern standing *Phlox* from
146 2017-2019, and we acquired additional samples from natural populations preserved in tissue
147 culture at the Ohio State University Ornamental Plant Germplasm Center (OPGC). Our sampling
148 includes seventeen recognized eastern standing *Phlox* species, representing thirty-two described
149 subspecies, and three outgroup taxa from the mat-forming *Phlox* (Fig. 1; Supporting Information
150 Table S1). We split taxa following Locklear, 2011b, except for the Texas annuals for which we
151 only designated at species level.

152 Plants were grown in the greenhouse under controlled conditions until flowering. We
153 extracted genomic DNA from bud tissue using a hybrid Omega Bio-Tek EZNA DNA kit and
154 CTAB/Chloroform extraction protocol. Genomic libraries were constructed with a double-digest
155 restriction-site-associated digestion (ddRAD) sequencing protocol and paired-end sequenced as
156 described in Goulet-Scott *et al.*, 2021. Raw sequence reads were demultiplexed, filtered, and *de*
157 *novo* assembled as described in Goulet-Scott *et al.*, 2021. Samples with minimal informative
158 shared loci were removed, resulting in a final dataset of 116 ingroup and 4 outgroup individuals.

159 For the 120 retained samples, filtered raw reads (average of 3,060,421 reads per
160 individual) were processed by iPyRAD v.0.9.50 (Eaton & Overcast, 2020) to construct four
161 datasets with varying levels of missingness, requiring a locus to be shared among a minimum of
162 N = 4, 10, 20, and 30 individuals, named MS4, MS10, MS20, and MS30 respectively. These
163 assembled datasets produced matrices with a total of 165,549 to 5,3871 loci and 1,884,423 to
164 105,363 SNP sites with a locus missingness rate of 90%, 83%, 72%, and 60% respectively.
165
166 *Phylogenetic inferences*
167 Phylogenetic relationships of our samples were inferred using concatenation and
168 coalescent-based methods. First, whole ddRAD loci from the MS10, MS20 and MS30 datasets
169 were individually concatenated into supermatrices. Maximum likelihood phylogenies were
170 estimated from each supermatrix using IQ-TREE v.1.6.10 (Nguyen *et al.*, 2015) with the GTR +
171 gamma nucleotide substitution model and 1000 bootstrap replicates using ultrafast bootstrap
172 approximation (Minh *et al.*, 2013; Hoang *et al.*, 2018)**.**
173 Second, we inferred a population-level species tree using SVDquartets (Chifman &
174 Kubatko, 2014), as implemented in PAUP v.4.0a166 (Swofford, 2002). This method uses
175 unlinked SNPs to infer relationships among quartets of taxa under a coalescent model and then
176 estimates a species tree using a quartet assembly method. We conducted the analysis on our
177 dataset with least missingness, MS30, with the mat-forming species removed and *P. stolonifera*
178 set as the outgroup. To avoid genetic linkage, we sampled a single SNP-site with the least
179 amount of missing data from each locus, totaling 5,306 variant sites. We used all 7,160,245
180 possible quartets and 100 non-parametric bootstrap replicates to assess topological support and to
181 generate a majority-rule consensus species tree.
182 Support values between these two inference methods are derived from different
183 estimation methods and cannot be compared one to one. Standard bootstrap supports (SBS), as
184 used with our SVDquartets inferred tree, underestimate the likelihood of relationships; SBS
185 supports of 80% have a 95% likelihood of being correct. Ultrafast bootstrap supports (UBS), as
186 used for our concatenation inference method, are unbiased when >70%, so a 95% support has a
187 0.95 probability of being correct (Minh *et al.*, 2013)**.**
188
189 *Tests for gene flow alongside reinforcement*

190 We tested for evidence of gene flow alongside the evolution of reinforcement in *P.*
191 *drummondii* and *P. pilosa* subsp. *pilosa*. First, we computed Patterson's D-statistic (Green *et al.*,
192 2010; Durand *et al.*, 2011) to infer if gene flow occurred between two lineages in a given
193 phylogeny. This test measures the asymmetry in the ratio of two discordant allele patterns,
194 ABBA and BABA, across the topology of a four-taxon pectinate tree ((P1, P2), P3), O). Under
195 stochastic lineage divergence without gene flow between lineages P3 and P1 or P3 and P2, the
196 proportion of discordant allele patterns from incomplete lineage sorting are unbiased
197 (ABBA=BABA); however, when introgression has occurred between P3 and P1 or P3 and P2,
198 one discordant allele pattern is more prevalent than the other (ABBA>BABA, BABA>ABBA).
199 We defined the P1, P2, P3, and O lineages as taxa and a "test" as a unique combination of
200 individuals from these taxa. We subset the complete MS4 data matrix to include the individuals
201 from a set of four taxa that fit the pectinate topology under our ML phylogenetic trees. P1 is the
202 taxon that diverged by reinforcement (*P. drummondii* and *P. pilosa* subsp. *pilosa*), P2 as a taxon
203 out to P1 but not involved in the reinforcement hypothesis, P3 as the taxon sympatric with P1
204 driving divergence by reinforcement (*P. cuspidata* and *P. glaberrima* subsp. *interior*), and O as
205 an individual from a proximal lineage out to all other lineages. Tests were iterated over all
206 possible combinations of all individuals of the P1 and P3 lineages, up to three randomly sampled
207 individuals of the P2 lineage not observed growing geographically near populations of P1 or P3,
208 and two individuals of the O lineage. The white flowered *P. pilosa* subsp. *pilosa* lineage that
209 evolved by reinforcement diverged from the lineage of northern *P. pilosa* subsp. *pilosa*
210 populations (Fig. 2), therefore, we only used *P. pilosa* subsp. *pilosa* North individuals in these
211 tests. We also considered two different species as the P2 lineage in the hypothesis concerning *P.*
212 *pilosa* subsp. *pilosa* North. For all possible tests, we implemented the D-statistic test for 1,000
213 bootstrap iterations with loci resampling with replacement, as described in (Eaton *et al.*, 2015).
214 Tests with |Z-score| >3 are statistically significant.
215 We complemented the D-statistic results with TreeMix v1.13 (Pickrell & Pritchard,
216 2012), a model-based method for inferring population splits and migration (gene flow) under a
217 tree framework, as implemented in iPyRAD. We targeted a potential signal of introgression by
218 modeling a subtree of our total phylogenetic sampling. We subset the complete MS4 data matrix
219 to include all individuals from all non-hybrid lineages in the clades containing the P1 and P3
220 lineages, described above, all intermediate clades, and from a lineage out to this subtree.

221 Individuals were grouped based on species identity with *P. drummondii* and *P. pilosa* subsp.
222 *pilosa* North split based on flower color phenotype. We denote these subtrees as "*P. pilosa*
223 subsp. *pilosa* North" and "*P. drummondii*" respectively. We required SNPs to be shared by at
224 least 20% of individuals from each species and randomly sampled one unlinked SNP from each
225 locus. The *P. drummondii* (4,638 unlinked SNPs) and *P. pilosa* subsp. *pilosa* North (3,383
226 unlinked SNPs) subtrees were modeled in TreeMix with m = 1-8 possible migration edges. The
227 best supported number of migration edges was qualitatively chosen as the value at which the log-
228 likelihood of the model began to increase only marginally.
229
230 *Testing origins of homoploid hybrid species*
231 To evaluate if the five putative homoploid hybrid species in the standing *Phlox* (Table 1)
232 have genetic support we leveraged our phylogenetic inferences and tests for gene flow to
233 evaluate four criteria: 1) the putative hybrid sits sister to or nested within one of its putative
234 parental taxon in our bifurcating phylogenetic reconstructions, 2) the putative hybrid shows a
235 signal of gene flow with its other parental taxon, 3) the signal of gene flow is exclusive to the
236 putative hybrid, and 4) the signal of gene flow goes into the putative hybrid.
237 For putative hybrids that are sister to or within one of their putative parent lineages, we
238 tested for the presence of gene flow with the second putative parental lineage using Patterson's
239 D-statistic, as described above. For each hybrid hypothesis, a four-taxon tree was generated by
240 subsetting the MS4 dataset to all individuals of the putative hybrid species and putative second
241 parent species, three individuals from a species lineage between the putative hybrid and putative
242 parent, and two individuals of a closely related outgroup. The putative hybrid and the putative
243 parent lineages served as either P1 or P3 based on the structure of our ML phylogenies. We also
244 considered multiple species as the P2 lineage. For putative hybrid lineages with significant
245 evidence of gene flow with their parental taxon, we evaluated if the signature of gene flow was
246 localized to the hybrid lineage by testing for any gene flow between the two putative parental
247 taxa. All hybrid hypotheses tested with D-statistics were also modeled with TreeMix v.1.13, as
248 described above. The *P. maculata* subsp. *pyramidalis*, *P. amoena* subsp. *lighthipei*, and *P. pilosa*
249 subsp. *detonsa* modeled subtrees used 4,490, 4,294, and 1,033 unlinked SNPs respectively. We
250 used the *P. pilosa* subsp. *pilosa* North modeled subtree for the *P. argillacea* hypothesis given
251 their identical sampling.

Inferring origins of allotetraploid hybrid species

We investigated the hypothesized ancestries of the four allotetraploid hybrid species (Table 1) using a consensus and comparative read alignment approach, similar to (Wang *et al.*, 2021)**.** Sequenced representatives of these taxa were previously confirmed to be tetraploid via flow cytometry at the OPGC.

First, we created reference consensus sequences from diploid lineages. Whole ddRAD loci present in at least 60% of diploid taxa from the MS4 dataset were concatenated to generate taxon-level consensus sequences using the iPyRAD window extractor toolkit, retaining the most common allele in the consensus sequence for species with multiple samples. We then mapped the filtered sequence reads for all allotetraploids to the diploid consensus sequences using bwa-mem (Li, 2013). We tallied the proportion of primary read alignments with a mapping quality ≥ 5 for the forward and reverse reads. We reasoned the diploid consensus receiving the highest proportion of quality read alignments would be most closely related to the ancestor of the mapped reads.

We tested this assumption by mapping one representative from each diploid taxon to the consensus sequences. We observed the consensus receiving the highest relative proportion of mapped reads corresponding to the known identity of the diploid individual the read came from, with some reads mapping to closely related sister taxa (Supporting Information Table S4). This method allows identification of small subclades of diploid taxa to which the reads are most similar, and we inferred the ancestry of the allopolyploid subgenomes as the diploid subclades receiving the highest rates of read mapping (Wang *et al.*, 2021).

RESULTS

Phylogenomic inferences of evolutionary relationships

Maximum likelihood (ML) trees inferred from the three concatenated datasets were highly congruent, despite variation in missing data (Fig. 2; Supporting Information Fig. S1, S2). Therefore, we focus our results to trends in interspecific relationships observed across phylogenetic inferences.

The base of the eastern standing *Phlox* is notably composed of long-styled taxa. We recover an early split between *P. stolonifera* and all other taxa (UFB=100), followed by a split of a well-supported clade (UFB=100) containing *P. ovata* out to *P. pulchra, P. glaberrima* subsp.

triflora, and the allotetraploid *P. buckleyi* (UFB=100). The remaining *P. glaberrima* subspecies are found in a neighboring clade with subspecies of *P. carolina* and *P. maculata* (UFB=100). Across all inferences, the *P. glaberrima* and *P. carolina* subspecies are not monophyletic within their species delineation. The next branch subtends a small clade including *P. amplifolia* and *P. paniculata* (UFB=100).

Across all datasets, we inferred all short-styled taxa form a monophyletic group evolving from the long-styled species (UFB=100). This short-styled group contains the *P. pilosa*, *P. divaricata, P. villosissima*, and *P. amoena* subspecies, *P. floridana*, and the three Texas annual species (*P. drummondii*, *P. roemeriana*, and *P. cuspidata*), Half of our *P. pilosa* subspecies samples, including *P. pilosa* subsp. *fulgida*, *P. pilosa* subsp. *pilosa*, and *P. pilosa* subsp. *deamii*, form a clade out to the remaining short-style species (UFB=100). Given the relative northern origin of these samples, we call this the *P. pilosa* "North" group. This group also contains the white flower morph of *P. pilosa* subsp. *pilosa* that has evolved by reinforcement, sometimes described as *P. argillacea*. Both *P. divaricata* and *P. amoena* species form monophyletic groups (UFB=100), although *P. divaricata* subspecies are interdigitated.

The remaining *P. pilosa* subspecies, Texas annuals, and the allotetraploids *P. floridana, P. villosissima* subsp. *villosissma* and *P. villosissima* subsp. *latisepala* form a large clade (UFB=100). We observe reduced branch supports along the backbone of this clade; however, the Texas annual species are monophyletic (UFB=100), with *P. roemeriana* sister to *P. drummondii.* Adjacent to the annuals lie the remaining *P. pilosa* subspecies. We refer to this *P. pilosa* group as "South", given the more southern origin of these taxa relative to the *P. pilosa* North group.

Our low support partitioning the *P. pilosa* South group from the Texas annuals may be due to the presence of the allotetraploid subspecies *P. villosissima* subsp. *latisepala* and *P. villosissima* subsp. *villosissima.* These taxa were inferred either out to the annuals or out to the *P. pilosa* South group with variable support (UFB=41-100). Variation in their inferred placement is consistent with their hypothesized hybrid ancestry between *P. pilosa* subsp. *pilosa* South and *P. drummondii* (Table 1). Similar phylogenetic uncertainty is observed for the allotetraploid *P. floridana* across analyses. This may be a result of its putative hybrid ancestry between *P. pilosa* subsp. *pilosa* South and *P. carolina* subsp. *angusta* (Table 1).

Coalescent-based species tree inference using SVDquartets recovered a species tree with nearly identical relationships to our ML inferred trees (Supporting Information Fig. S3);

however, some interspecific positions varied in their branch supports. We inferred strong support for most nodes along the backbone of the tree (SBS > 80) and splits between major species groupings, except for early splits differentiating relative placement within the long-style species. *P. pulchra, P. glaberrima* subsp. *triflora,* and *P. buckleyi* remained monophyletic (SBS=84) and separate from the remaining monophyletic *P. glaberrima*/*P.maculata/P.carolina* subspecies (SBS=100). We also recovered the polyphyletic relationships of the *P. pilosa* North and *P. pilosa* South groups. Unlike our ML analyses, our coalescent inferences showed support for partitioning the clade of *P. pilosa* South and the allotetraploids *P. floridana*, *P. villosissima* subsp. *villosissima* and *P. villosissima* subsp. *latisepala* sister to the Texas annuals (SBS=89).

Evidence of gene flow alongside reinforcement

We applied D-statistics and TreeMix v.1.13 to infer if gene flow occurred between sympatric populations of focal species in two cases of reinforcement. All D-statistic tests were structured so a negative D supports a history of introgression between these focal species.

In *P. drummondii*, reinforcement against hybridization with *P. cuspidata* has favored the divergence of light-blue to dark-red flower color in sympatry (Fig. 3a). D-statistic tests between allopatric *P. drummondii* and *P. cuspidata* show no evidence of gene flow; however, all 48 tests between sympatric dark-red *P. drummondii* and every *P. cuspidata* were negative, with 24 being significant (Table 2). Our best fit model (m=2) of the *P. drummondii* subtree in TreeMix recovered *P. cuspidata* out to *P. drummondii* and *P. roemeriana,* with a migration event going from *P. cuspidata* into dark-red sympatric *P. drummondii* (Fig. 3c). This migration edge remained present for all models m ≥2 (Supporting Information Fig. S4, S9).

In *P. pilosa* subsp. *pilosa* North, reinforcement against hybridization with *P. glaberrima* subsp. *interior* has favored the divergence of flower color from pink to white (Fig. 3b). Unlike reinforcement in *P. drummondii*, the ranges of *P. pilosa* subsp. *pilosa* North and *P. glaberrima* subsp. *interior* are mosaic and cannot be distinctly divided as geographically allopatric or sympatric (Levin & Kerster, 1967). Therefore, we grouped our tests for gene flow based on the flower color of the *P. pilosa* subsp. *pilosa* North individual used, pink or white, instead of by geography. Our tests show no support for gene flow between pink (N=360) or white (N=180) flowered *P. pilosa* subsp. *pilosa* North and *P. glaberrima interior* (Table 2). TreeMix modeled *P. pilosa* subsp. *pilosa* North subtrees produced similar topologies to our ML phylogenetic

inferences but did not infer migration events involving *P. pilosa* subsp. *pilosa* North (Fig. 3d; Supporting Information Fig. S5, S9).

Evidence of divergence and gene flow underlying hypotheses of putative homoploid hybrid species

We leveraged our inferred phylogenies with tests for gene flow to evaluate support for the five hypothesized cases of homoploid hybrid speciation in the eastern standing *Phlox* (Table 1). Specifically, we asked if a putative hybrid species sits nested within or sister to one of its hypothesized parental species and if a signature of gene flow exists from the other putative parental species into the putative hybrid.

Our phylogenetic inferences find support for four of the five putative hybrids being nested or within one of their putative parents (Fig. 2, Supporting Information Fig. S1, S2, S3); *P. amoena* subsp. *lighthipei* within *P. amoena* subsp. *amoena*, *P. maculata* subsp. *pyramidalis* within *P. maculata* subsp. *maculata*, *P. argillacea* within *P. pilosa* subsp. *pilosa* North*,* and *P. pilosa* subsp. *detonsa* in *P. pilosa* subsp. *pilosa* South*. P. pilosa* subsp. *deamii* was not found out or within *P. pilosa* subsp. *pilosa* South or *P. amoena* subsp. *amoena*, consistent with (Goulet-Scott *et al.*, 2021)**.**

For *P. amoena* subsp. *lighthipei, P. argillacea, and P. pilosa* subsp. *detonsa,* D-statistic tests and TreeMix analyses did not find support for gene flow between the putative hybrids and their second hypothesized parental species (Table 3; Supporting Information Fig. S5, S6, S7, S9). However, D-statistic tests do support gene flow between *P. glaberrima* subsp. *interior* and *P. maculata* subsp. *pyramidalis,* with 63 of 70 tests being significantly negative. Importantly, no evidence of gene flow was found between *P. glaberrima* subsp. *interior* and *P. maculta* subsp. *maculta,* N=160 (Table 3)*.* All models of the *P. maculata* subsp. *pyramidalis* subtree in TreeMix (best fit m=4) confirmed this result, with a migration edge from *P. glaberrima* subsp. *interior* into *P. maculta* subsp. *pyramidalis* (Fig. 4, Supporting Information Fig. S8, S9). These results are consistent with *P. maculata* subsp. *pyramidalis* as a hybrid lineage arising from *P. maculata* subsp. *maculata* and *P. glaberrima* subsp. *interior* parental lineages.

Inferring origins of allotetraploid hybrid species

375 We leveraged comparative read mapping to infer the subgenome ancestries of four
376 allotetraploid *Phlox* species (Table 1) (Levin, 1966b, 1968; Smith & Levin, 1967). *P.*
377 *villosissima* subsp. *villosissima* and *P. villosissima* subsp. *latisepala* are thought to be
378 independent products between *P. pilosa* subsp. *pilosa* South and *P. drummondii*. *P. floridana* is
379 hypothesized to be the product of *P. pilosa* subsp. *pilosa* South and *P. carolina* subsp. *angusta***,**
380 and *P. buckleyi* contains two discordant diploid karyotypes hypothesized to be from extinct taxa
381 (Wherry, 1955)**.**
382 Concatenated ddRAD loci were used to generate 439,271 bp long reduced consensus
383 sequences for each of the 28 diploid species, with a locus missingness rate of 25% among them.
384 Mapping of reads to this consensus dataset resulted in differential assignment of sequence
385 ancestry for each tetraploid sample to subclades of diploid taxa (Fig 5.).
386 Reads from *P. villosissima* subsp. *villosissima* and *P. villosissima* subsp. *latisepala*
387 mapped primarily to the *P. pilosa* South group and the Texas annuals, with each receiving 30-
388 34% and 22-26% respectively. Within these two groups the top recipients of mapped reads were
389 *P. pilosa* subsp. *pilosa* South and *P. cuspidata* for all three samples. *P. floridana* showed a
390 similar high mapping rate to the *P. pilosa* South group (34%), with most reads mapping to *P.*
391 *pilosa* subsp. *pilosa* South and second most to *P. pilosa* subsp. *detonsa*. Contrary to the
392 hypothesized origins of this species, we did not observe elevated read mapping to the *P. carolina*
393 *subspecies* consensus sequence. Instead, we observed elevated read mapping to *P. amoena*
394 subsp. *amoena*. Finally, *P. buckleyi* reads mapped with the highest rate to the *P. ovata*, *P.*
395 *pulchra*, *P. glaberrima* subsp. *triflora* clade (38%), with the highest recipients being *P. pulchra*
396 and *P. ovata.*
397
398 **DISCUSSION**
399 With the accessibility of genomic data and modern phylogenomic analyses, evolutionary
400 biology is well-positioned to understand the presence, extent, and consequences of hybridization
401 and gene flow across large clades of organisms. Here, we present well-resolved phylogenetic
402 relationships of the eastern standing *Phlox*, demonstrate clear support for most described species
403 relationships, and reveal novel non-monophyletic relationships of subspecies in historically
404 taxonomically difficult species complexes. Using this phylogenetic framework, we determine

405 hybridization has generated new species in this clade, although maybe not as extensively as
406 previously hypothesized. Our results support one case of hypothesized homoploid hybrid
407 speciation, identify putative ancestries of multiple polyploid species, and find evidence of gene
408 flow in one of two cases of reinforcement. However, we also find no support for many
409 hypothesized cases of gene flow in the formation of new lineages. Our findings demonstrate the
410 utility and importance of phylogenomics in confirming hypothesized evolutionary histories of
411 non-model systems and add to the growing evidence that hybridization and gene flow across
412 species boundaries can play diverse roles in generating novel biodiversity.
413
414 *Evolutionary relationships of the eastern standing Phlox*
415 Previous phylogenetic inference of the eastern standing *Phlox* either relied on a handful
416 of genetic markers or limited taxonomic sampling, leaving the evolutionary relationships of this
417 group unclear (Ferguson *et al.*, 1999; Ferguson & Jansen, 2002; Roda *et al.*, 2017; Landis *et al.*,
418 2018; Goulet-Scott *et al.*, 2021). Our phylogenomic inferences on genome-wide ddRAD markers
419 from a broad taxonomic sampling clarify the evolutionary relationships of the eastern standing
420 *Phlox* and provide novel insight into their diversification.
421 Taxonomic treatments of the eastern standing *Phlox* have grouped these taxa by
422 conspicuous differences in style length (Wherry, 1930, 1931, 1932, 1955). Unlike prior
423 phylogenetic studies, our inferences support a large monophyletic clade of short-styled taxa
424 subtended by multiple paraphyletic clades of long-styled taxa (Fig. 2). Divergence in style-length
425 can generate reproductive isolation (Kay, 2006; Brothers & Delph, 2017), and this transition
426 from long to short style-length may have reduced competition and stimulated the diversification
427 of the short-styled clade.
428 Our analyses also reveal unprecedented resolution into relationships within species
429 groups. Our inferences support *P. drummondii* and *P. roemeriana* are sister taxa within the
430 Texas annuals (Fig. 2). This topology is supported in Roda *et al.*, 2017 but not supported in
431 studies not using genome-wide markers (Ferguson *et al.*, 1999; Ferguson & Jansen, 2002; Landis
432 *et al.*, 2018). In the *P. glaberrima*/ *P. carolina*/ *P. maculata* complex, we find support for non-
433 monophyletic relationships among subspecies (Fig. 2). Additionally, the subspecies of the *P.*
434 *pilosa* complex form distinct polyphyletic groups coinciding with their relative northern or
435 southern location of origin, as suggested in Goulet-Scott *et al.*, 2021. The one exception is *P.*

436 *pilosa* subsp. *sangamonensis* which sits in the southern clade but is found in the north (Fig. 1), a
437 discovery consistent with the hypothesis of *P. pilosa* subsp. *sangamonensis* arising by long-
438 distance dispersal from southern populations of *P. pilosa* subsp. *pilosa* (Levin & Smith, 1965;
439 Levin, 1984). The non-monophyletic relationship among the northern and southern *P. pilosa* taxa
440 and within *P. glaberrima* were suggested in previous phylogenetic studies, but remained
441 uncertain due to low phylogenetic supports and reliance on few genetic loci (Ferguson *et al.*,
442 1999; Ferguson & Jansen, 2002; Landis *et al.*, 2018). Only with modern phylogenomics are we
443 able to definitively show support for these relationships.
444 Classic taxonomic delineations within *Phlox* are largely based on morphological
445 characteristics (Wherry, 1955; Locklear, 2011b), yet our phylogenetic inferences demonstrate
446 current taxonomy may not best reflect the true evolutionary relationships of some taxa within
447 this group. Discovery of these new phylogenetic relationships begs reconsideration of the
448 taxonomy of the group and motivates future research into why morphological variation is
449 inconsistent with phylogenetic relationships.
450
451 *Evolution of reinforcement with and without gene flow*
452 Limited empirical study has evaluated if interspecific hybridization generating selection
453 for reinforcement also results in reinforcement evolving in the face of gene flow (Garner *et al.*,
454 2018). We investigated if gene flow accompanied two cases of divergence by reinforcement in
455 *Phlox* (Fig. 3).
456 *P. drummondii* has diverged from light-blue to dark-red flower color to prevent
457 hybridization with *P. cuspidata* (Levin, 1985; Hopkins & Rausher, 2012). Our geographically
458 widespread sampling revealed evidence of gene flow between *P. cuspidata* and multiple
459 sympatric dark-red *P. drummondii* individuals but not between *P. cuspidata* and any allopatric
460 light-blue *P. drummondii,* consistent with previous observations (Roda *et al.*, 2017). A history of
461 gene flow in sympatry but not allopatry mirrors the model of divergence by reinforcement to
462 reduce hybridization in sympatry but not in allopatry (Garner *et al.*, 2018). This pattern also
463 suggests the signal of gene flow is the product of hybridization in contemporary sympatry and
464 not due to ancient admixture between *P. cuspidata* and *P. drummondii*. Although F1 hybrids are
465 highly sterile (Suni & Hopkins, 2018), our findings indicate hybrids do backcross in nature,

resulting in interspecific gene flow. Therefore, reinforcement in *P. drummondii* evolved despite gene flow in sympatry.

Conversely, our analyses do not support a history of gene flow coinciding with reinforcement in *P. pilosa* subsp. *pilosa* North. *P. pilosa* subsp. *pilosa* North has evolved from pink to white colored flowers to prevent hybridization where it co-occurs with *P. glaberrima* subsp. *interior* in high frequency (Fig. 2) (Levin, 1966b; Levin & Kerster, 1967; Levin & Schaal, 1970a,b, 1972)**.** Reproductive isolation between these taxa is high but field experiments and experimental crosses demonstrate these species can generate hybrid seed**,** with divergence in flower color reducing the formation of hybrid seed by half in nature (Levin, 1966b; Levin & Kerster, 1967; Levin & Schaal, 1970a,b, 1972)(Fig. 2). However, our analyses do not detect gene flow between these species (Fig. 3; Table 2). This result suggests hybrid offspring between these taxa may be completely inviable, lethally maladapted, or have exceedingly high sterility and that reinforcement may have occurred after gene flow had ceased.

The presence/absence of gene flow in these two cases of reinforcement present a powerful opportunity to investigate the dynamics by which reproductive trait divergence, and flower color specifically, evolves by reinforcing selection. For reproductive trait divergence to evolve by reinforcement, alleles conferring increased assortative mating within a species must remain associated with alleles causing costly hybridization between species (Servedio & Kirkpatrick, 1997; Kirkpatrick, 2000). When gene flow between species is present, recombination can disassociate these alleles, impeding successful divergence. Yet, we find the same trait evolving due to reinforcing selection in two *Phlox* species despite gene flow occurring alongside one case but not the other. This result may be the consequence of differing genetic architectures and genetic linkage underlying the cost of hybridization and flower color between these cases, varying strengths of reproductive isolating barriers between the species pairs upon their secondary contact, and/or differences in the functional cost of hybridization (i.e. offspring or gamete loss). Further study of the strength and genetic architecture of reproductive isolating barriers between these *Phlox* species will better inform how reinforcement evolves.

Varying support for hypothesized homoploid and allotetraploid hybrid species

Within the eastern standing *Phlox* there are five hypothesized homoploid hybrid species and four hypothesized allopolyploid species (Table 1). We only observed genomic support for *P.*

497 *maculata* subsp. *pyramidalis* being a homoploid hybrid species between *P. maculata* subsp.
498 *maculata* and *P. glaberrima* subsp. *interior. P. maculata* subsp. *pyramidalis* is found
499 phylogenetically closely related to multiple *P. maculata* subsp. *maculata* individuals (Fig. 2)
500 with a strong signal of receiving gene flow from *P. glaberrima* subsp. *interior* (Fig. 4; Table
501 3.). We can conclude our sampled *P. maculata* subsp. *pyramidalis* is of hybrid origin, however
502 future work will be necessary to discern if the hybrid origin of *P. maculata* subsp. *pyramidalis*
503 actually gave rise to reproductive isolation of its lineage from its putative parental species
504 (Schumer *et al.*, 2018).

505 The remaining four hypothesized cases of homoploid hybrid speciation were not
506 supported by our genomic investigation, despite extensive phenotypic and biomolecular
507 evidence. In the cases of *P. amoena* subsp. *lighthipei*, *P. argillacea,* and *P. pilosa* subsp. *detonsa*
508 the lineages thought to be hybrid in origin may simply be divergent geographically isolated
509 populations from one of their hypothesized parental lineages. Yet in the case of *P. pilosa* subsp.
510 *deamii*, the lineage is distantly related to both hypothesized parents and may possesses
511 phenotypic traits resembling these species through convergence or incomplete lineage sorting.

512 Polyploid hybrid lineages can be significantly easier to identify than homoploid hybrids
513 through chromosome structure and counts. Despite this advantage, inferring the evolutionary
514 origin of these lineages remains challenging (Rothfels, 2021). Although the signals from our
515 comparative read mapping are coarse, our approach suggests the identity of the progenitor clades
516 that gave rise to the polyploid hybrid species in this group. Our evidence supports *P. villosissima*
517 subsp. *latisepala* and *P. villosissima* subsp. *villosissima* are derived from *P. pilosa* (*P. pilosa*
518 subsp. *pilosa* South) and a Texas annual species. *P. drummondii* was previously hypothesized as
519 the parent but our analyses cannot resolve which of the Texas annual species or ancestor is the
520 parent. As previously hypothesized, we find support for one parent of *P. floridana* to be *P. pilosa*
521 subsp. *pilosa* South, yet we find evidence that *P. amoena* subsp. *amoena* may be the second
522 parent instead of the *P. carolina subspecies*. We also observe *P. buckleyi* is genetically similar to
523 the early-diverging long-styled *Phlox*, but we cannot confirm the specific parental lineages from
524 this group. Future work using longer haplotype phase may help resolve the evolutionary origins
525 of these species and better inform the dynamics and evolutionary trajectory of these
526 allopolyploid species.

527 Across both the hypothesized homoploid and polyploid hybrid lineages, the *P. pilosa*
528 group has been a potential and realized source of hybridization activity. Four of the five
529 hypothesized homoploid hybrid lineages and three of the four polyploid lineages were thought to
530 include *P. pilosa* subsp. *pilosa* as a parent. While our analyses indicate none of the hypothesized
531 homoploid hybrid lineages have hybrid origin with *P. pilosa* subspecies, three of the four
532 hypothesized polyploid hybrid lineages are derived from *P. pilosa* subsp. *pilosa* South
533 *subspecies* ancestry. *P. pilosa*'s developmental biology may make it prone to evolutionary
534 innovation, with many populations having high rates of unreduced gametes and whole genome
535 duplications across their range (Worcester, *et al*, 2012). Future work may help address what
536 factors underly this developmental instability and if it has an outsized role in the generation of
537 novel evolutionary lineages relative to other *Phlox* species.
538
539 *Implications on Evolutionary History of Trait Variation*
540 Much of the data supporting the role of hybridization and gene flow in the speciation of
541 the eastern standing *Phlox* stemmed from comparisons of trait variation between lineages found
542 in sympatry. Our findings support hybridization has likely contributed to some of the phenotypic
543 variation observed across taxa in these cases, both as a source of genetic variation and force for
544 selective divergence. However, our findings also indicate a lack of interspecific genetic exchange
545 for many hypothesized cases of hybrid speciation. Further study of these confirmed cases may
546 inform how specific traits and even genes move between species and contribute to reproductive
547 isolation between lineages. While our lack of support in some cases suggests forces other than
548 hybridization may underlie patterns of trait variation observed across taxa in this group, this
549 finding also motivates a strong need to reevaluate our previous evidence for the existence of
550 gene flow, and to rethink our expectations of how and why traits evolve within and between
551 closely related species. Future investigation into the phenotypic variation of these taxa may
552 provide new insights into how differences in ecological selective pressures across overlapping
553 species ranges, effective population size, and incomplete lineage sorting contribute to phenotypic
554 divergence within and between species.
555
556 **FUNDING**

This study was supported by the National Science Foundation (DEB-1844906 to R.H., and Graduate Research Fellowship to A.G.G.); the Harvard Graduate School of Arts and Sciences' Herchel Smith Graduate Research Fellowship in Science (to A.G.G. and B.E.G.S.); and the Harvard University Herbaria Expedition Grant (to A.G.G.).

ACKNOWLEDGEMENTS

We are grateful to S.V. Edwards, E.M. Kramer, J. Wakeley, and J. Mallet for thoughtful discussions that improved this study, and to C.J. Ferguson, G.A. Burgin, and A.E. Berardi for their helpful comments on this manuscript. We are also thankful to P.S. Jourdan and the OPGC staff for sharing their *Phlox* collections with us.

AUTHOR CONTRIBUTIONS

A.G.G. and R.H. conceptualized the project. A.G.G., B.E.G.S., and R.H. collected and analyzed the data. A.G.G. and R.H. wrote the manuscript.

DATA ACCESSIBILITY

Supporting data underlying this article is found on Dryad Digital Repository: [insert rep ID}

REFERENCES

Abbott R, **Albach D**, **Ansell S**, **Arntzen JW**, **Baird SJE**, **Bierne N**, **Boughman J**, **Brelsford A**, **Buerkle CA**, **Buggs R**, ***et al.*** **2013**. Hybridization and speciation. *Journal of Evolutionary Biology* **26**: 229–246.

Anderson E. **1949**. Introgressive hybridization. *Introgressive Hybridization.*

Anderson E, **Gage A**. **1952**. Introgressive hybridization in *Phlox bifida*. *American Journal of Botany* **39**: 399–404.

Andrews KR, **Good JM**, **Miller MR**, **Luikart G**, **Hohenlohe PA**. **2016**. Harnessing the power of RADseq for ecological and evolutionary genomics. *Nature Reviews Genetics* **17**: 81–92.

Barker MS, **Arrigo N**, **Baniaga AE**, **Li Z**, **Levin DA**. **2016**. On the relative abundance of autopolyploids and allopolyploids. *New Phytologist* **210**: 391–398.

Bombonato JR, **do Amaral DT**, **Silva GAR**, **Khan G**, **Moraes EM**, **da Silva Andrade SC**, **Eaton DAR**, **Alonso DP**, **Ribolla PEM**, **Taylor N**, ***et al.*** **2020**. The potential of genome-wide

589 RAD sequences for resolving rapid radiations: a case study in Cactaceae. *Molecular*
590 *Phylogenetics and Evolution* **151**: 106896.

591 **Brothers AN**, **Delph LF**. **2017**. Divergence in style length and pollen size leads to a postmating-
592 prezygotic reproductive barrier among populations of *Silene latifolia*. *Evolution* **71**: 1532–1540.

593 **Chifman J**, **Kubatko L**. **2014**. Quartet inference from SNP data under the coalescent model.
594 *Bioinformatics (Oxford, England)* **30**: 3317–3324.

595 **Durand EY**, **Patterson N**, **Reich D**, **Slatkin M**. **2011**. Testing for ancient admixture between
596 closely related populations. *Molecular Biology and Evolution* **28**: 2239–2252.

597 **Dyer KA**, **Bewick ER**, **White BE**, **Bray MJ**, **Humphreys DP**. **2018**. Fine-scale geographic
598 patterns of gene flow and reproductive character displacement in *Drosophila subquinaria* and
599 *Drosophila recens*. *Molecular Ecology* **27**: 3655-3670.

600 **Eaton DAR**, **Hipp AL**, **González-Rodríguez A**, **Cavender-Bares J**. **2015**. Historical
601 introgression among the American live oaks and the comparative nature of tests for
602 introgression. *Evolution* **69**: 2587–2601.

603 **Eaton DAR**, **Overcast I**. **2020**. ipyrad: Interactive assembly and analysis of RADseq datasets.
604 *Bioinformatics (Oxford, England)* **36**: 2592–2594.

605 **Eaton DAR**, **Ree RH**. **2013**. Inferring phylogeny and introgression using RADseq data: an
606 example from flowering plants (*Pedicularis*: Orobanchaceae). *Systematic Biology* **62**: 689–706.

607 **Erbe L**, **Turner BL**. **1962**. A biosystematic study of the *Phlox cuspidata-Phlox drummondii*
608 complex. *The American Midland Naturalist* **67**: 257.

609 **Excoffier L**, **Dupanloup I**, **Huerta-Sánchez E**, **Sousa VC**, **Foll M**. **2013**. Robust demographic
610 inference from genomic and SNP data. *PLoS Genetics.* **9**: e1003905.

611 **Fehlberg SD**, **Ty MC**, **Ferguson CJ**. **2014**. Reexamination of a putative diploid hybrid taxon
612 using genetic evidence: The distinctiveness of *Phlox pilosa* subsp. *deamii* (Polemoniaceae).
613 *International Journal of Plant Sciences* **175**: 781–793.

614 **Felsenstein J**. **1981**. Skepticism Towards Santa Rosalia, or Why are There so Few Kinds of
615 Animals? *Evolution* **35**: 124.

616 **Ferguson CJ**, **Jansen RK**. **2002**. A chloroplast DNA phylogeny of eastern *Phlox*
617 (Polemoniaceae): implications of congruence and incongruence with the ITS phylogeny.
618 *American Journal of Bbotany* **89**: 1324–1335.

619 **Ferguson CJ**, **Kramer F**, **Jansen RK**. **1999**. Relationships of eastern north American *Phlox*
620 (Polemoniaceae) based on ITS sequence data. *Systematic Botany* **24**: 616.

Fishman L, **Wyatt R**. **1999**. Pollinator-mediated competition, reproductive character displacement, and the evolution of selfing in *Arenaria uniflora* (Caryophyllaceae). *Evolution* **53**: 1723–1733.

Garner AG, **Goulet BE**, **Farnitano MC**, **Molina-Henao YF**, **Hopkins R**. **2018**. Genomic signatures of reinforcement. *Genes* **9**.

Gottlieb LD. **1972**. Levels of confidence in the analysis of hybridization in plants. *Annals of the Missouri Botanical Garden* **59**: 435.

Goulet BE, **Roda F**, **Hopkins R**. **2017**. Hybridization in plants: Old ideas, new techniques. *Plant Physiology* **173**: 65–78.

Goulet-Scott BE, **Garner AG**, **Hopkins R**. **2021**. Genomic analyses overturn two long-standing homoploid hybrid speciation hypotheses. *Evolution* **75**: 1699–1710.

Grant V. **1981**. *Plant Speciation*. New York Chichester, West Sussex: Columbia University Press.

Green RE, **Krause J**, **Briggs AW**, **Maricic T**, **Stenzel U**, **Kircher M**, **Patterson N**, **Li H**, **Zhai W**, **Fritz MH-Y**, *et al.* **2010**. A draft sequence of the neandertal genome. *Science* **328**: 710–722.

Guo C, **Ma P-F**, **Yang G-Q**, **Ye X-Y**, **Guo Y**, **Liu J-X**, **Liu Y-L**, **Eaton DAR**, **Guo Z-H**, **Li D-Z**. **2021**. Parallel ddRAD and genome skimming analyses reveal a radiative and reticulate evolutionary history of the temperate bamboos. *Systematic Biology* **70**: 756–773.

Gutenkunst RN, **Hernandez RD**, **Williamson SH**, **Bustamante CD**. **2009**. Inferring the joint demographic history of multiple populations from multidimensional SNP frequency data. *PLoS Genetics* **5**: e1000695.

Hadley EB, **Levin DA**. **1969**. Physiological evidence of hybridization and reticulate evolution in *Phlox maculata. American Journal of Botany* **56**: 561–570.

Hibbins M, **Hahn MW**. **2021**. Phylogenomic approaches to detecting and characterizing introgression. *Genetics* **220**: iyab173.

Hoang DT, **Chernomor O**, **von Haeseler A**, **Minh BQ**, **Vinh LS**. **2018**. UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution* **35**: 518–522.

Hopkins R. **2013**. Reinforcement in plants. *New Phytologist* **197**: 1095–1103.

Hopkins R, **Rausher MD**. **2012**. Pollinator-mediated selection on flower color allele drives reinforcement. *Science (New York, N.Y.)* **335**: 1090–1092.

Howard DJ. **1993**. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. *Hybrid Zones and the Evolutionary Process*: 46–69.

Kay KM. **2006**. Reproductive isolation between two closely related hummingbird-pollinated neotropical gingers. *Evolution* **60**: 538–552.

Kirkpatrick M. **2000**. Reinforcement and divergence under assortative mating. *Proceedingsof the Royal Society B: Biological Sciences* **267**: 1649–1655.

Kulathinal RJ, **Stevison LS**, **Noor MAF**. **2009**. The genomics of speciation in *Drosophila*: diversity, divergence, and introgression estimated using low-coverage genome sequencing. *PLoS Genetics* **5**: e1000550.

Landis JB, **Bell CD**, **Hernandez M**, **Zenil-Ferguson R**, **McCarthy EW**, **Soltis DE**, **Soltis PS**. **2018**. Evolution of floral traits and impact of reproductive mode on diversification in the *Phlox* family (Polemoniaceae). *Molecular Phylogenetics and Evolution* **127**: 878–890.

Lemmon EM, **Juenger TE**. **2017**. Geographic variation in hybridization across a reinforcement contact zone of chorus frogs (*Pseudacris*). *Ecology and Evolution* **7**: 9485–9502.

Léveillé-Bourret É, **Chen B-H**, **Garon-Labrecque M-È**, **Ford BA**, **Starr JR**. **2020**. RAD sequencing resolves the phylogeny, taxonomy and biogeography of Trichophoreae despite a recent rapid radiation (Cyperaceae). *Molecular Phylogenetics and Evolution* **145**: 106727.

Levin DA. **1963**. Natural hybridization between *Phlox maculata* and *Phlox glaberrima* and its evolutionary significance. *American Journal of Botany* **50**: 714.

Levin DA. **1966a**. Chromatographic evidence of hybridization and evolution in *Phlox maculata*. *American Journal of Botany* **53**: 238–245.

Levin DA. **1966b**. The *Phlox pilosa* complex: crossing and chromosome relationships. *Brittonia* **18**: 142.

Levin DA. **1968**. The genome constitutions of eastern north american *Phlox* amphiploids. *Evolution* **22**: 612.

Levin DA. **1969**. The challenge from a related species: A stimulus for saltational speciation. *The American Naturalist* **103**: 316–322.

Levin DA. **1984**. Genetic variation and divergence in a disjunct *Phlox*. *Evolution* **38**: 223–225.

Levin DA. **1985**. Reproductive character displacement in *Phlox*. *Evolution* **39**: 1275.

Levin DA, **Kerster HW**. **1967**. Natural selection for reproductive isolation in *Phlox*. *Evolution;* **21**: 679–687.

Levin DA, **Schaal BA**. **1970a**. Corolla color as an inhibitor of interspecific hybridization in *Phlox*. *The American Naturalist* **104**: 273–283.

Levin DA, **Schaal BA**. **1970b**. Reticulate evolution in *Phlox* as seen through protein electrophoresis. *American Journal of Botany* **57**: 977–987.

686 **Levin DA**, **Schaal BA**. **1972**. Seed protein polymorphism in *Phlox pilosa* (Polemoniaceae).
687 *Brittonia* **24**: 46.

688 **Levin DA**, **Smith DM**. **1965**. An enigmatic *Phlox* from Illinois. *Brittonia* **17**: 254.

689 **Levin DA**, **Smith DM**. **1966**. Hybridization and evolution in the *Phlox pilosa* complex. *The*
690 *American Naturalist* **100**: 289–302.

691 **Levy M**, **Levin DA**. **1974**. Novel flavonoids and reticulate evolution in the *Phlox pilosa–P.*
692 *drummondii* complex. *American journal of botany* **61**: 156–167.

693 **Levy M**, **Levin DA**. **1975**. The novel flavonoid chemistry and phylogenetic origin of *Phlox*
694 *floridana*. *Evolution* **29**: 487–499.

695 **Li H**. **2013**. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM.
696 *arXiv [q-bio.GN]*.

697 **Locklear JH**. **2011a**. *Phlox ovata* L. (Polemoniaceae): Clarification of the nomenclature of the
698 Allegheny *Phlox*. *Castanea* **76**: 116–117.

699 **Locklear JH**. **2011b**. *Phlox a natural history and gardener's guide*. Portland, OR: Timber Press.

700 **Marques DA**, **Meier JI**, **Seehausen O**. **2019**. A combinatorial view on speciation and adaptive
701 radiation. *Trends in ecology & evolution* **34**: 531–544.

702 **McNeilly T**, **Antonovics J**. **1968**. Evolution in closely adjacent plant populations IV. Barriers to
703 gene flow. *Heredity* **23**: 205–218.

704 **Minh BQ**, **Nguyen MAT**, **von Haeseler A**. **2013**. Ultrafast approximation for phylogenetic
705 bootstrap. *Molecular Biology and Evolution* **30**: 1188–1195.

706 **Nguyen L-T**, **Schmidt HA**, **von Haeseler A**, **Minh BQ**. **2015**. IQ-TREE: a fast and effective
707 stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and*
708 *Evolution* **32**: 268–274.

709 **Pease JB**, **Haak DC**, **Hahn MW**, **Moyle LC**. **2016**. Phylogenomics reveals three sources of
710 adaptive variation during a rapid radiation. *PLoS biology* **14**: e1002379.

711 **Pickrell JK**, **Pritchard JK**. **2012**. Inference of population splits and mixtures from genome-
712 wide allele frequency data. *PLoS genetics* **8**: e1002967.

713 **Roda F**, **Mendes FK**, **Hahn MW**, **Hopkins R**. **2017**. Genomic evidence of gene flow during
714 reinforcement in Texas *Phlox*. *Molecular Ecology* **26**: 2317–2330.

715 **Rothfels CJ**. **2021**. Polyploid phylogenetics. *New Phytologist* **230**: 66–72.

716 **Schumer M**, **Rosenthal GG**, **Andolfatto P**. **2014**. How common is homoploid hybrid
717 speciation? *Evolution* **68**: 1553–1560.

Schumer M, **Rosenthal GG**, **Andolfatto P**. **2018**. What do we mean when we talk about hybrid speciation? *Heredity* **120**: 379–382.

Servedio MR, **Kirkpatrick M**. **1997**. The effects of gene flow on reinforcement. *Evolution* **51**: 1764.

Servedio MR, **Noor MAF**. **2003**. The role of reinforcement in speciation: Theory and data. *Annual Review of Ecology, Evolution, and Systematics* **34**: 339–364.

Smith DM, **Levin DA**. **1967**. Karyotypes of eastern north American *Phlox*. *American Journal of Botany* **54**: 324.

Soltis PS, **Soltis DE**. **2009**. The role of hybridization in plant speciation. *Annual Review of Plant Biology* **60**: 561–588.

Stebbins GL. **1959**. The role of hybridization in evolution. *Proceedings of the American Philosophical Society* **103**: 231–251.

Suissa JS, **Kinosian SP**, **Schafran PW**, **Bolin JF**, **Taylor WC**, **Zimmer EA**. **2022**. Homoploid hybrids, allopolyploids, and high ploidy levels characterize the evolutionary history of a western North American quillwort (*Isoëtes*) complex. *Molecular Phylogenetics and Evolution* **166**: 107332.

Suni SS, **Hopkins R**. **2018**. The relationship between post-mating reproductive isolation and reinforcement in *Phlox*. *Evolution* **72.** 1387-1398.

Swofford DL. **2002**. PAUP*. Phylogenetic analysis using parsimony (* and other methods). *Vol. Sinauer Associates, Sunderland, MA*.

Taylor SA, **Larson EL**. **2019**. Insights from genomes into the evolutionary importance and prevalence of hybridization in nature. *Nature Ecology & Evolution* **3**: 170–177.

Turissini DA, **Matute DR**. **2017**. Fine scale mapping of genomic introgressions within the *Drosophila yakuba* clade. *PLoS Genetics* **13**: e1006971.

Turner BL. **1998**. Atlas of the Texas species of *Phlox* (Polemoniaceae). *Phytologia* **85**: 309–326.

Wang N, **Kelly LJ**, **McAllister HA**, **Zohren J**, **Buggs RJA**. **2021**. Resolving phylogeny and polyploid parentage using genus-wide genome-wide sequence data from birch trees. *Molecular Phylogenetics and Evolution* **160**: 107126.

Wherry ET. **1930**. The eastern short-styled Phloxes. *Bartonia* **12**: 24–53.

Wherry ET. **1931**. The eastern long-styled Phloxes, part I. *Bartonia* **13***:* 18–37.

Wherry ET. **1932**. The eastern long-styled Phloxes, part II. *Bartonia* **14**: 14–26.

Wherry ET. **1955**. The genus *Phlox*, Monograph III. *Morris Arbor, Philadelphia, USA*.

751 **Wherry ET**. **1956**. The genus *Phlox*. *The American Midland Naturalist* **56**: 508.

752 **Wherry ET**. **1965**. The genus *Phlox*, Ten Years After. *Bartonia* **35***:* 13–16.

753 **Whitehouse E**. **1945**. Annual *Phlox* Species. *The American Midland Naturalist* **34**: 388.

754 **Worcester L, Mayfield MH, Ferguson CJ**. **2012**. Cytotypic variation in *Phlox pilosa* ssp.
755 *pilosa* (Polemoniaceae) at the western edge of its range in the central United States. *Journal of*
756 *the Botanical Research Institute of Texas* **6**: 443–451.

757 **Yakimowski SB**, **Rieseberg LH**. **2014**. The role of homoploid hybridization in evolution: a
758 century of studies synthesizing genetics and ecology. *American Journal of Botany* **101**: 1247–
759 1258.

760 **SUPPLEMENTARY INFORMATION:**

761 **Figure S1.** Maximum likelihood phylogenetic inference from whole concatenated loci, with a
762 locus shared among at least 10 individuals.

763 **Figure S2.** Maximum likelihood phylogenetic inference from whole concatenated loci, with a
764 locus shared among at least 20 individuals.

765 **Figure S3.** Coalescent-based phylogenetic inference using SVDquartets, with a locus shared
766 among at least 30 individuals.

767 **Figure S4.** TreeMix models for the *P. drummondii* subtree.

768 **Figure S5.** TreeMix models for the *P. pilosa subsp. pilosa* North subtree.

769 **Figure S6.** TreeMix models for the *P. pilosa subsp. detonsa* subtree.

770 **Figure S7.** TreeMix models for the *P. amoena subsp. lighthipei* subtree.

771 **Figure S8.** TreeMix models for the *P. maculate subsp. pyramidalis* subtree.

772 **Figure S9**. TreeMix model best fit estimation.

773 **Table S1.** Species sampling.

774 **Table S2.** All D-statistic test results for introgression in two cases of reinforcement.

775 **Table S3.** All D-statistic test results for introgression in four hypothesized cases of hybrid
776 speciation.

777 **Table S4.** Summary of comparative read mapping of diploid and allotetraploid species to diploid
778 consensus sequences.

779

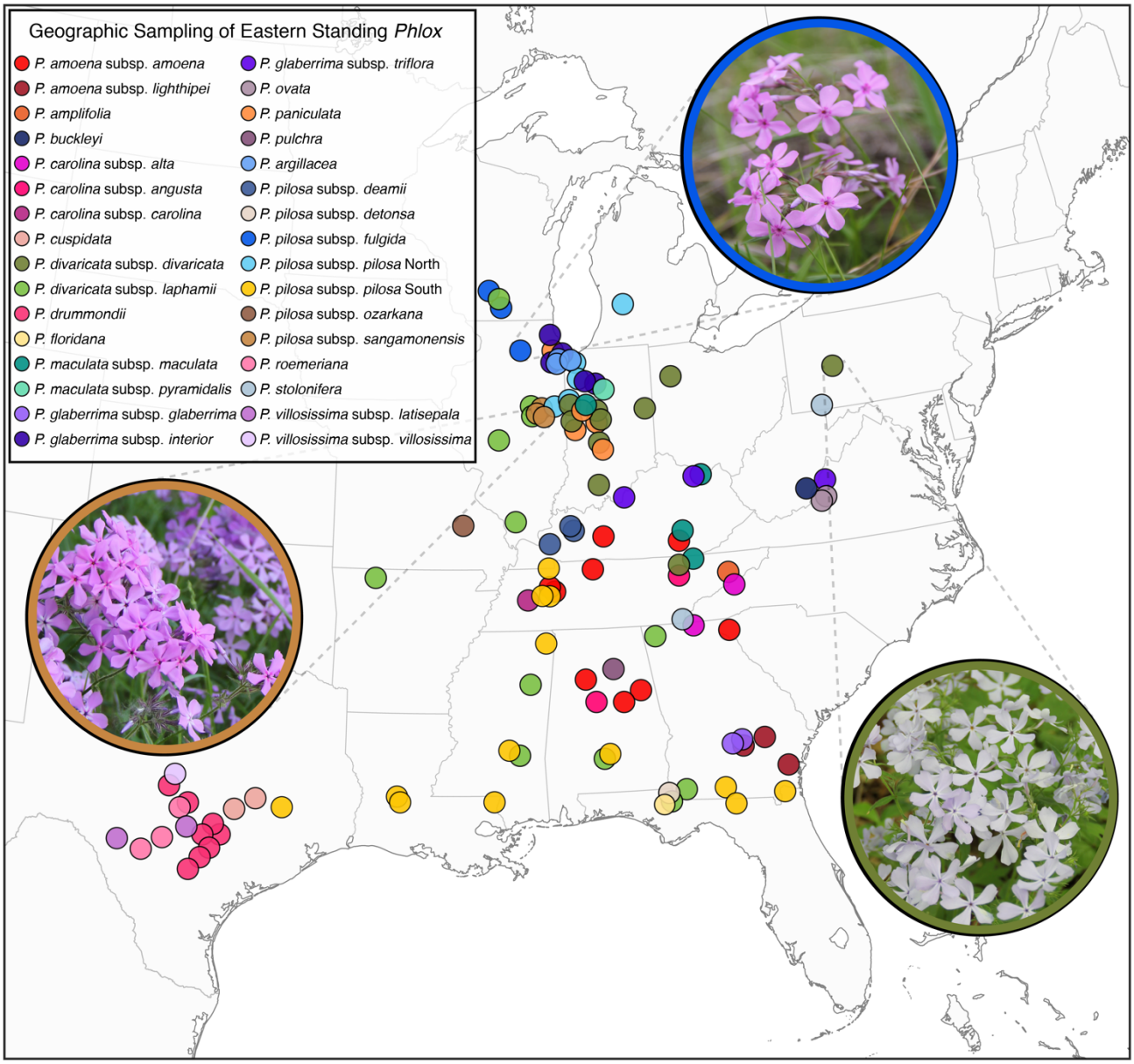
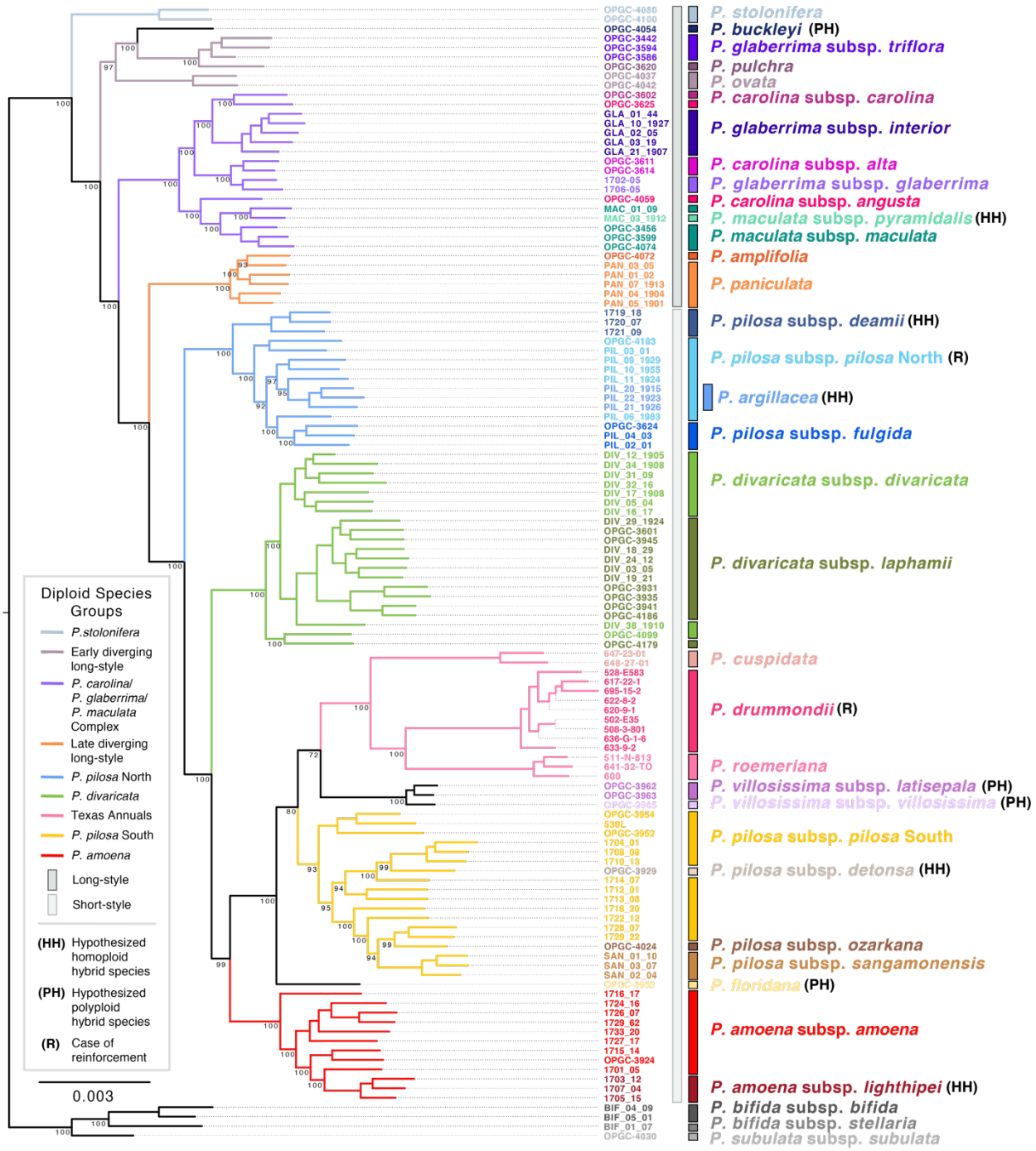


Figure 1. Map of individuals sampled across the interdigitating ranges of the thirty-two eastern standing *Phlox* taxa included for phylogenetic analyses. Each dot represents the locality of a single individual with the color corresponding to an individual's taxonomic identity. Three insets show *P. pilosa* subsp. *sangamonensis* (brown), *P. pilosa* subsp. *fulgida* (blue), and *P. divaricata* subsp. *divaricata* (green) in their natural habitat.

Figure 2. Maximum likelihood phylogenetic inference of eastern standing *Phlox* from whole concatenated ddRAD loci. Loci were required to be shared by at least 30 individuals. Numbers on branches represent bootstrap supports from 100 bootstrap replicates using ultrafast bootstrap approximation (UFB). Bootstrap supports are shown for all nodes differentiating between taxa.



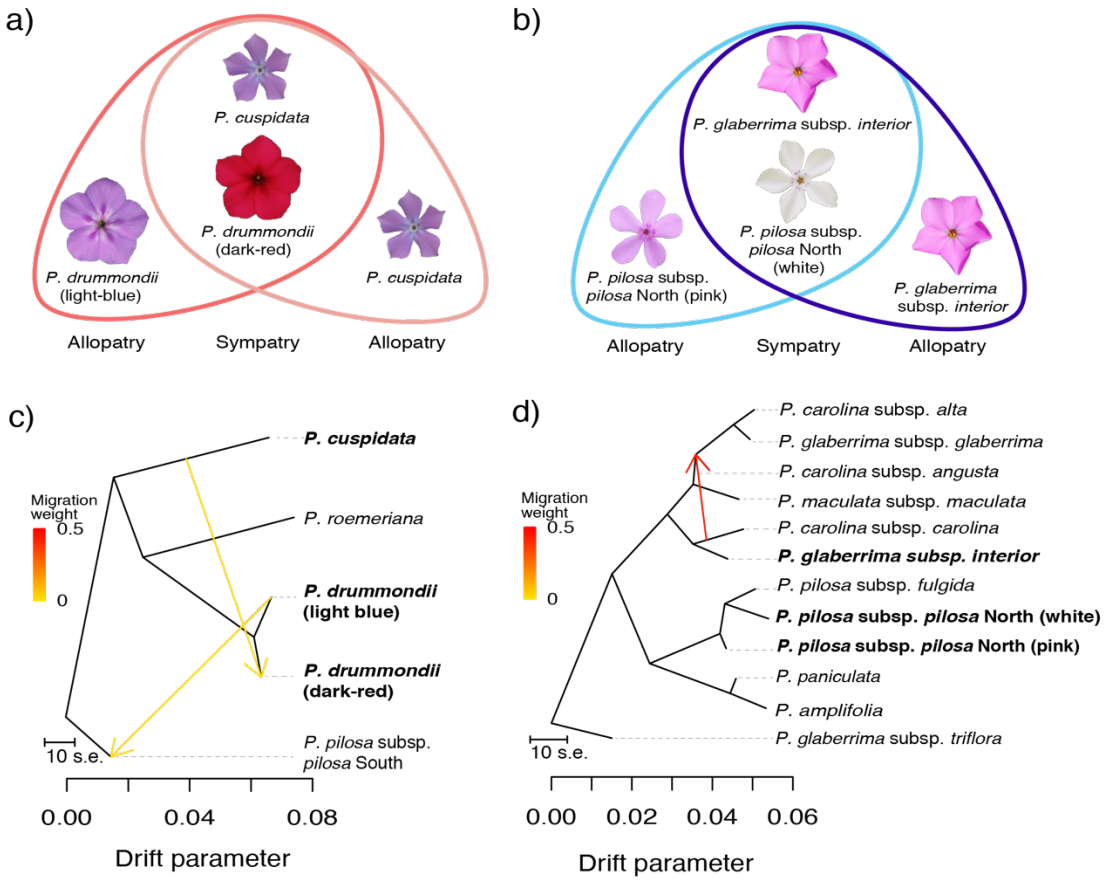


Figure 3. (a and b) Schematics of flower color divergence due to reinforcement in two species of *Phlox* to prevent maladaptive hybridization with another species in sympatry. (a) *P. drummondii* (red) has similar light-blue colored flowers to *P. cuspidata* (pink) in allopatry but *P. drummondii* has evolved dark-red flowers in sympatry to reduce hybridizing with *P. cuspidata*. (b) In allopatry *P. pilosa* subsp. *pilosa* North (light blue) has similar pink colored flowers to *P. glaberrima* subsp. *interior* (dark blue), but where they co-occur in high frequency in sympatry, *P. pilosa* subsp. *pilosa* North has evolved white flowers to reduce hybridizing with *P. glaberrima* subsp. *interior*. (c and d) Best fit models from TreeMix inferred gene flow between *P. cuspidata* and dark-red *P. drummondii* (c) (best fit m=2) but did not support evidence of gene flow between *P. glaberrima* subsp. *interior* and either color morph of *P. pilosa* subsp. *pilosa* North (d) (best fit m=1).

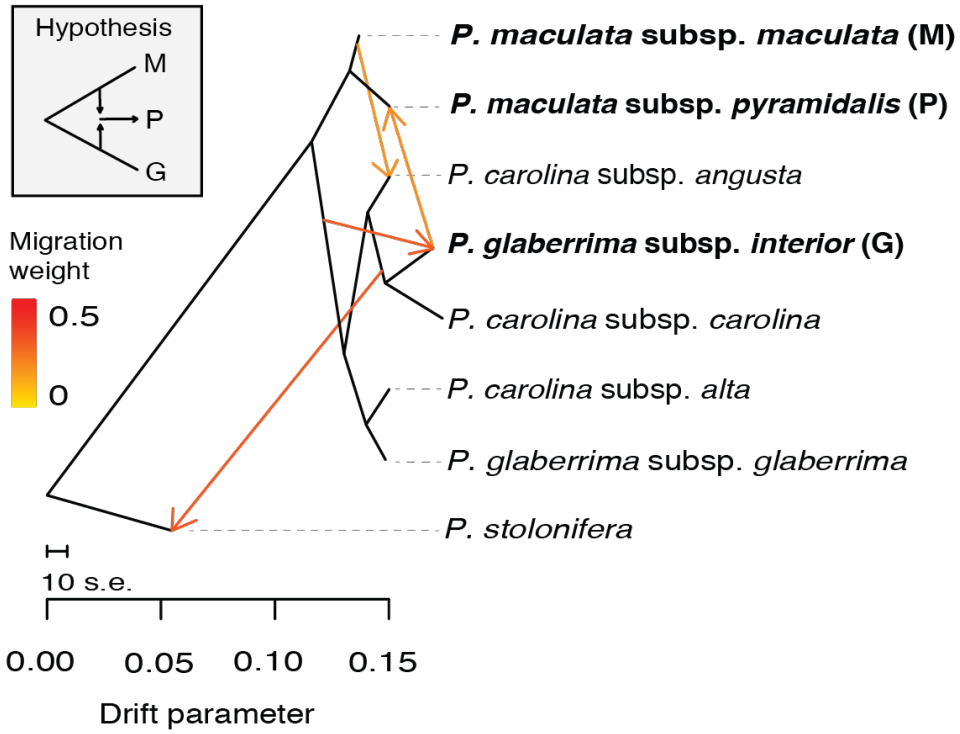


Figure 4. Results from TreeMix support the hybrid speciation hypothesis for *P. maculata pyramidalis* in a pruned subtree (best fit m=4). Modeling of 4,490 unlinked SNPs for individuals clustered by taxon identity inferred *P. maculata* subsp. *pyramidalis* sister to *P. maculata* subsp. *maculata* and receiving a migration event (gene flow) from *P. glaberrima* subsp. *interior*.

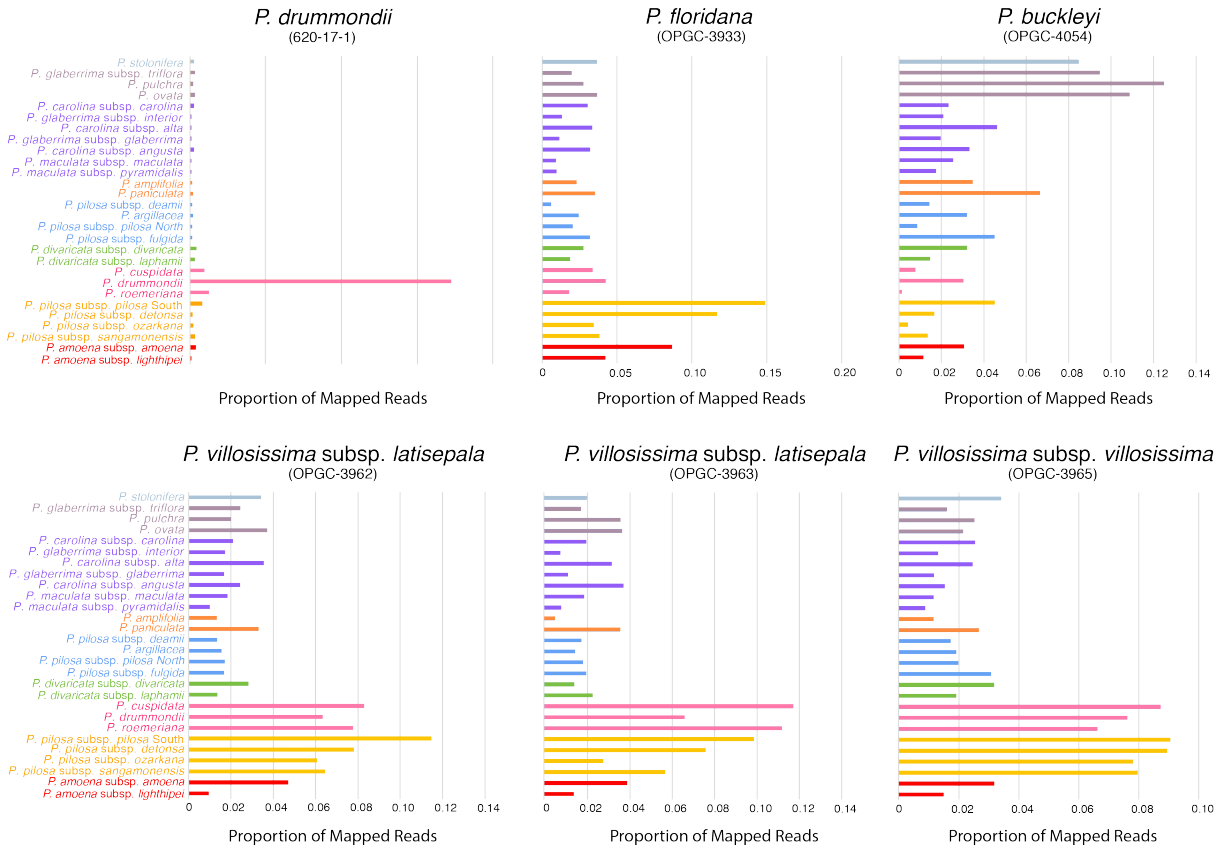


Figure 5. Proportion of reads mapped to diploid species-level consensus sequences for a diploid representative of *P. drummondii* and all allotetraploid hybrid individuals. Bar colors correspond to diploid species groups in Figure 2.

Table 1. Hybrid speciation hypotheses in the eastern standing *Phlox*

Hypothesized Hybrid Species	Ploidy	Putative Parent Species 1	Putative Parent Species 2	Motivating Previous Evidence
P. argillacea	2N	*P. glaberrima* subsp. *interior*	*P. pilosa* subsp. *pilosa* North	morphology[A], physiology[A], seed protein polymorphism[B], experimental crosses[C]
P. maculata subsp. *pyramidalis*	2N	*P. glaberrima* subsp. *interior*	*P. maculata* subsp. *maculata*	morphology[D], physiology[E], seed protein polymorphism[F], experimental crosses[C]
P. amoena subsp. *lighthipei*	2N	*P. pilosa* subsp. *pilosa* South	*P. amoena* subsp. *amoena*	morphology[G], seed protein polymorphism[F], experimental crosses[CG]
P. pilosa subsp. *deamii*	2N	*P. pilosa* subsp. *pilosa* South	*P. amoena* subsp. *amoena*	morphology[G], experimental crosses[CG], microsatellites[H]
P. pilosa subsp. *detonsa*	2N	*P. pilosa* subsp. *pilosa* South	*P. carolina*	seed protein polymorphism[F], experimental crosses[C]
P. floridana	4N	*P. pilosa* subsp. *pilosa* South	*P. carolina* subsp. *angusta*	karyology[IJ], flavonoids[K], seed protein polymorphism[F], experimental crosses[C]
P. villosissima subsp. *villosissima*	4N	*P. pilosa* subsp. *pilosa* South	*P. drummondii*	karyology[J], flavonoids[L], seed protein polymorphism[F], experimental crosses[C]
P. villosissima subsp. *latisepala*	4N	*P. pilosa* subsp. *pilosa* South	*P. drummondii*	karyology[IJ], flavonoids[L], seed protein polymorphism[F], experimental crosses[C]
P. buckleyi	4N	unknown	unknown	karyology[I]

Supporting Literature: A (Levin 1969), B (Levin an Schaal 1972), C (Levin 1966b), D (Levin 1963), E (Hadley Levin 1969), F (Levin an Schaal 1970), G (Levin and Smith 1966), H (Fehlberg et al. 2014), I (Smith and Levin 1967), J (Levin 1968), K (Levy and Levin 1975), L (Levy and Levin 1974).

Table 2. Summary results for four taxon D-statistic tests for introgression in two cases of reinforcement

Reinforcement Scenario	P1	P2	P3	Range D	Range Z†	nSig/N‡ -D	nSig/N‡ +D	Test ID
P. drummmondii	*P. drummmondii* (light-blue)	*P. roemariana*	*P. cuspidata*	(-0.12. -0.01)	(0.187, 2.479)	0/24	0/24	T2:1 - T2:24
	P. drummmondii (dark-red)	*P. roemariana*	*P. cuspidata*	(-0.16. -0.01)	(0.341, **4.692**)	**24**/48	0/48	T2:25 - T2:72
P. pilosa subsp. *pilosa* North								
	P. pilosa subsp. *pilosa* North (pink)	*P. divaricata* subsp. *divaricata*	*P. glaberrima* subsp. *interior*	(-0.10. 0.08)	(0.008, 2.771)	0/180	0/180	T2:73 - T2:252
	P. pilosa subsp. *pilosa* North (white)	*P. divaricata* subsp. *divaricata*	*P. glaberrima* subsp. *interior*	(-0.08. 0.09)	(0.016, 2.418)	0/90	0/90	T2:253 - T2:342
	P. pilosa subsp. *pilosa* North (pink)	*P. paniculata*	*P. glaberrima* subsp. *interior*	(-0.09. 0.05)	(0.025, 2.707)	0/180	0/180	T2:343 - T2:522
	P. pilosa subsp. *pilosa* North (white)	*P. paniculata*	*P. glaberrima* subsp. *interior*	(-0.09. 0.10)	(0.026, 2.566)	0/90	0/90	T2:523 - T2:612

P1, P2, P3 refer to the three lineages used for the ABBA-BABA D statistic test (See Methods). Outgroups not shown. All tests can be found in Table S2.

† Z-score to assess significance of whether D deviates from zero. Bold indicates significance at α=0.01.

‡ Number of significant tests over all possible sampled individuals. Significant -D identifes gene flow between P1 and P3, while significant +D identifies gene flow between P2 and P3.

Table 3. Summary results for four taxon D-statistic tests for introgression in four hypothesized cases of hybrid speciation

Hypothesized Hybrid	P1	P2	P3	Range D	Range Z[†]	nSig/N[‡] -D	nSig/N[‡] +D	Test ID
P. argillacea								
	P. argillacea	*P. pilosa* subsp. *pilosa* North	*P. glaberrima* subsp. *interior*	(-0.09, 0.08)	(0.023, 2.115)	0/90	0/90	T3:1 - T3:90
	P. argillacea	*P. divaricata* subsp. *divaricata*	*P. glaberrima* subsp. *interior*	(-0.08. 0.09)	(0.016, 2.418)	0/90	0/90	T3:91 - T3:180
	P. argillacea	*P. paniculata*	*P. glaberrima* subsp. *interior*	(-0.09. 0.10)	(0.026, 2.566)	0/90	0/90	T3:181 - T3:270
P. pilosa subsp. *detonsa*								
	P. pilosa subsp. *detonsa*	*P. pilosa* subsp. *pilosa* South	*P. carolina* subsp. *angusta*	(-0.14. 0.01)	(0.033, 2.383)	0/12	0/12	T3:271 - T3:282
	P. pilosa subsp. *detonsa*	*P. pilosa* subsp. *pilosa* South	*P. carolina* subsp. *alta*	(-0.09. 0.02)	(0.042, 1.520)	0/12	0/12	T3:283 - T3:294
	P. pilosa subsp. *detonsa*	*P. pilosa* subsp. *pilosa* South	*P. carolina* subsp. *carolina*	(-0.13. 0.06)	(0.207, 1.794)	0/6	0/6	T3:295 - T3:300
	P. pilosa subsp. *detonsa*	*P. drummondii*	*P. carolina* subsp. *angusta*	(-0.08. -0.01)	(0.223, 1.880)	0/12	0/12	T3:301 - T3:312
	P. pilosa subsp. *detonsa*	*P. drummondii*	*P. carolina* subsp. *alta*	(-0.05. 0.05)	(0.164, 1.238)	0/12	0/12	T3:313 - T3:324
	P. pilosa subsp. *detonsa*	*P. drummondii*	*P. carolina* subsp. *carolina*	(-0.04. 0.06)	(0.024, 1.147)	0/6	0/6	T3:325 - T3:330
	P. pilosa subsp. *detonsa*	*P. roemeriana*	*P. carolina* subsp. *angusta*	(-0.05, 0.06)	(0.055, 1.266)	0/12	0/12	T3:331 - T3:342
	P. pilosa subsp. *detonsa*	*P. roemeriana*	*P. carolina* subsp. *alta*	(-0.05, 0.13)	(0.024, 2.696)	0/12	0/12	T3:343 - T3:354
	P. pilosa subsp. *detonsa*	*P. roemeriana*	*P. carolina* subsp. *carolina*	(-0.05, 0.02)	(0.188, 1.102)	0/6	0/6	T3:355 - T3:360
P. amoena subsp. *lighthipei*								
	P. amoena subsp. *lighthipei*	*P. amoena amoena*	*P. pilosa* subsp. *pilosa* South	(-0.14. 0.08)	(0.005, 2.887)	0/234	0/234	T3:361 - T3:594
	P. pilosa subsp. *pilosa* South	*P. drummondii*	*P. amoena* subsp. *lighthipei*	(-0.13, 0.06)	(0.007, 2.631)	0/234	0/234	T3:595 - T3:828
	P. pilosa subsp. *pilosa* South	*P. cuspidata*	*P. amoena* subsp. *lighthipei*	(-0.10, 0.13)	(0.007, 2.559)	0/156	0/156	T3:829 - T3:984
P. maculata subsp. *pyramidalis*								
	P. maculata subsp. *pyramidalis*	*P. maculata* subsp. *maculata*	*P. glaberrima* subsp. *interior*	(-0.50, 0.08)	(1.869, **20.718**)	**27**/30	0/30	T3:985 - T3:1014
	P. glaberrima subsp. *interior*	*P. carolina* subsp. *alta*	*P. maculata* subsp. *pyramidalis*	(-0.34, -0.06)	(2.002, **13.515**)	**17**/20	0/20	T3:1015 - T3:1034
	P. glaberrima subsp. *interior*	*P. glaberrima* subsp. *glaberrima*	*P. maculata* subsp. *pyramidalis*	(-0.31, -0.06)	(2.167, **12.277**)	**19**/20	0/20	T3:1035 - T3:1054
	P. glaberrima subsp. *interior*	*P. carolina* subsp. *alta*	*P. maculata* subsp. *maculata*	(0.01, 0.20)	(0.299, **6.323**)	0/80	**62**/80	T3:1055 - T3:1134
	P. glaberrima subsp. *interior*	*P. glaberrima* subsp. *glaberrima*	*P. maculata* subsp. *maculata*	(-0.02, 0.19)	(0.090, **6.313**)	0/80	**59**/80	T3:1135 - T3:1214

P1, P2, P3 refer to the three lineages used for the ABBA-BABA D statistic test (See Methods). Outgroups not shown. All tests can be referenced by Test ID in Table S3.

[†] Z-score to assess significance of whether D deviates from zero. Bold indicates significance at α=0.01.

[‡] Number of significant tests over all possible sampled individuals. Significant -D identifes gene flow between P1 and P3, while significant +D identifies gene flow between P2 and