American Journal of Botany

RESEARCH ARTICLE

Differences in flowering time maintain species boundaries in a continental radiation of *Viburnum*

Elizabeth L. Spriggs[1,4,5], Caroline Schlutius[1], Deren A. Eaton[2], Brian Park[1], Patrick W. Sweeney[3], Erika J. Edwards[1,3], and Michael J. Donoghue[1,3]

Manuscript received 26 September 2018; revision accepted 19 March 2019.

[1] Department of Ecology and Evolutionary Biology, Yale University, P.O. Box 208106, New Haven, Connecticut 06520, USA

[2] Department of Ecology, Evolution, and Environmental Biology, Columbia University, New York, New York 10027, USA

[3] Division of Botany, Peabody Museum of Natural History, Yale University, P.O. Box 208118, New Haven, Connecticut 06520, USA

[4] Current address: The Arnold Arboretum of Harvard University, 1300 Centre Street, Boston, Massachusetts 02131, USA

[5] Author for correspondence (e-mail: espriggs@fas.harvard.edu)

Citation: Spriggs, E. L., C. Schlutius, D. A. Eaton, B. Park, P. W. Sweeney, E. J. Edwards, and M. J. Donoghue. 2019. Differences in flowering time maintain species boundaries in a continental radiation of *Viburnum*. *American Journal of Botany* 106(6): 833–849.

doi:10.1002/ajb2.1292

PREMISE: We take an integrative approach in assessing how introgression and Pleistocene climate fluctuations have shaped the diversification of the core *Lentago* clade of *Viburnum*, a group of five interfertile species with broad areas of sympatry. We specifically tested whether flowering time plays a role in maintaining species isolation.

METHODS: RAD-seq data for 103 individuals were used to infer the species relationships and the genetic structure within each species. Flowering times were compared among species on the basis of historical flowering dates documented by herbarium specimens.

RESULTS: Within each species, we found a strong relationship between flowering date and latitude, such that southern populations flower earlier than northern ones. In areas of sympatry, the species flower in sequence rather than simultaneously, with flowering dates offset by ≥9 d for all species pairs. In two cases it appears that the offset in flowering times is an incidental consequence of adaptation to differing climates, but in the recently diverged sister species *V. prunifolium* and *V. rufidulum*, we find evidence that reinforcement led to reproductive character displacement. Long-term trends suggest that the two northern-most species are flowering earlier in response to recent climate change.

CONCLUSIONS: We argue that speciation in the *Lentago* clade has primarily occurred through ecological divergence of allopatric populations, but differences in flowering time were essential to maintain separation of incipient species when they came into secondary contact. This combination of factors may underlie diversification in many other plant clades.

KEY WORDS Adoxaceae; flowering; phenology; reinforcement; reproductive isolation; speciation; *Viburnum*.

Members of closely related species in many plant lineages are able to successfully interbreed, but hybrids may nevertheless be rare in the wild. Studies of reproductive isolation in plants have shown that multiple barriers to gene flow act to reduce the likelihood of introgression and together these can confer nearly total isolation (Husband and Schemske, 2000; Ramsey et al., 2003; Lowry et al., 2008; Dell'Olivo et al., 2011; Runquist et al., 2014; Ostevik et al., 2016). Often, early acting barriers are the most important (Rieseberg and Willis, 2007), and some of the best examples of prezygotic isolating mechanisms have involved changes in floral morphology that alter pollinator interactions, for example *Phlox* (Levin, 1985) and *Mimulus* (Schemske and Bradshaw, 1999). Shifts in flowering time may be an equally effective and even more general mechanism of ensuring reproductive isolation, and indeed offset flowering times have been documented in pairs of closely related species from a wide range of lineages (Hurlbert, 1970; Husband and Schemske, 2000; Savolainen et al., 2006; Lowry et al., 2008; Papadopulos et al., 2011; Runquist et al., 2014; Ostevik et al., 2016). In these cases, close relatives have staggered flowering periods that result in reduced rates of interspecific pollen transfer and, ultimately, gene flow.

Divergence in flowering time between closely related species could be specifically selected to reduce gene flow or could arise incidentally as populations adapt to local environmental conditions (McNeilly and Antonovics, 1968). In temperate climates, tightly regulated flowering times are essential to coordinate the flowering of individuals of the same species, to synchronize their timing with pollinators, and to avoid unfavorable conditions (e.g., late frosts in the spring; Tang et al., 2016). Unsurprisingly, species in colder climates (e.g., at higher latitudes) typically flower later in the year, and there is significant variation in flowering time across

the geographic ranges of species whose distributions span multiple climates (Turesson, 1930; McMillan, 1959; Weber and Schmid, 1998; Olsson and Ågren, 2002; Kollmann and Bañuelos, 2004; Montesinos-Navarro et al., 2011; Huang et al., 2012; Prendeville et al., 2013; Prevéy et al., 2017).

Here, using RAD-seq data, we analyze the diversification of a lineage within the *Lentago* clade of *Viburnum* (Adoxaceae). This "core *Lentago*" lineage contains five interfertile species distributed in eastern North America across a wide range of environments. We document phylogenetic relationships, introgression, and patterns of genetic structure within these species as well as differences in flowering times that we argue play a crucial role in maintaining species boundaries. With herbarium records that span the geographic range of each species, we evaluate how flowering time varies across environmental gradients and within the regions where closely related species are sympatric. Because these species are well represented by collections that span >150 yr, we are also able to test whether flowering times have shifted in this clade in response to anthropogenic climate change. Finally, we evaluate how shifts in phenology driven by climate change might affect the likelihood of interspecific gene flow in the future.

MATERIALS AND METHODS

Study clade

The *Lentago* clade consists of eight North American species and has been recognized as distinct since the earliest taxonomic treatments of *Viburnum* (e.g., Oersted, 1861). There are two major lineages within the group, which are estimated to have split from one another some 35–45 mya (Spriggs et al., 2015; M. Landis et al., unpublished data): the *V. nudum* complex (with three species; Spriggs et al., 2019a) and the core *Lentago* clade (with five species; Jones, 1983). Although molecular phylogenetic studies based on chloroplast and nuclear DNA have strongly supported these two subgroups (Winkworth and Donoghue, 2004; Clement and Donoghue, 2011; Clement et al., 2014; Spriggs et al., 2015; Eaton et al., 2017), the monophyly of the *Lentago* clade as a whole remains somewhat unclear, with some possibility that the Asian species *V. puncatum* is closely related to the *V. nudum* complex. Species relationships within the core *Lentago* clade are also uncertain because datasets dominated by chloroplast sequences resolve *V. lentago* as sister to *V. prunifolium* (Donoghue et al., 2004; Clement and Donoghue, 2011; Clement et al., 2014) while nuclear data support *V. lentago* as sister to all other species in the core *Lentago* clade (Winkworth and Donoghue, 2004, 2005; Eaton et al., 2017; M. Landis et al., unpublished data).

Although there are scattered reports of hybrids among species in the *Lentago* clade (Brumbaugh and Guard, 1956; McAtee, 1956; Jones, 1983), only one controlled set of hybridization experiments has been conducted (Egolf, 1956). Egolf (1956) did not have access to all members of the core *Lentago* clade, but he found that crosses between *V. lentago* and *V. prunifolium* yielded seedlings, while those between *V. lentago* and the more distantly related *V. cassinoides* (part of the *V. nudum* complex) did not. These findings suggest that all members of the core *Lentago* clade are likely to be interfertile, but that hybridization between these species and members of the *V. nudum* complex is probably not possible.

Sample collection and RAD sequencing

In 2013–2016, we collected 204 individuals from across eastern North America. Leaf material for each individual was dried in silica gel, and voucher specimens were later deposited at the Yale University Herbarium (YU) (for full details, see Appendix 1). Eighty-three individuals were chosen from these collections for sequencing. These individuals were supplemented with material from 10 herbarium specimens (from NYBG and TEX) with collection dates 1980–2007 and four *V. elatum* specimens collected in 1979–1981. Six individuals from the *V. nudum* complex were also included and used to root the core *Lentago* clade. Genomic DNA was extracted using either a DNEasy kit (Qiagen, Valencia, California) or following the CTAB protocol of Doyle and Doyle (1987). Each sample for this study was included in one of four separate but identical RAD library preparations (Floragenex, http://floragenex.com) using *PstI* for the initial digestion followed by sonication and size selection for a mean fragment length of 400 bp. Each library was sequenced at the University of Oregon GC3F facility (http://gc3f.uoregon.edu) on an Illumina HiSeq2000 or Hiseq2500. Although the total number of reads per sample varied, the identity of the loci recovered was highly repeatable across libraries, and there was no evidence of a plate bias.

Data Assembly

Raw sequence data were demultiplexed and assembled using the software ipyrad (http://github.com/dereneaton/ipyrad; Eaton, 2014). All reads with Phred scores <20 at more than five bases were excluded, and a minimum depth of six was required for statistical base calling. Reads were grouped into loci within species using a clustering threshold of 0.88. This parameter determines the level of sequence similarity necessary for two sequences to be considered homologous and clustered together. Because all species in the *Lentago* clade are known to be diploid (Egolf, 1962), any putative loci with more than two alleles were removed because these are likely to be derived from paralogous or repetitive genomic regions. Finally, datasets were constructed with different levels of missing data, requiring that all loci be shared across ≥25% of individuals (min25, 65,036 loci, 50.5% missing data), 50% of individuals (min50, 38,442 loci, 40.5% missing data), or 75% of individuals (min75, 4099 loci, 25.7% missing data). For some analyses, smaller versions of these datasets were also created (e.g., of only *V. prunifolium* samples) and filtered such that all loci were shared across ≥75% of the individuals.

Phylogenetic inference

Maximum likelihood trees were inferred using RAxML version 8.2.9 (Stamatakis, 2014) based on concatenated supermatrices of each of the three most inclusive datasets (min25, min50, min75). For all RAxML analyses, the GTR+Γ substitution model was used with 20 tree searches with 100 rapid boostraps to assess node support.

Tests of introgression

Potential scenarios of gene flow among the species of the *Lentago* clade were evaluated using the *D*-statistic. The standard *D*-statistic test describes the relative frequency of two discordant

site patterns—ABBA and BABA in a four-taxon tree (Durand et al., 2011). We used this version of the test to investigate potential gene flow between *V. lentago* and each of the other species (*V. obovatum*, *V. elatum*, *V. prunifolium*, and *V. rufidulum*). We then used the five-taxon extension of the test (Eaton and Ree, 2013) to look for gene flow between *V. obovatum*, *V. elatum*, *V. prunifolium*, and *V. rufidulum*. All tests were performed with the ipyrad Python API (http://github.com/dereneaton/ipyrad) using the best-supported tree topology, which unites *V. elatum* and *V. obovatum* as sister species. The standard deviation of *D* was measured on the basis of 1000 bootstrap replicates in which RAD loci were resampled as in Eaton and Ree (2013). Similar to the approach described in Eaton et al. (2015), all individuals of each species were pooled and an outgroup was created by combining three *V. nudum* complex samples—one each from *V. nudum*, *V. nitidum*, and *V. cassinoides* (E. L. Spriggs et al., 2019a). Three putative hybrid individuals (ELS523, ELS524, ELS455) were removed prior to these analyses and were tested separately (see below).

To investigate potential gene flow between the sister species *V. prunifolium* and *V. rufidulum*, we divided the individuals of those species into two groups based on whether they were collected from populations in the area of sympatry or outside it. For these tests we again used the pooled *V. nudum* complex samples as an outgroup, but we randomly sampled individuals from each other group because we expected that the level of introgression could vary among individuals, particularly if the area of sympatry has shifted over time. First we tested for gene flow from *V. prunifolium* into sympatric *V. rufidulum* as compared to allopatric *V. rufidulum*; then we tested the reverse—from *V. rufidulum* into sympatric *V. prunifolium* as compared to allopatric *V. prunifolium*. A Bonferroni correction for 100 tests was applied to these replicated tests. Finally we tested whether two specific individuals (ELS523, ELS524) that are sister to the rest of *V. rufidulum* were admixed. For these tests we again sampled random individuals of each species.

Phylogenetic networks

To further evaluate past introgression in the core *Lentago* clade, we inferred phylogenetic networks with the software PhyloNet (Than et al., 2008; Wen et al., 2018). PhyloNet is a phylogenetic network method based on the frequencies of rooted triples that accounts for incomplete lineage sorting and infers a specified number of hybridization edges. To run PhyloNet, we first created five datasets that each contained a single representative of each species and no missing data. We chose individuals for these datasets semi-randomly such that each set of individuals shared >500 loci. We then used the TIQR pipeline (Stenz et al., 2015) to run MrBayes (Ronquist and Huelsenbeck, 2003) for each locus with three runs, three chains, 1 million generations, a sample frequency of 200, and a burn-in of 0.25. Consensus trees for all loci were rooted in R with the *V. nudum* complex and used as the input for PhyloNet. Finally, maximum pseudo-likelihood networks with one and two hybridization edges were inferred for each dataset.

Population clustering

We examined population structure within each species individually using the Bayesian clustering algorithm STRUCTURE (Pritchard et al., 2000). For each species, we constructed a dataset with only loci shared across ≥75% of the individuals of that species and considered clustering scenarios with up to four groups (K = 1–4). For each value of K, we ran 10 replicates with 100,000 generations and discarded the first 10,000 as burn-in. We then used CLUMPP (Jakobsson and Rosenberg, 2007) to combine replicate runs and evaluated convergence by comparing the alpha parameter, $P(X|K)$, and the variance of $P(X|K)$. We used Structure Harvester to compare alternative values of K based on the log probability of the data ($\log P(X|K)$) and the ΔK statistic (Evanno et al., 2005). To further assess gene flow between *V. prunifolium* and *V. rufidulum*, we also conducted a joint analysis of those two species alone.

Species distribution data and range maps

Occurrence data for the species in the core *Lentago* clade were obtained from several sources. The majority was from herbarium specimens complied from online databases and in-person herbarium visits. Each specimen was georeferenced to the county level. These data were supplemented with GBIF (http://gbif.org/) records of specimens from North America after removing any records derived from herbaria already in our dataset. The combined dataset includes 4525 specimens from 58 herbaria. To remove misidentified specimens or individuals that were cultivated outside of their native range, localities that fell outside of the USGS range map of each species in the core *Lentago* clade (Little, 1971) were removed, but the range maps were buffered so that points in the areas directly adjacent to range boundaries were also included. This procedure removed 1–5% of the records per species. A USGS range map is unavailable for the Mexican *V. elatum*; so for this species only a handful of errant localities on the Baja peninsula were removed. Finally, our own field collections were added. A final range map was then constructed for each species including all areas within 75 km of an accepted occurrence.

Species distribution modeling

Current and past distributions of the species in the core *Lentago* clade were estimated with the maximum entropy method MAXENT (Phillips et al., 2006) implemented in the R package "dismo" (Hijmans et al., 2017). Climatic data (19 bioclimatic variables) were downloaded from Worldclim (http://www.worldclim.org) at a 2.5 arc-minute resolution for the present and for the Last Glacial Maximum under the PCCSM model. To assess correlations among the bioclim variables, we extracted the environmental variables at 1000 random points in the study area (15° to 65° latitude, −130° to −52° longitude). We removed one variable in each pair of variables with a Pearson correlation coefficient ≥0.5, leaving five variables (bio01 = annual mean temperature, bio02 = mean diurnal temperature range, bio04 = temperature seasonality, bio12 = annual precipitation, bio15 = precipitation seasonality). We also performed a second analysis with a less stringent cutoff of ≥0.8 that left nine variables.

For each species, the county means of the environmental variables were compiled for all counties with an occurrence record. We chose to use county means rather than county centroids because mean values more accurately consider georeferencing uncertainty and have been shown to perform better in species distribution modeling (Park and Davis, 2017). Models were fit with 1000 random background points sampled from across the study area. For each species, 10 replicates were performed with 80% of the data

used to train the model and the other 20% used for testing. Model fit was then evaluated using the area under the ROC curve (AUC). These models were projected onto current and LGM climate data to predict the potential distribution of each species.

Estimates of flowering time

As in most of *Viburnum*, inflorescences in the core *Lentago* clade are flat-topped, umbel-like compound corymbs that contain 25–200 small white flowers (Donoghue, 1980, 1982). Species in the core *Lentago* clade typically flower once per year in late spring and bear large, bird-dispersed fruits that take several months to mature (Jones, 1983). Flowering is highly synchronized within populations and on a single plant, and most individuals flower for a period of 7–10 d (Donoghue, 1980).

To estimate flowering times across the range of each species, we scored the phenological condition of herbarium specimens by inspecting photos of specimens taken in person or images of specimens available in online databases. Each specimen was georeferenced to the county level, the collection date was recorded, and the phenology was scored as nonreproductive, immature flowers, flowering, or past flowering/fruiting. We converted flowering dates to numeric days since January 1 (Julian days); hereafter we refer to this simply as "flowering time." Even though the flowering period is relatively short, flowering specimens are overrepresented in herbaria and we were able to score 1379 flowering specimens (Appendix S1 and Appendix S2; see the Supplemental Data with this article; full dataset available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.t7r6kg5 [Spriggs et al., 2019b]). To exclude aberrant fall-flowering specimens, we removed eight specimens with a flowering date >200 (i.e., after July 19). A small number of samples included in our dataset might be duplicates of single collecting events shared between herbaria. In some cases these might even be replicates taken from a single individual on a particular day. We did not attempt to remove these, but the resampling procedure we describe below ensures that these did not drive the larger patterns in the data.

Comparison of flowering times among species

We used a multiple regression framework to test how species, latitude, and collection year affected flowering date. Latitude and mean annual temperature are highly correlated in our dataset (Pearson correlation coefficient = 0.985), so we focused only on latitude. *Viburnum elatum* was excluded from these analyses because there are few collections of this species (only 11 flowering specimens in our sample), and a substantial portion of the climatic variation across the range of *V. elatum* is related to altitude, which we are unable to characterize without more precise locality information. All statistical analyses were conducted in R (R Core Team, 2017).

We first tested the relationship between latitude and flowering time across our whole dataset. A simple model in which flowering time depended only on latitude was compared to more complex models that considered "species" and "latitude by species" interaction terms using analysis of variance. We then separately compared each pair of species that have overlapping geographic ranges, testing first whether the flowering times were significantly different between the species (i.e., whether a model that included "species" as a predictor was significantly better than a model based on latitude alone), and again tested whether there was a significant interaction between species and latitude. We then asked whether the flowering times of each species were different where they are sympatric versus allopatric with the second species. For this we added a "location" term that provided information on whether or not each data point was located in the area of sympatry between the species or outside of it (allopatry). To ensure that these analyses were not biased by outlier points, we bootstrapped each dataset 1000 times, randomly sampling the flowering date records with replacement until we obtained a dataset the same size as the original. Note that this procedure resulted in varying numbers of observations for each species between replicates.

To better visualize variation in flowering time across the range of each species, we mapped flowering date using inverse distance weighting in the R package "gstat" (Benedikt et al., 2016). To compare flowering times in regions where the ranges of species overlap, we used only the points that occur in the zone of overlap. We calculated a continuous flowering-time map for each species in the region with inverse distance weighting to interpolate between points. We then subtracted one surface from the other to calculate the difference in flowering date in each grid cell. Because inverse distance weighting interpolates the flowering date for grid cells that lack data on the basis of nearby values, it is possible to compare flowering dates of species that were not consistently collected in the same counties. For instance, although *V. lentago* and *V. prunifolium* are well represented throughout their area of sympatry (171 specimens of *V. lentago* from 78 different counties and 237 specimens of *V. prunifolium* from 115 counties), there are only 28 counties from which flowering specimens of both species have been collected.

Flowering time and climate change

We also assessed long-term changes in flowering time using linear models. For each species, we compared a model in which flowering time was predicted only by latitude to models that included the collection year and an interaction term for collection year by latitude. Because we found significant climate change trends in some species, we repeated most of the phenological analyses described above only on the basis of flowering records before 1950 and observed no significant changes.

RESULTS

Data assembly

For most individuals, 1–3 million reads were recovered after the initial quality-filtering steps. Older collections and individuals that were sampled from herbarium specimens had consistently fewer reads (mean = 0.75 million) than recently collected individuals (mean = 2.3 million) and had correspondingly fewer loci in our final data assemblies. Raw (demultiplexed) sequence data have been deposited in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra), and assembled sequence data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.t7r6kg5 (Spriggs et al., 2019b).

Phylogenetic inference

Most relationships within the *Lentago* clade were well supported (Fig. 1), and all species were inferred to be monophyletic. The

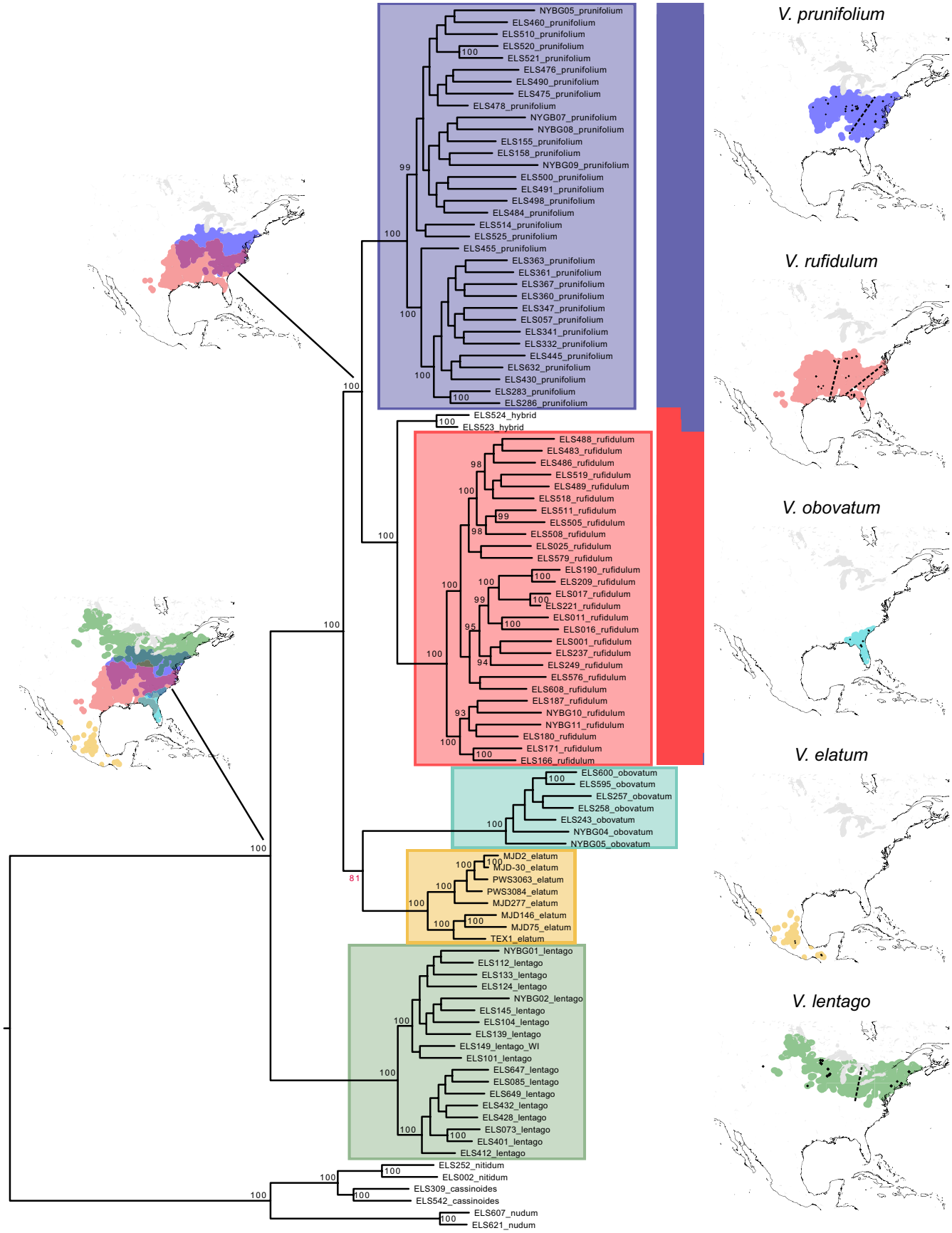
NYBG05_prunifolium
ELS460_prunifolium
ELS510_prunifolium
ELS520_prunifolium
ELS521_prunifolium
ELS476_prunifolium
ELS490_prunifolium
ELS475_prunifolium
ELS478_prunifolium
NYGB07_prunifolium
NYBG08_prunifolium
ELS155_prunifolium
ELS158_prunifolium
NYBG09_prunifolium
ELS500_prunifolium
ELS491_prunifolium
ELS498_prunifolium
ELS484_prunifolium
ELS514_prunifolium
ELS525_prunifolium
ELS455_prunifolium
ELS363_prunifolium
ELS361_prunifolium
ELS367_prunifolium
ELS360_prunifolium
ELS347_prunifolium
ELS057_prunifolium
ELS341_prunifolium
ELS332_prunifolium
ELS445_prunifolium
ELS632_prunifolium
ELS430_prunifolium
ELS283_prunifolium
ELS286_prunifolium
ELS524_hybrid
ELS523_hybrid
ELS488_rufidulum
ELS483_rufidulum
ELS486_rufidulum
ELS519_rufidulum
ELS489_rufidulum
ELS518_rufidulum
ELS511_rufidulum
ELS505_rufidulum
ELS508_rufidulum
ELS025_rufidulum
ELS579_rufidulum
ELS190_rufidulum
ELS209_rufidulum
ELS017_rufidulum
ELS221_rufidulum
ELS011_rufidulum
ELS016_rufidulum
ELS001_rufidulum
ELS237_rufidulum
ELS249_rufidulum
ELS576_rufidulum
ELS608_rufidulum
ELS187_rufidulum
NYBG10_rufidulum
NYBG11_rufidulum
ELS180_rufidulum
ELS171_rufidulum
ELS166_rufidulum
ELS600_obovatum
ELS595_obovatum
ELS257_obovatum
ELS258_obovatum
ELS243_obovatum
NYBG04_obovatum
NYBG05_obovatum
MJD2_elatum
MJD-30_elatum
PWS3063_elatum
PWS3084_elatum
MJD277_elatum
MJD146_elatum
MJD75_elatum
TEX1_elatum
NYBG01_lentago
ELS112_lentago
ELS133_lentago
ELS124_lentago
NYBG02_lentago
ELS145_lentago
ELS104_lentago
ELS139_lentago
ELS149_lentago_WI
ELS101_lentago
ELS647_lentago
ELS085_lentago
ELS649_lentago
ELS432_lentago
ELS428_lentago
ELS073_lentago
ELS401_lentago
ELS412_lentago
ELS252_nitidum
ELS002_nitidum
ELS309_cassinoides
ELS542_cassinoides
ELS607_nudum
ELS621_nudum
V. prunifolium
V. rufidulum
V. obovatum
V. elatum
V. lentago

FIGURE 1. Maximum likelihood (RAxML) phylogeny for the *Lentago* clade of *Viburnum* based on the dataset with only loci shared across ≥50% of the individuals. Bootstrap support values >90 are displayed. Range maps for each species are at the right with the collection locations for all sequenced individuals plotted. Dotted lines on these maps represent the approximate locations of genetic breaks inferred using STRUCTURE (see Appendix S5). Bar graph displays the results of a STRUCTURE analysis that included all individuals of *V. prunifolium* and *V. rufidulum*. Colors indicate the posterior probability of assignment of each individual to a particular cluster. The two maps on the left show the range overlap of all species descended from particular nodes. Note that these are not meant to be reconstructions of the ancestral ranges.

three species of the *V. nudum* complex form a clade that is sister to the core *Lentago* clade, which contains *V. lentago*, *V. obovatum*, *V. elatum*, *V. prunifolium*, and *V. rufidulum*. Within core *Lentago* there is strong support for a first split between *V. lentago* and the rest, and for a sister relationship between *V. prunifolium* and *V. rufidulum*. The placement of *V. obovatum* and *V. elatum* is less certain. The most common resolution is one in which *V. obovatum* and *V. elatum* form a clade, but the two species are paraphyletic in as many as 19% of the bootstrap replicates (depending on the size of the dataset examined), with *V. elatum* being more closely related to *V. prunifolium* + *V. rufidulum* than to *V. obovatum*.

Two individuals (ELS523, ELS524) that were collected in Kentucky in a sympatric population of *V. prunifolium* and *V. rufidulum* were found to be sister to all other *V. rufidulum* (Fig. 1). This placement suggests that these individuals may be hybrids of *V. prunifolium* and *V. rufidulum*, and a STRUCTURE analysis that included both *V. prunifolium* and *V. rufidulum* estimated the ancestry of these two individuals to be 52% *V. rufidulum* and 48% *V. prunifolium* (Fig. 1; Appendix S3). By comparison, the estimated proportion of admixture in all other individuals was very low (<3%).

Introgression and phylogenetic networks

D-statistic tests identified a significant signal of introgression between *V. lentago* and *V. prunifolium* ($Z = 8.513$, $P < 1e\text{-}16$). They also identified weaker evidence of gene flow between *V. lentago* and *V. rufidulum* ($Z = 3.347$, $P = 0.0008$), but this is probably not due to actual genetic exchange between these species but likely reflects the shared ancestry of *V. rufidulum* and *V. prunifolium*. If introgression occurred from *V. prunifolium* into *V. lentago*, some genes that are shared between *V. prunifolium* and *V. rufidulum* would also be introgressed and would create this signal. There was no evidence of gene flow between *V. lentago* and either *V. obovatum* or *V. elatum*. The five-taxon *D*-statistic tests revealed a complex pattern of introgression among *V. obovatum*, *V. elatum*, *V. prunifolium*, and *V. rufidulum*, with at least two instances of gene flow. Overall the tests support a scenario where gene flow occurred from *V. obovatum* into *V. rufidulum* and from *V. prunifolium* into *V. elatum*; however, other patterns of introgression could lead to similar genetic patterns, and we cannot confidently state that these are true introgression events or that they are the only ones that occurred. Tests of introgression between the sister species *V. prunifolium* and *V. rufidulum* found no evidence of gene flow between them. The only exceptions were the two individuals collected in Kentucky (ELS523, ELS524) that were confirmed to be hybrids. All *D*-statistic results are summarized in Figure 2.

For each of our five datasets, PhyloNet identified multiple phylogenetic networks with only slightly different log probabilities (Appendix S4). In many of these, *V. elatum* formed a clade with *V. prunifolium* and *V. rufidulum* and was admixed with *V. obovatum*. Gene flow between *V. lentago* and *V. prunifolium* was also commonly identified, and networks with two admixture edges usually displayed both patterns (Appendix S4).

Population structure

We recovered significant east-west population structure within three species in the core *Lentago* clade (Fig. 1). We found support for two genetic clusters within *V. lentago* and *V. prunifolium*, and for three clusters within *V. rufidulum*. The eastern and western collections of *V. lentago* were significantly different, but sampling in the middle of the range of this species is insufficient to more precisely characterize this split (Appendix S5). In *V. prunifolium* we found distinct clusters on either side of the Appalachian Mountains (Appendix S5), while in *V. rufidulum* the major split corresponds to the Mississippi River drainage, with some sign of north-south differentiation in the eastern portion of its range (Appendix S5).

Species distribution models

The species distribution models for all species performed well regardless of whether nine or five environmental predictor variables were used (AUC ≥0.93). When these models were hind-cast onto climatic reconstructions for the LGM, we found that the three more northern species (*V. lentago*, *V. prunifolium*, and *V. rufidulum*) all had significantly different ranges compared to their current distributions (Fig. 3; Appendix S6). We note that there is relatively little area estimated to be highly suitable for *V. lentago* (>0.5), and the most suitable regions for *V. prunifolium* are disjunct between Texas and a region along the Atlantic coast. By contrast, it appears that both *V. obovatum* and *V. elatum* may have survived the LGM more or less in place in Florida and Mexico, respectively (Fig. 3; Appendix S6).

Phenology

There is clear variation in flowering time across the geographic range of all species (Fig. 4). In each, plants in southern portions of the range flowered first, with the earliest-flowering individual flowering 54–134 d ahead of the latest-flowering individual of the same species. Flowering time in all species was significantly correlated with latitude (*V. lentago*: $df = 358$, $P < 0.0001$, Pearson's correlation coefficient [cor] = 0.364; *V. prunifolium*: $df = 488$, $P < 0.0001$, cor = 0.716; *V. rufidulum*: $df = 430$, $P < 0.0001$, cor = 0.802; *V. obovatum*: $df = 86$, $P < 0.0001$, cor = 0.656), and the slope of the relationship varied among species (Table 1; Appendix S7). The slopes of the relationship were steepest in the southern *V. obovatum*, shallowest in the northern *V. lentago*, and statistically indistinguishable between *V. prunifolium* and *V. rufidulum* (Appendix S7).

To further investigate flowering times, we fit regression models to data for each pair of species that are partially sympatric (*V. lentago*/*V. prunifolium*, *V. rufidulum*/*V. obovatum*, and *V. prunifolium*/*V. rufidulum*). These results are summarized in Table 1,

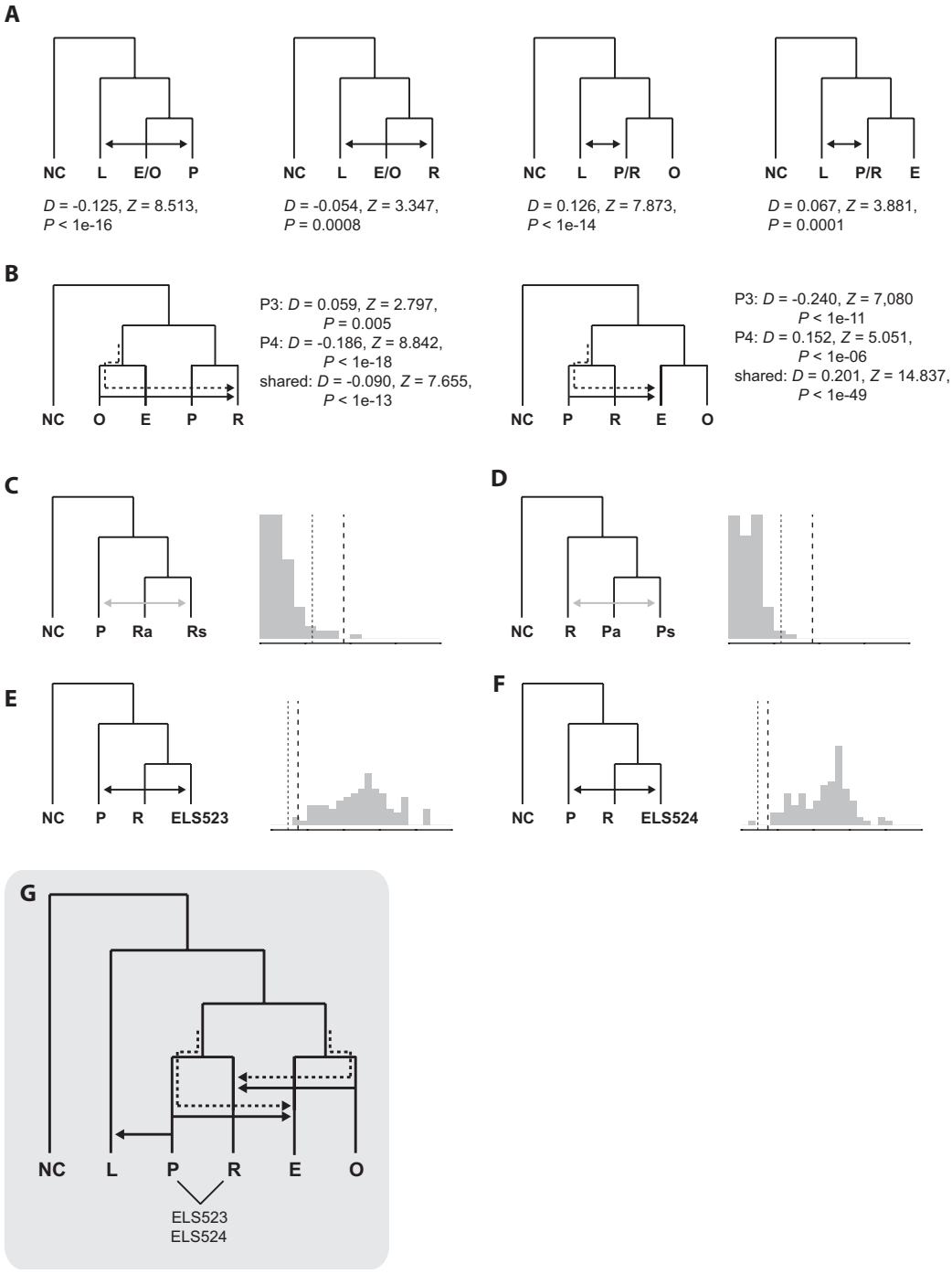

FIGURE 2. *D*-statistic tests reveal multiple instances of past introgression in the core *Lentago* clade. Taxa are abbreviated as follows: NC = *Viburnum nudum* complex, L = *V. lentago*, E = *V. elatum*, O = *V. obovatum*, P = *V. prunifolium*, R = *V. rufidulum*, Ra = *V. rufidulum* allopatric with *V. prunifolium*, Rs = *V. rufidulum* sympatric with *V. prunifolium*, Pa = *V. prunifolium* allopatric with *V. rufidulum*, Ps = *V. prunifolium* sympatric with *V. rufidulum*. The phylogeny in each panel illustrates the design of a specific test. Black arrows show where a significant signal of introgression was detected with gray arrows for tests that did not identify ingression. (A) Four-taxon *D*-statistic tests for gene flow between *V. lentago* and each of the other species. (B) Five-taxon tests for gene flow among *V. obovatum*, *V. elatum*, *V. prunifolium*, and *V. rufidulum*. (C, D) Four-taxon tests for introgression between *V. prunifolium* and *V. rufidulum* where the two species are sympatric. Graph to the right of each tree shows a histogram of *Z*-scores for 100 replicate tests with different individuals sampled. Higher *Z*-scores (lower *P*-values) are located to the right of each graph. Smaller dotted line is at $P = 0.01$, larger dotted line is at $P = 0.01$ with a bonferroni correction for 100 tests. (E, F) Tests for putative hybrid individuals (histograms as in C and D). (G) Summary of all *D*-statistic results shows full species tree with all inferred instances of introgression. Solid lines show instances of introgression supported by *D*-statistic tests with dotted lines illustrating ancestral alleles that would have been transferred with these events. ELS523 and ELS524 are the two individuals supported to be hybrids between *V. prunifolium* and *V. rufidulum*.

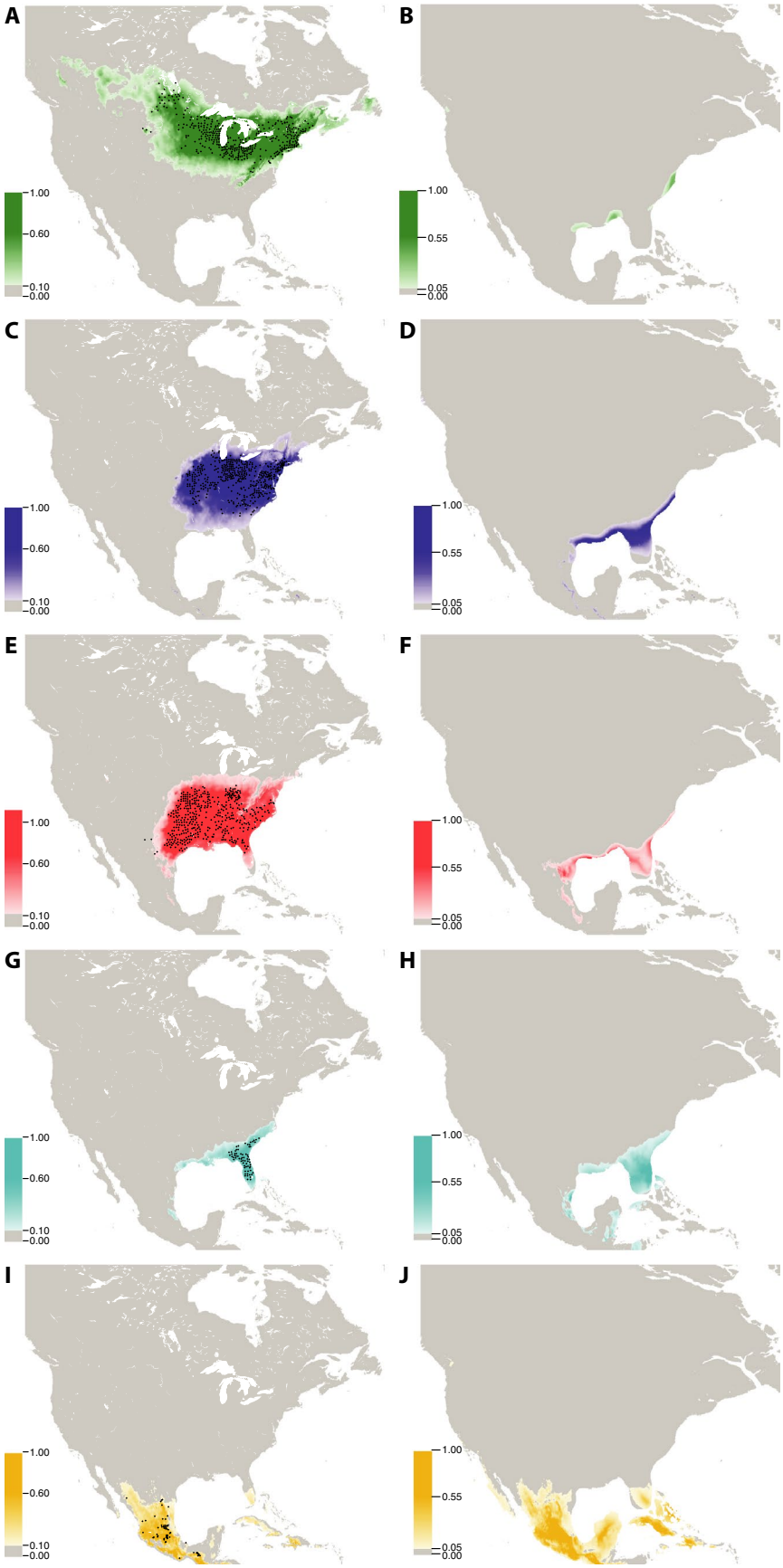

FIGURE 3. Species distribution models based on nine climatic variables predict differences between current (A–E) and LGM (F–J) distributions of species in the core *Lentago* clade. For each species, occurrence data are plotted on the current map, and the relative probability of occurrence at each grid cell is indicated by shading. (A, F) *Viburnum lentago*, (B, G) *V. prunifolium*, (C, H) *V. rufidulum*, (D, I) *V. obovatum*, and (E, J) *V. elatum*.

Figure 5, and Appendix S7. The first two cases (*V. lentago*/*V. prunifolium* and *V. rufidulum*/*V. obovatum*) were similar to one another. In both, the species had significantly different relationships between latitude and flowering time (Fig. 5A, D), and the southern species flowered first in the area of sympatry (Fig. 5A, C, D, E). For *V. lentago*/*V. prunifolium* flowering times in the zone of sympatry were significantly different from those in allopatry (Fig. 5B). For these, the species' flowering dates were more similar to one another in their zone of sympatry than would be expected on the basis of the flowering dates where they are allopatric; however, there was still a consistent difference between them, with the southern *V. prunifolium* flowering first. These patterns were consistent across the bootstrapped replicates (Table 1; Appendix S7). Maps of interpolated flowering time confirmed these patterns. For *V. lentago*/*V. prunifolium*, *V. prunifolium* flowered first in 99.9% of the area of sympatry, >5 d ahead of *V. lentago* in 98.2%, and >7 d ahead in 96.2% (Fig. 5C). Overall these maps predict a 15.27 d average difference in flowering time between *V. lentago* and *V. prunifolium*. For *V. rufidulum*/*V. obovatum*, *V. obovatum* flowered first in 99.9% of the area, >5 d ahead of *V. rufidulum* in 97.3%, and >7 d ahead in 95.7% (Fig. 5E). For this species pair, interpolation of flowering times predicts *V. obovatum* to flower an average of 22.12 d ahead of *V. rufidulum*.

The case of *V. prunifolium*/*V. rufidulum* was different from the other two pairs, because for this pair the *northern* species flowered first. Linear models that considered all data for each species predicted a 9.2 d difference, with the more northern *V. prunifolium* flowering ahead of the more southern *V. rufidulum*. Adding location (allopatry/sympatry) significantly improved the model and showed that the difference in flowering time between these

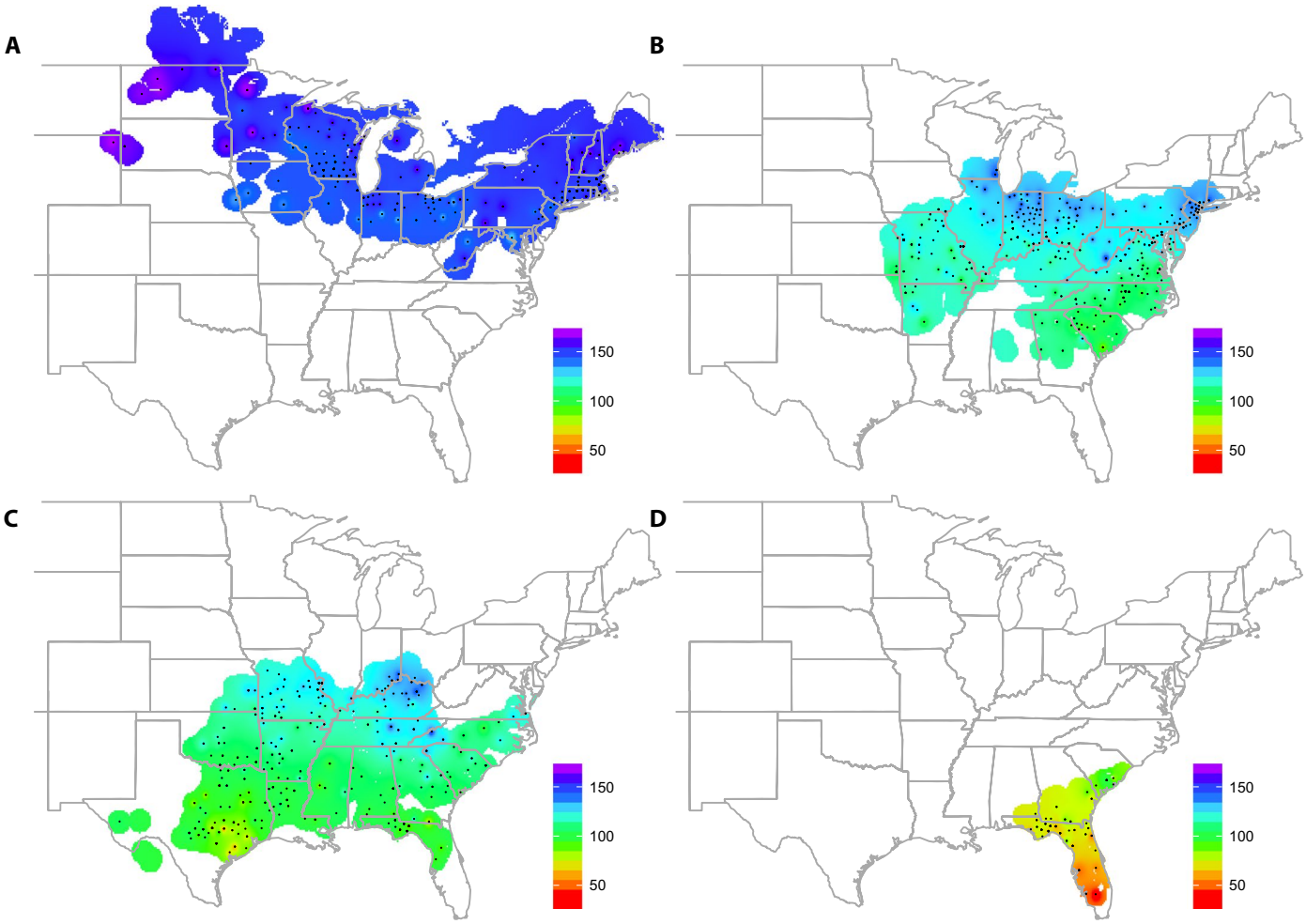

FIGURE 4. Within each species, populations in the south flower earlier than those in the north. The location (county centroids) of flowering specimens used to generate each map is indicated by points, and the color of each grid cell indicates the flowering time predicted on the basis of inverse distance weighting. (A) *Viburnum lentago*, (B) *V. prunifolium*, (C) *V. rufidulum*, and (D) *V. obovatum*.

species occurs only in the area where they are sympatric (Fig. 5F, G). In other words, data from regions where the species are allopatric suggest that flowering time as predicted by latitude is not significantly different between the species (Fig. 5G). Where the species are sympatric, however, *V. prunifolium* flowers 4.2 d earlier than expected on the basis of its allopatric range and *V. rufidulum* flowers 6.3 d later than expected on the basis of its allopatric range. These shifts combine to create a >10 d difference in expected flowering time between the two species in their area of sympatry. These findings were also consistent across our 1000 bootstrap analyses (Table 1; Appendix S7). As in the previous comparisons, the differences in flowering time between *V. prunifolium* and *V. rufidulum* were confirmed by interpolated flowering-time maps. These maps predict that *V. prunifolium* flowers first in 91.4% of the area of sympatry, >5 d ahead of *V. rufidulum* in 70.3%, and >7 d ahead in 58.4% (Fig. 5H). There is an average difference of 8.2 d predicted for these species across their area of sympatry.

To test whether the differences in flowering time detected among species were affected by anthropogenic climate change, we conducted a second analysis with only specimens collected before 1950. Although this cutoff dramatically decreased our sample size, we observed the same trends (Appendix S8). Our tests for long-term shifts in flowering phenology as a potential consequence of climate change found a significant effect of collection year only for the two northern-most species, *V. lentago* and *V. prunifolium* ($P < 0.001$). The collection year by latitude term was nonsignificant for all species (*V. lentago* $P = 0.076$, *V. prunifolium* $P = 0.53$). Our models show that flowering time has shifted over the past century to be an average of 5.23 d earlier in *V. lentago* and 6.18 d earlier in *V. prunifolium* (Fig. 6).

DISCUSSION

The core *Lentago* clade is a North American radiation of five clearly (though subtly) differentiated species that diverged from one another primarily along a north-south axis. Despite significant range movements in the past, and many opportunities for hybridization, species boundaries are clear and appear to be maintained today by multiple factors including allopatry (*V. obovatum* vs. *V. elatum*; *V. lentago* vs. *V. rufidulum*) and differences in flowering phenology (*V. lentago* vs. *V. prunifolium*; *V. prunifolium* vs.

TABLE 1. Linear models used to compare flowering time and latitude for the *Lentago* clade of the *Viburnum*. For each set of comparisons the best model is in bold, and the letter in the left-hand column indicates the panel where the model is plotted in Figure 6. Significance values (column S) refer to the comparison of each model with the last significant simpler model in each set. For example, "flowering date ~ latitude + species" compared to "flowering date ~ latitude" (*** indicates significance at $P \leq 0.001$). The final three columns report the proportion of bootstrapped datasets where the model was significantly better than the last significant simpler model for three values of *P*.

			R^2	S	$P \leq 0.05$	$P \leq 0.01$	$P \leq 0.001$
	All species (1370 specimens)						
	Flowering date ~ latitude	$F_{1,1368} = 5066.695$	0.787				
	Flowering date ~ latitude + species	$F_{4,1365} = 1767.393$	0.838	***			
	Flowering date ~ latitude * species	$F_{7,1362} = 1081.894$	0.847	***			
	V. lentago + *V. prunifolium* (850 specimens)						
	Flowering date ~ latitude	$F_{1,848} = 1885.634$	0.689				
	Flowering date ~ latitude + species	$F_{2,847} = 1234.086$	0.744	***	1	1	1
A	Flowering date ~ latitude * species	$F_{3,846} = 893.398$	0.759	***	1	1	1
B	Flowering date ~ latitude * species + species:location	$\mathbf{F_{5,844} = 575.361}$	**0.772**	***	1	1	1
	V. rufidulum + *V. obovatum* (520 specimens)						
	Flowering date ~ latitude	$F_{1,518} = 1079.357$	0.675				
	Flowering date ~ latitude + species	$F_{2,517} = 779.934$	0.75	***	1	1	1
D	Flowering date ~ latitude * species	$\mathbf{F_{3,516} = 535.102}$	**0.755**	***	0.826	0.705	0.523
	Flowering date ~ latitude * species + species:location	$F_{5,514} = 324.016$	0.757	NS	0.533	0.293	0.104
	V. prunifolium + *V. rufidulum* (922 specimens)						
	Flowering date ~ latitude	$F_{1,920} = 1481.105$	0.616				
F	Flowering date ~ latitude + species	$F_{2,919} = 875.104$	0.655	***	1	1	1
	Flowering date ~ latitude * species	$F_{3,918} = 583.29$	0.655	NS	0.143	0.05	0.008
G	Flowering date ~ latitude + species + species:location	$\mathbf{F_{4,917}} = 458.542$	**0.665**	***	1	0.997	0.982

V. rufidulum; *V. rufidulum* vs. *V. obovatum*). In zones of overlap between pairs of more distantly related species—*V. lentago* and *V. prunifolium* and *V. rufidulum* and *V. obovatum*—the offset in flowering times appears to be incidental, resulting from divergence in the relationship between latitude and flowering time in each species. However, in the case of the sister species *V. prunifolium* and *V. rufidulum*, flowering times are further displaced in their zone of sympatry, consistent with the predicted outcome of reinforcing selection to reduce hybridization through increased premating isolation.

The two northern-most species, *V. lentago* and *V. prunifolium*, have significantly advanced the onset of flowering over the past century in response to climate change, while the more southern species show no such shift (Fig. 6). Although the warmer and less predictable spring temperatures associated with climate change will affect both the relative and absolute timing of flowering in the core *Lentago* clade, we argue below that the direction and magnitude of these changes is not likely to result in increased gene flow among the species.

Phylogenetic relationships and introgression

Although RAD-seq data for the core *Lentago* clade revealed a complex history of gene flow among multiple pairs of species, we were able to resolve relationships that were previously unsupported or in conflict (Winkworth and Donoghue, 2004, 2005; Clement and Donoghue, 2011; Clement et al., 2014) and to infer specific introgression events in some cases. Most notably, we found no evidence for the hypothesis that *V. prunifolium* originated through hybridization between *V. lentago* and *V. rufidulum* (Rader, 1976; Donoghue et al., 2004), or the hypothesis that *V. lentago*, *V. prunifolium*, and *V. rufidulum* form an introgressive cline (Brumbaugh and Guard, 1956). Instead, our analyses strongly support an early split between *V. lentago* and the rest of core *Lentago*. *D*-statistic tests identified a significant signal of past gene flow between *V. lentago* and *V. prunifolium*, the two northern species of the clade. Introgression between these species could explain a major conflict between chloroplast and nuclear data, namely that chloroplast phylogenies consistently group *V. lentago* and *V. prunifolium* as sister species (Clement et al., 2014). RADseq data likely reflect the species tree for these taxa better than the chloroplast data, and past introgression may have led to a chloroplast capture event (Rieseberg and Soltis, 1991) in which *V. lentago* acquired a *V. prunifolium* chloroplast after the divergence of *V. prunifolium* and *V. rufidulum*.

Disentangling the history of divergences and gene flow among the other species of core *Lentago* is more complicated. Although *V. obovatum* differs significantly in morphology from the other species (small leaves, short petioles, small inflorescences, large corollas, short stamens; Appendix S9), our analyses find that it is squarely nested within the core *Lentago* clade. The four- and five-taxon *D*-statistic tests identified significant introgression in the past between multiple pairs of taxa (Fig. 2). These patterns were largely confirmed in our phylogenetic network analysis; however, the inference of the phylogenetic network in this case is limited by identifiability problems associated with few taxa and many instances of introgression (Pardi and Scornavacca, 2015; Solís-Lemus and Ané, 2016) and by the extremely short lengths of our loci (Roch et al., 2018).

It is worth noting here that the phenological arguments we make below do not depend on the exact topology of the core *Lentago* clade. For these purposes it does not matter whether *V. obovatum* and *V. elatum* are truly sister species, or even if *V. rufidulum* and *V. prunifolium* are sister species. Regardless, they are closely related to one another and are capable of hybridizing in their areas of sympatry.

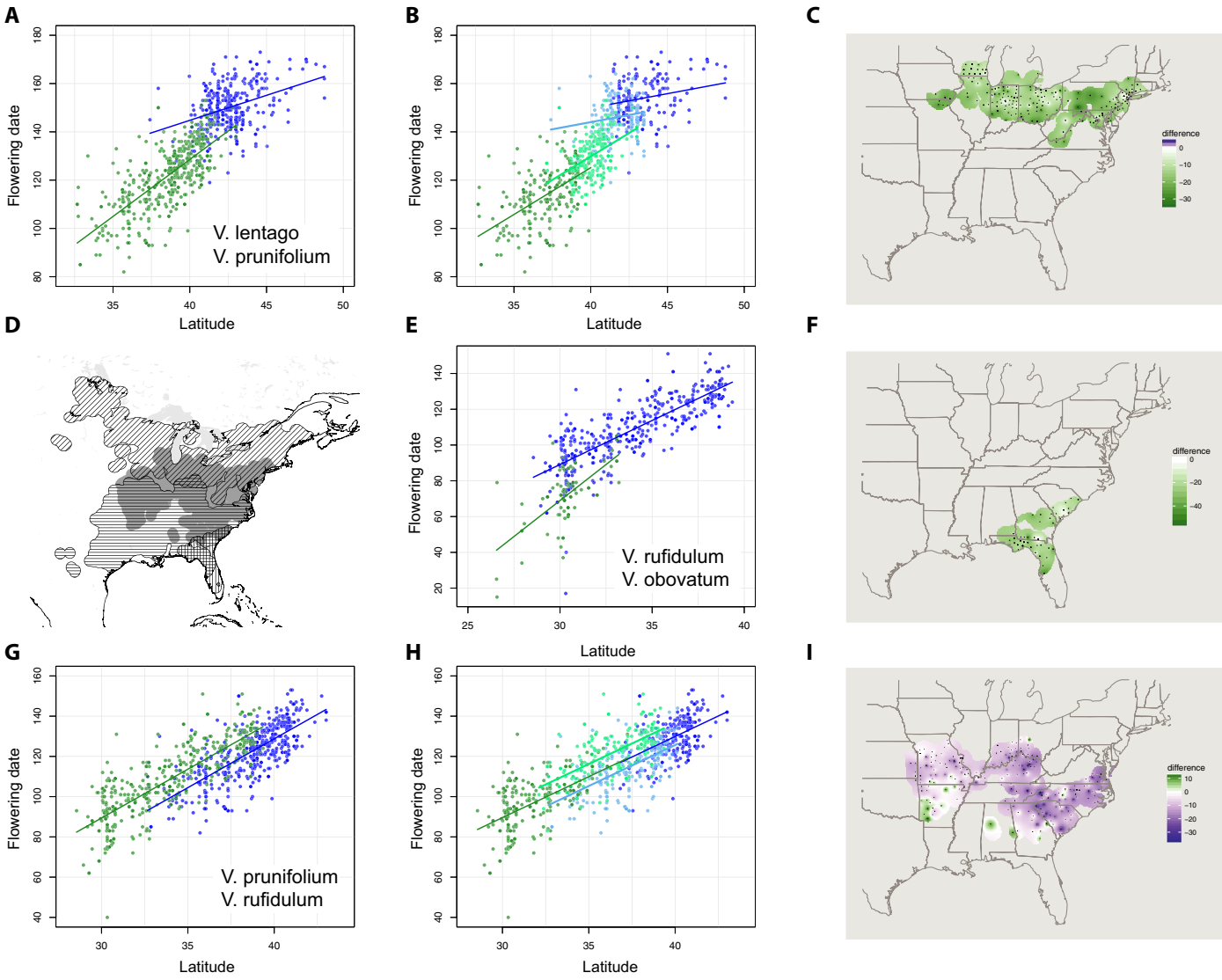

FIGURE 5. Within each species there is a significant relationship between latitude and flowering time. Analyses for each species pair are displayed with the more northern species in blue and the more southern species in green. When applicable, sympatric regions are indicated by lighter shades of the same colors. Points indicate locations of flowering specimens and linear models plotted in each panel correspond to specific lines of Table 1. (A–C) Analyses of *Viburnum lentago* (blue) and *V. prunifolium* (green). (A) All flowering records for *V. lentago* and *V. prunifolium*. (B) Flowering time for *V. lentago* and *V. prunifolium* separated into regions where the species are sympatric (light blue, light green) and allopatric (blue, green). (C) Where *V. lentago* and *V. prunifolium* are sympatric, *V. prunifolium* flowers earlier. Points indicate the locations of flowering specimens, and shading ranges from green (*V. prunifolium* flowers first) to white (no difference) to blue (*V. lentago* flowers first). (D) Range maps for all four species: diagonal lines mark *V. lentago*, solid gray marks *V. prunifolium*, horizontal lines mark *V. rufidulum*, and vertical lines mark *V. obovatum*. (E–F) Analyses of *V. rufidulum* (blue) and *V. obovatum* (green). (E) All flowering records for *V. rufidulum* and *V. obovatum*. (F) Where *V. rufidulum* and *V. obovatum* are sympatric, *V. obovatum* flowers earlier. Points indicate the locations of flowering specimens, and shading ranges from green (*V. obovatum* flowers first) to white (no difference) to blue (*V. rufidulum* flowers first). (G–I) Analyses of *V. prunifolium* (blue) and *V. rufidulum* (green). (G) All flowering records for *V. prunifolium* and *V. rufidulum*. (H) Flowering time for *V. prunifolium* and *V. rufidulum* separated into regions where the species are sympatric (light blue, light green) and allopatric (blue, green). (I) Where *V. prunifolium* and *V. rufidulum* are sympatric, *V. prunifolium* flowers earlier. Points indicate the locations of flowering specimens, and shading ranges from green (*V. rufidulum* flowers first) to white (no difference) to blue (*V. prunifolium* flowers first).

Population structure

In three of the species, we identified population structure along an east-west axis. In *V. lentago*, our sampling in the middle portion of the range is insufficient to fully describe the split; however, in *V. prunifolium* there is differentiation on the two sides of the Appalachian Mountains, and in *V. rufidulum* on the two sides of the Mississippi River. Both of these splits are correlated with intervening areas of unsuitable habitat. For *V. prunifolium* high-elevation

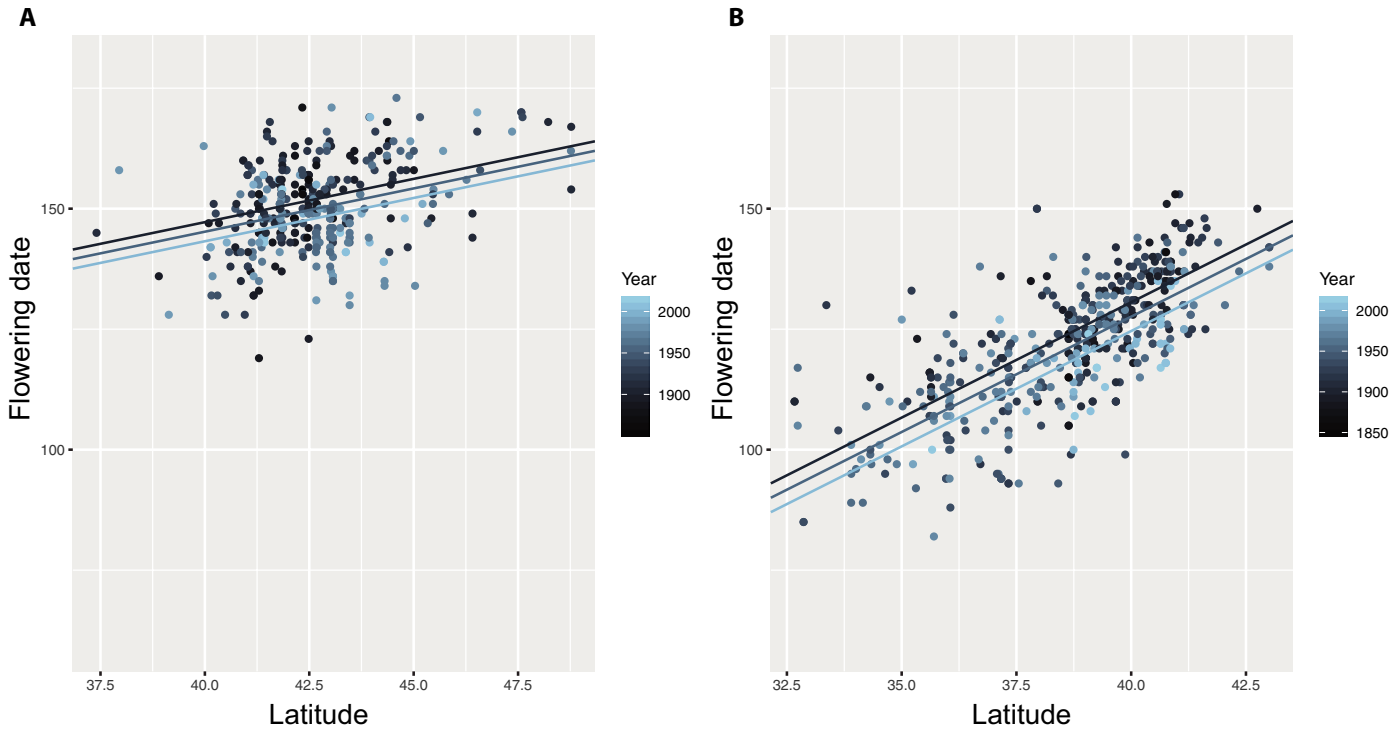

FIGURE 6. Average flowering time in *Viburnum lentago* and *V. prunifolium* had advanced with climate change. Trend lines reflect either the overall relationship between latitude and flowering time (*V. obovatum* and *V. rufidulum*) or for *V. lentago* and *V. prunifolium*, the relationship at three contrasting time points: the years 1900 (black), 1950 (dark blue) and 2000 (light blue).

regions of the Appalachians are too cold, and for *V. rufidulum* the lowland forests that border the Mississippi River are too wet. Both of these discontinuities are common North American phylogeographic patterns that have been identified in other plant and animal taxa (Soltis et al., 2006).

Range maps and historical distributions

Distribution models for the core *Lentago* clade predict that the three northern species have undergone large range shifts since the LGM. *V. prunifolium* and *V. rufidulum* are each predicted to have occupied a rather wide, more or less continuous band along the coastal plain during the LGM (Fig. 3). These distributions seem plausible given the few known occurrences of *Viburnum* pollen at that time (neotomaDB.org; Spriggs et al., 2019a), but they do not provide clear insight into how the east-west population structure arose. One possibility is that the structure originated when populations were forced into disjunct refugia within these ranges. Our distribution models likely overpredict the available area at the LGM because they do not include soil, microhabitat, or biotic information. Alternatively, the structure might have arisen as populations migrated north after the LGM, or it might have arisen because of natural barriers within the species' ranges such as rivers or drainage systems (Soltis et al., 2006).

In contrast to the northern species, the ranges of the two southern species, *V. elatum* and *V. obovatum*, appear to have undergone little change since the LGM. The climate tolerances of these two species appear to be relatively similar, and models for each species identify parts of the range of the other species as potentially suitable. The distribution models for *V. elatum*, however, are relatively imprecise because they are based on few occurrence records, and the steep topography throughout its range in Mexico adds considerable variance to the estimates.

Diversification and gene flow in the core *Lentago* clade

Most of the speciation events in the diversification of the core *Lentago* clade appear to represent north-south divisions. For instance, the earliest split in the clade separates the northern *V. lentago* from the remaining species, all of which occur to its south. Likewise, the *V. prunifolium*–*V. rufidulum* split occurred along a north-south axis. The only exception to this pattern is the east-west split between *V. elatum* and *V. obovatum*, which have entirely disjunct ranges (Fig. 1). Within all species, southern populations flower earlier than northern ones, and we might expect to see corresponding north-south population structure. However, the within-species population structure that we have identified in all cases is oriented east-west. We hypothesize that the east-west structure was driven by historical events, most likely related to range contraction and expansion during glacial cycles (not necessarily the LGM).

Why, then, have speciation events in the *Lentago* clade occurred primarily along a north-south axis rather than along an east-west axis? At first glance, it seems most likely that speciation patterns would match patterns of within-species genetic structure; that is, divergent populations might eventually become separate species. However, it is important to appreciate that the geographic ranges of species in the core *Lentago* clade have changed considerably, and that only population divisions that are associated with both the evolution of different climate tolerances and strong reproductive

barriers (i.e., different flowering times; see below) are likely to have resulted in the origin of species.

The climatic differences associated with north-south splits are stronger in eastern North America than those associated with east-west splits. Each of the five species in the core *Lentago* clade occupies a different environment with respect to temperature, but also probably with respect to soil type and/or microhabitat. Our experience in the field suggests that all species can be found at the borders of streams or mesic woods, but *V. lentago* in the north commonly occurs in extremely wet places such as bogs or pond margins, *V. prunifolium* commonly occupies limestone regions, *V. rufidulum* tends to occur in warmer but more xeric woodlands, and *V. obovatum* in drier sandy soils along river banks. For both temperature and microhabitat, the differences among the species are subtle and there is significant overlap between them. The north-south range limits of the species, however, appear to be constrained by climatic tolerances as opposed to geographic barriers. Our distribution models for each of the species in the core *Lentago* clade are north-south restricted and predict no potentially suitable habitat north of known occurrences (Fig. 3). By contrast, east-west population divisions are clearly correlated with geographic barriers, and the climatic differences between the eastern and western portions of the ranges appear to be minor. Species distribution models based on observations in only the eastern or the western portions of the ranges also identify the other half of the range as suitable (Appendix S10). Species that are separated along the north-south axis appear to be adapted to different climates, and, consequently, hybrids between them may have lower fitness than east-west hybrids, in which case reinforcement to maintain the separation is more likely for north-south pairs.

Stronger reproductive barriers are also more likely to exist in north-south splits than in east-west splits. The only east-west speciation event in the core *Lentago* clade is the one involving *V. obovatum* and *V. elatum*. These species have disjunct ranges that have probably been maintained since the drying that created the plains habitat of southern Texas (Prothero, 1998). For the rest of the species, it seems that barriers separating eastern and western populations are not strong enough, or have not persisted long enough to lead to speciation. By contrast, as we argue below, in north-south divisions, local adaptation lead naturally to phenological shifts, which would in turn provide the basis for speciation. When previously isolated populations come back into contact, their flowering times might already be different or, as we will argue below for the *V. prunifolium*–*V. rufidulum* split, hybridization with negative consequences might lead to reinforcement and the evolution of staggered flowering times.

Phenology and latitude

Flowering time varied by 54–134 d within species, but most of this variation is geographically structured such that southern populations flower predictably earlier than northern ones. While herbarium specimens are an excellent resource for understanding flowering time, particularly long-term trends in flowering time (Primack et al., 2004; Panchen et al., 2012; Calinger et al., 2013; Matthews and Mazer, 2016; Munson and Long, 2017; Willis et al., 2017), there are many potential sources of noise in these datasets (Daru et al., 2017), and a large number of specimens are needed to detect significant patterns. Fortunately, most of the species in the core *Lentago* clade are common in herbaria, and there appears to be little temporal or geographic collecting bias, although (as is often the case) most specimens in our dataset were collected relatively near major population centers (Appendices S1, S2). The Mexican species, *V. elatum*, is the clear exception; here we would need ≥100 additional flowering specimens to conduct similar analyses.

The slope of the relationship between latitude and flowering time varies significantly among species; it is steepest in the southern *V. obovatum*, intermediate in *V. rufidulum* and *V. prunifolium*, and shallowest in the northern *V. lentago*. We focused on latitude for these analyses because it is a convenient proxy for several aspects of climate that have been identified as important in regulating phenology in other temperate plant lineages, namely spring temperatures, vernalization, and day length (Körner and Basler, 2010; Polgar et al., 2014). The relative importance of these environmental factors for flowering in *Viburnum* is not fully established, but evidence from multiyear observations at the Arnold Arboretum in Massachusetts implicate spring temperatures as the most important component (Donoghue, 1980; L. M. Garrison et al., unpublished data). The latitudinal trends we observe are also correlated with spring temperature variability, which might be particularly important in the evolution of species-level differences in flowering time. Zohner et al. (2017) observed greater chilling requirements and later leaf-out dates in species adapted to regions with high levels of spring temperature variability, and we see a parallel trend: the species that occurs in the region with the highest spring temperature variability (*V. lentago*) flowers later than more southern species, even where they are sympatric. Adaptation to different levels of temperature variability could be a mechanism that underlies the "incidental" differences that we observe among species.

Phenology and species interactions

In all three zones of geographic overlap between species in the core *Lentago* clade, we estimate that flowering times are offset between the species pairs by ≥9 d. In two of the cases (*V. lentago* and *V. prunifolium*; *V. rufidulum* and *V. obovatum*) the more southern species flowers many days ahead of the northern species (13.4 and 17.1 d, respectively). In these pairs, the two species appear to have evolved different relationships between latitude and flowering time (Fig. 5), and this translates into different flowering times where they are sympatric. In these cases, the simplest explanation appears to be that the offset in flowering times is an incidental consequence of the adaptation of these non-sister species to different climatic regimes.

In the case of the *V. prunifolium*–*V. rufidulum* sister species pair, however, both species seem to have shifted their flowering times in the zone of sympatry, with the result that the northern species, *V. prunifolium*, actually flowers first. Based only on their allopatric occurrence records, the flowering times of *V. prunifolium* and *V. rufidulum* are not significantly different, and they would be predicted to flower concurrently in sympatry. Instead, we estimate that *V. prunifolium* has shifted to flowering ~4 d earlier, while *V. rufidulum* has shifted to flowering ~6 d later. Thus, where they co-occur there is, on average, a 10 d difference in flowering time. This pattern of character displacement (*sensu* Stuart et al., 2017) might arise through reinforcement to reduce hybridization, through competition for pollinators, or via ecological displacement if the species occupy different habitats in their area of sympatry. In general, these processes are not mutually exclusive and several may be operating simultaneously (Pfennig and Pfennig, 2009).

We argue that the differentiation of *V. prunifolium* and *V. rufidulum* is a case of reproductive character displacement driven by reinforcement. Our evidence suggests that hybridization between these species can and does occur in the places where they are sympatric, but that the hybrids have a low fitness (and may even be sterile). Low hybrid fitness is suggested by the observation that hybrid individuals are rare and that there is little or no backcrossing or gene flow with the parental species. We find that both species have shifted their reproductive timing in the zone of sympatry, and we think that this is most likely due to selection to avoid wasteful hybridization. It is unlikely that this differentiation is driven by competition for pollinators, because these species do not have specialized pollinators, and, because these *Viburnum* species are rarely abundant, competition for pollinators is probably far greater with distantly related plants (e.g., *Cornus* species). We are also confident that *V. prunifolium* and *V. rufidulum* are not flowering at different times only because they are living in different microhabitats within the region of sympatry (a phenomenon we suggested elsewhere for *V. nudum* and *V. nitidum*; Spriggs et al., 2019a). Two populations that contained intermingled individuals of both species were observed at different reproductive stages in Ohio and Kentucky in April 2015 (E. L. Spriggs, personal observation). Flowering times were clearly separated in these populations, with *V. prunifolium* approximately a week further along in floral development than *V. rufidulum* (*V. prunifolium* inflorescences were fully expanded or in flower, while *V. rufidulum* inflorescences were only partially expanded with green flower buds). Differences in flowering time between these species are maintained in cultivated plants at the Arnold Arboreum (Donoghue, 1982; L. M. Garrison et al., unpublished data) and were also observed in wild populations in North Carolina (Rader, 1976). This system fits the most common definition of reinforcement (*sensu* Howard, 1993; Servedio and Noor, 2003; Hopkins, 2013), although narrower definitions that require evidence of ongoing or past introgessive hybridization between species might favor "reproductive character displacement" for this particular example (Butlin, 1987).

We do not expect there to be entirely non-overlapping flowering periods in these *Viburnum* species in every location in every year, and there are clearly other mechanisms at work that promote isolation, including low fruit set in hybrid crosses (Egolf, 1956). Indeed, it is likely that particular climatic circumstances will occasionally create overlap in flowering time. However, our data indicate that normally the vast majority of the individuals of *V. prunifolium* will flower ahead of *V. rufidulum*, and that this offset will reduce gene flow between them. Comprehensive studies of reproductive isolation in other systems have found that even minor phenological differences have a significant effect on isolation (McNeilly and Antonovics, 1968; Ostevik et al., 2016). Our case is, by comparison, an extreme example in which flowering times appear to be almost completely distinct.

Phenology as an isolating mechanism

Similar differences in flowering time between closely related species have been observed elsewhere in *Viburnum*. In the montane Neotropical *Oreinotinus* clade, Donoghue (1982) described two cases in which species appear to have shifted from the typical summer flowering time to a winter flowering time in regions where they are sympatric with close relatives (*V. blandum* in relation to *V. jucundum* in southern Mexico; *V. venustum* in relation to *V. stellato-tomentosum* and *V. costaricanum* in Costa Rica). Phenological differentiation (together with specialization on different soil types) has also been proposed as an isolating mechanism in the case of *V. nudum* and *V. nitidum* in the southeastern United States (Spriggs et al., 2019a). Such phenological isolation might be a particularly effective mechanism in *Viburnum* because the individual flowering period is predictably short, and a shift in flowering time of just 10–14 d would completely eliminate interspecific pollen transfer in most cases.

It seems likely that species isolation mechanisms will vary predictably—and phylogenetically—depending on whether temporal or pollinator based reproductive switches are easier to evolve. Pollinator shifts seem to evolve readily in lineages such as *Penstemon* (Wilson et al., 2006), *Iochroma* (Smith et al., 2008), *Gladiolus* (Valente et al., 2012), *Aquilegia* (Hodges and Arnold, 1995), and *Disa* (Johnson and Steiner, 1997). However, many lineages are evidently far less likely to evolve major shifts in pollinators and floral morphology. *Viburnum* appears to be a case in point, as there are only a few major differences in flower morphology and little specialization for particular pollinators, but potentially multiple instances of temporal isolation. At the extreme end of the spectrum, wind-pollinated lineages need to rely on temporal separation to avoid interspecific pollen transfer. Indeed, several of the best-known examples of flowering-time divergence are in wind-pollinated clades (McNeilly and Antonovics, 1968; Savolainen et al., 2006). It is also noteworthy that in many cases, differences in flowering phenology are associated with ecological differentiation, and there appears to be a general pattern in which populations on warmer, drier soils tend to flower first (McNeilly and Antonovics, 1968). Evidence suggests that under these circumstances, differentiation associated with distinct flowering times can evolve over very short distances and despite gene flow (McNeilly and Antonovics, 1968; Savolainen et al., 2006; Papadopulos et al., 2011). There are also many descriptions of ecotypes that are adapted to coastal or alpine areas and flower at different times (e.g., Turesson, 1925; Clausen and Hiesey, 1958). This raises the possibility that ecotypes with phenological differences are more likely to become separate species, perhaps through later reinforcement in areas of sympatry. In this way, minor shifts in flowering time associated with different soil or environmental conditions could end up playing a large role in generating species diversity in heterogeneous environments.

Phenology and climate change

A combination of distinct climatic tolerances and staggered flowering times has enabled species in the core *Lentago* clade to remain distinct despite a recent history of range shifts in relation to glaciation and many opportunities for contact. Through these mechanisms, the core *Lentago* clade has diversified across a continuous area, dividing up eastern North America by differentiating along the major north-south climatic axis.

Anthropogenic climate change is already changing flowering phenology in many plants (Fitter and Fitter, 2002; Wolkovich et al., 2012; Ellwood et al., 2013; Matthews and Mazer, 2016; Munson and Long, 2017), and these changes have the potential to drastically alter patterns of intraspecific and interspecific gene flow (Prevéy et al., 2017). In the case of the core *Lentago* clade, we have documented significant changes in flowering phenology in the northern-most species over the past century. However, given the current sequence of flowering times and the particular shifts that are occurring, it appears unlikely that continuing climate change will create new opportunities for interbreeding and the breakdown

of species boundaries in this group. Instead, our analyses suggest that increased warming will cause both *V. lentago* and *V. prunifolium* to flower earlier, probably preserving the relative difference in their flowering times. In areas where *V. rufidulum* and *V. prunifolium* co-occur, *V. rufidulum* seems likely to maintain its current pattern of flowering while *V. prunifolium* will probably flower even earlier, thus accentuating the displaced flowering times of these sister species.

ACKNOWLEDGEMENTS

This work was supported by National Science Foundation (NSF) grants IOS-1256706 to M.J.D., IOS-1257262 to E.J.E., DGE 1122492 to E.L.S., and NSF 1501188 to E.L.S. and M.J.D. Additional funding was provided by the Torrey Botanical Society, the American Society of Plant Taxonomists, the Yale Peabody Museum of Natural History, and the Yale Institute for Biospheric Studies. We thank Bruce Sorrie, Alan Weakley, Miranda Sinnott-Armstrong, and the Donoghue-Near lab group for helpful discussions. We are also grateful to Jannice Friedman and two anonymous reviewers for their thoughtful comments on the manuscript. We are also grateful to the Florida State Parks, the North Carolina State Parks, the Illinois State Parks, the Minnesota State Parks, Tall Timbers Research Station, Ordway-Swisher Biological Station, the Cloquet Forestry Center, and the Kickapoo Valley Reserve for assistance collecting. Finally, we thank several herbaria for providing specimen images or occurrence data through in-person visits or online databases. The Index Herbariorum herbarium codes (Thiers, 2018) or names of these herbaria are as follows: A, ACAD, B, Black Rock Forest Consortium Herbarium, BM, BRIT, CAN, CAS, CBS, CHAP, CHIP, CIB, CICY, CIMI, CONN, CS, E, ENCB, F, FCME, FITECMA, FLAS, FSU, GH, HEM, IEB, IND, KHD, KR, KU, LEA, LSU, M, MA, MEXU, MIL, MO, MSC, MT, NCU, NEBC, NY, OS, QFA, QMEX, TEX, TRT, TTRS, UBC, UNA, US, USF, W, WIN, WIS, XAL, YU, ZEA.

AUTHOR CONTRIBUTIONS

E.L.S., M.J.D., and E.J.E. designed the study. E.L.S. and C.S. collected the data. E.L.S., D.A.E., B.P., and P.W.S. analyzed the data. All the authors contributed to writing the manuscript.

DATA ACCESSIBILITY

Sequences generated in this study are available from the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra). Alignments of filtered loci and all phenology datasets are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.t7r6kg5 (Spriggs et al., 2019b).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Histogram of collection year for all herbarium specimens used to score phenology.

APPENDIX S2. Map of the counties where herbarium specimens used to score phenology were collected.

APPENDIX S3. Results of a joint structure analysis for *V. prunifolium* and *V. rufidulum* highlight two admixed individuals, but little other introgression across a large area of range overlap.

APPENDIX S4. Phylogenetic networks inferred for each of five datasets using PhyloNet.

APPENDIX S5. Population structure within *V. lentago*, *V. prunifolium*, and *V. rufidulum*.

APPENDIX S6. Species distribution models based on nine climatic variables predict differences between current and LGM distributions of species in the core *Lentago* clade.

APPENDIX S7. Full linear model results for Table 1.

APPENDIX S8. Table identical to Table 1 except that it is based only on specimens dated before 1950.

APPENDIX S9. Morphological variation among species in the core *Lentago* clade.

APPENDIX S10. Species distribution models based on only the eastern or western portion of the species.

LITERATURE CITED

Benedikt, G., E. Pebesma, and G. Heuvelink. 2016. Spatio-temporal interpolation using gstat. *The R Journal* 8: 204–218.

Brumbaugh, J. H., and A. T. Guard. 1956. A study of evidence of introgession among *Viburnum lentago*, *V. prunifolium* and *V. rufidulum* based on leaf characteristics. *Indiana Acadamy of Sciences* 66: 300.

Butlin, R. 1987. Speciation by reinforcement. *Trends in Ecology and Evolution* 2: 8–13.

Calinger, K. M., S. Queenborough, and P. S. Curtis. 2013. Herbarium specimens reveal the footprint of climate change on flowering trends across north-central North America. *Ecology Letters* 16: 1037–1044.

Clausen, J. W., and M. Hiesey. 1958. Experimental studies on the nature of species. IV. Genetic structure of ecological rases. Carnegie Institution of Washington Publication 615.

Clement, W. L., and M. J. Donoghue. 2011. Dissolution of *Viburnum* section Megalotinus (Adoxaceae) of Southeast Asia and its implications for morphological evolution and biogeography. *International Journal of Plant Sciences* 172: 559–573.

Clement, W. L., M. Arakaki, P. W. Sweeney, E. J. Edwards, and M. J. Donoghue. 2014. A chloroplast tree for *Viburnum* (Adoxaceae) and its implications for phylogenetic classification and character evolution. *American Journal of Botany* 101: 1029–1049.

Daru, B. H., D. S. Park, R. B. Primack, C. G. Willis, D. S. Barrington, T. J. S. Whitfeld, T. G. Seidler, et al. 2017. Widespread sampling biases in herbaria revealed from large-scale digitization. *New Phytologist* 2017: 939–955.

Dell'Olivo, A., M. E. Hoballah, T. Gübitz, and C. Kuhlemeier. 2011. Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). *Evolution* 65: 1979–1991.

Donoghue, M. J. 1980. Flowering times in Viburnum. *Arnoldia* 40: 2–22.

Donoghue, M. J. 1982. Systematic studies in the genus *Viburnum*. Biology. Harvard University.

Donoghue, M. J., B. G. Baldwin, J. Li, and R. C. Winkworth. 2004. *Viburnum* phylogeny based on chloroplast *trnK* intron and nuclear riboxomal ITS DNA sequences. *Systematic Botany* 29: 188–198.

Doyle, J., and J. L. Doyle. 1987. Genomic plant DNA preparation from fresh tissue–CTAB method. *Phytochemical Bulletin* 19: 11–15.

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

Eaton, D. A. R. 2014. PyRAD: assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics* 30: 1844–1849.

Eaton, D. A. R., and R. H. Ree. 2013. Inferring phylogeny and introgression using RADseq data: an exaple from flowering plants (*Pedicularis*: Orobanchaceae). *Systematic Biology* 62: 689–706.

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

Eaton, D. A. R., E. L. Spriggs, B. Park, and M. J. Donoghue. 2017. Misconceptions on missing data in RAD-seq phylogenetics with a deep-scale example from flowering plants. *Systematic Biology* 66: 399–412.

Egolf, D. R. 1956. Cytological and interspecific hybridization studies in the genus *Viburnum*. Cornell University.

Egolf, D. R. 1962. A cytological study of the genus *Viburnum*. *Journal of the Arnold Arboretum* 43: 132–172.

Ellwood, E. R., S. A. Temple, R. B. Primack, N. L. Bradley, and C. C. Davis. 2013. Record-breaking early flowering in the Eastern United States. *PloS One* 8: e53788.

Evanno, G., S. Regnaut, and nad J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14: 2611–2620.

Fitter, A. H., and R. S. R. Fitter. 2002. Rapid changes in flowering time in British plants. *Science* 296: 1689–1691.

Hijmans, R. J., S. Phillips, J. Leathwick, and J. Elith. 2017. dismo: species distribution modeling. R package version 1.1-4.

Hodges, S. A., and M. L. Arnold. 1995. Spurring plant diversification: are floral nectar spurs a key innovation? *Proceedings of the Royal Society of London B, Biological Sciences* 262: 343–348.

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

Howard, D. J. 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. *In* R. G. Harrison, ed. Hybrid Zones and the Evolutionary Process, 46–69, Oxford University Press.

Huang, H.-R., P.-C. Yan, M. Lascoux, and X.-J. Ge. 2012. Flowering time and transcriptome variation in *Capsella bursa-pastoris* (Brassicaceae). *New Phytologist* 194: 676–689.

Hurlbert, S. H. 1970. Flower number, flowering time, and reproductive isolation among ten species of Solidago (Compositae). *Journal of the Torrey Botanical Society* 97: 189–195.

Husband, B. C., and D. W. Schemske. 2000. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology* 88: 689–701.

Jakobsson, M., and N. A. Rosenberg. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23: 1801–1806.

Johnson, S. D., and K. E. Steiner. 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* 51: 45–53.

Jones, T. H. 1983. A revision of the genus *Viburnum* section Lentago (Caprifoliaceae). Ph.D. North Carolina State University at Raleigh.

Kollmann, J., and M. J. Bañuelos. 2004. Latitudinal trends in growth and phenology of the invasive alien plant *Impatiens glandulifera* (Balsaminaceae). *Diversity and Distributions* 10: 377–385.

Körner, C., and D. Basler. 2010. Phenology under global warming. *Science* 327: 1461–1462.

Levin, D. A. 1985. Reproductive character displacement in Phlox. *Evolution* 39: 1275–1281.

Little, E. L. 1971. Atlas of the United States trees. U.S. Department of Agriculture Miscellaneous Publication 991: 1–97.

Lowry, D. B., R. C. Rockwood, and J. H. Willis. 2008. Ecological reproductive isolation of coast and inland races of *Mimulus guttatus*. *Evolution* 62: 2196–2214.

Matthews, E. R., and S. J. Mazer. 2016. Historical changes in flowering phenology are governed by temperature × precipitation interactions in a widespread perennial herb in western North America. *New Phytologist* 210: 157–167.

McAtee, W. L. 1956. A review of the Neartic Viburnum. Privately printed, Chapel Hill, NC.

McMillan, C. 1959. The role of ecotypic variation in the distribution of the central grassland of North America. *Ecological Monographs* 29: 285–308.

McNeilly, T., and J. Antonovics. 1968. Evolution in closely adjacent plant populations. *Heredity* 23: 205–218.

Montesinos-Navarro, A., J. Wig, F. X. Pico, and S. J. Tonsor. 2011. *Arabidopsis thaliana* populations show clinal variation in a climatic gradient associated with altitude. *New Phytologist* 189: 282–294.

Munson, S. M., and A. L. Long. 2017. Climate drives shifts in grass reproductive phenology across the western USA. *New Phytologist* 213: 1945–1955.

Oersted, A. S. 1861. Til belysning af slaegten *Viburnum*. *Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening I Kjobenhavn* 13: 267–305.

Olsson, K., and J. Ågren. 2002. Latitudinal population differentiation in phenology, life history and flower morphology in the perennial herb *Lythrum salicaria*. *Journal of Evolutionary Biology* 15: 983–996.

Ostevik, K. L., R. L. Andrew, S. P. Otto, and L. H. Rieseberg. 2016. Multiple reproductive barriers separate recently diverged sunflower ecotypes. *Evolution* 70: 2322–2335.

Panchen, Z. A., R. B. Primack, T. Anisko, and R. E. Lyons. 2012. Herbarium specimens, photographs, and field observations show Philadelphia area plants are responding to climate change. *American Journal of Botany* 99: 751–756.

Papadopulos, A. S. T., W. J. Baker, D. Crayn, R. K. Butlin, R. G. Kynast, I. Hutton, and V. Savolainen. 2011. Speciation with gene flow on Lord Howe Island. *Proceedings of the National Academy of Sciences, USA* 108: 13188–13193.

Pardi, F., and C. Scornavacca. 2015. Reconstructible phylogenetic networks; do not distinguish the indistinguishable. *PloS Computational Biology* 11: e1004135.

Park, D. S., and C. C. Davis. 2017. Implications and alternatives of assigning climate data to geographical centroids. *Journal of Biogeography* 44: 2188–2198.

Pfennig, K. S., and D. W. Pfennig. 2009. Character displacement: ecological and reproductive responses to a common evolutionary problem. *The Quarterly Review of Biology* 84: 253–276.

Phillips, S., R. P. Anderson, and R. E. Schapire. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* 190: 231–259.

Polgar, C., A. Gallinat, and R. B. Primack. 2014. Drivers of leaf-out phenology and their implications for species invations: insights from Thoreau's Concord. *New Phytologist* 202: 106–115.

Prendeville, H. R., K. B. Barnard-Kubow, C. Dai, B. C. Barringer, and L. F. Galloway. 2013. Clinal variation for only some phenological traits across a species range. *Oecologia* 173: 421–430.

Prevéy, J., M. Vellend, N. Rüger, R. D. Hollister, A. D. Bjorkman, I. H. Myers-Smith, S. C. Elmendor, et al. 2017. Greater temperature sensitivity of plant phenology at colder sites: implications for convergence across northern latitudes. *Global Change Biology* 23: 2660–2671.

Primack, D., C. Imbres, R. B. Primack, A. J. Miller-rushing, and P. Del Tredici. 2004. Herbarium specimens demonstrate earlier flowering times in response to warming in Boston. *American Journal of Botany* 91: 1260–1264.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.

Prothero, D. R. 1998. The chronostratigraphic, paleogeographic and paleoclimatic background to North American mammalian evolution. *In* C. Janis, M. Scott, and L. Jacobs, eds. Evolution of tertiary mammals of North America. Cambridge University Press, Cambridge.

R Core Team. 2017. R: a language and environmental for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.

Rader, L. L. 1976. A biosystematic study of Viburnum prunifolium and Viburnum rufidulum (Caprifolicaeae). University of Tennessee, Knoxville.

Ramsey, J., H. D. J. Bradshaw, and D. W. Schemske. 2003. Components of reproductive isolation between the Monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* 57: 1520–1534.

Rieseberg, L. H., and D. E. Solits. 1991. Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants*, 5(1): 65–84.

Rieseberg, L. H., and J. H. Willis. 2007. Plant speciation. *Science* 317: 910–914.

Roch, S., M. Nute, and T. Warnow. 2018. Long-branch attraction in species tree estimation: inconsistency of partitioned likelihood and topology-based summary methods. arXiv:1803.02800 [q-bio.PE].

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Runquist, R. B. B., E. Chu, J. L. Iverson, J. C. Kopp, and D. A. Moeller. 2014. Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution* 68: 2885–2900.

Savolainen, V., M.-C. Anstett, C. Lexer, I. Hutton, J. J. Clark, M. V. Norup, M. P. Powell, et al. 2006. Sympatric speciation in palms on an oceanic island. *Nature* 44: 210–213.

Schemske, D. W., and H. D. J. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the Indiana Academy of Science* 96: 11910–11915.

Servedio, M. R., and M. A. F. Noor. 2003. The role of reinforcement in speciation: theory and data. *Annual Review of Ecology Evolution and Systematics* 34: 339–364.

Smith, S. D., C. Ané, and D. A. Baum. 2008. The role of pollinator shifts int he floral diversification of *Iochroma* (Solanaceae). *Evolution* 62: 793–806.

Solís-Lemus, C., and abd C. Ané. 2016. Inferring phylogenetic networks with maximum psuedolikelihood under incomplete lineage sorting. *PLoS Genetics* 12: e1005896.

Soltis, D. E., A. B. Morris, J. S. McLachlan, P. S. Manos, and P. S. Soltis. 2006. Comparative phylogeography of unglaciated eastern North America. *Molecular Ecology* 15: 4261–4293.

Spriggs, E. L., W. L. Clement, P. W. Sweeney, S. Madriñán, E. J. Edwards, and M. J. Donoghue. 2015. Temperate radiations and dying embers of a tropical past: the diversification of *Viburnum*. *New Phytologist* 207: 340–354.

Spriggs, E. L., D. A. R. Eaton, P. W. Sweeney, C. Schlutius, E. J. Edwards, and M. J. Donoghue. 2019a. RAD-seq data reveal a cryptic *Viburnum* species on the North American Coastal Plain. *Systematic Biology* 68: 187–203.

Spriggs, E. L., C. Schlutius, D. A. Eaton, B. Park, P. W. Sweeney, E. J. Edwards, and M. J. Donoghue. 2019b. Data from: Differences in flowering time maintain species boundaries in a continental radiation of *Viburnum*. *Dryad Digital Repository*. https://doi.org/10.5061/dryad.t7r6kg5.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313.

Stenz, N. W., B. Larget, D. A. Baum, and C. Ané. 2015. Exploring tree-like and non-tree-like patterns using genome sequences: an example using the inbreeding plant species *Arabidopsis thaliana* (L.) Heynh. *Systematic Biology* 64: 809–823.

Stuart, Y. E., S. A. Inkpen, R. Hopkins, and D. I. Bolnick. 2017. Character displcaement is a pattern: so what causes it? *Biological Journal of the Linnean Society* 121: 711–715.

Tang, J., C. Körner, H. Muraoka, S. Piao, M. Shen, S. Thackeray, and X. Yang. 2016. Emerging opportunities and challences in phenology: a review. *Ecosphere* 7: e01436.

Than, C., D. Ruths, and L. Nakhleh. 2008. PhyloNet: a software package for analyzing and reconstructing reticulate evolutionary histories. *BMC Bioinformatics* 9: 322.

Thiers, B. 2018. Index Herbariorum: A global directory of pubic herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. http://sweetgum.nybg.org/science/ih/ (accessed 14 February 2018).

Turesson, G. 1925. The plant species in relation to habitat and climate. *Hereditas* 6: 147–236.

Turesson, G. 1930. The selective effect of climate upon the plant species. *Hereditas* 14: 99–152.

Valente, L. M., J. C. Manning, P. Goldblatt, and P. Vargas. 2012. Did pollination shifts drive diversification in Southern African *Gladiolus*? Evaluating the model of pollinator-driven speciation. *American Naturalist* 180: 83–98.

Weber, E., and B. Schmid. 1998. Latitudinal population differentiation in two species of *Solidago* (Asteraceae) introduced into Europe. *American Journal of Botany* 85: 1110–1121.

Wen, D., Y. Yu, J. Zhu, and L. Nakhleh. 2018. Inferring phylogenetic networks using PhyloNet. *Systematic Biology* 67: 735–740.

Willis, C. G., E. R. Ellwood, R. B. Primack, C. C. Davis, K. D. Pearson, A. S. Gallinat, J. M. Yost, et al. 2017. Old plants, new tricks: phenological research using herbarium specimens. *Trends in Ecology and Evolution* 32: 531–546.

Wilson, P., M. C. Castellanos, A. D. Wolfe, and J. D. Thomson. 2006. Shifts between bee and bird pollinaton in Penstemons. *In* N. M. Waser and J. Ollerton, eds. Plant-pollinator interactions: from specialization to generalization. The University of Chicago Press, Chicago, IL.

Winkworth, R. C., and M. J. Donoghue. 2004. *Viburnum* phylogeny: evidence from the duplicated nuclear gene *GBSSI*. *Molecular Phylogenetics and Evolution* 33: 109–126.

Winkworth, R. C., and M. J. Donoghue. 2005. *Viburnum* phylogeny based on combined molecular data: implications for taxonomy and biogeography. *American Journal of Botany* 92: 653–666.

Wolkovich, E. M., B. I. Cook, J. M. Allen, T. M. Crimmins, J. L. Betancourt, S. E. Travers, S. Pau, et al. 2012. Warming experiments underpredict plant phenological responses to climate change. *Nature* 485: 494.

Zohner, C. M., B. M. Benito, J. D. Fridley, J. C. Svennig, and S. S. Renner. 2017. Spring predictability explains different leaf-out strategies in the woody floras of North America, Europe and East Asia. *Ecology Letters* 20: 452–460.

APPENDIX 1. List of *Viburnum* material studied. Index Herbariorum (Thiers, 2018) abbreviations are in brackets.

***Viburnum prunifolium*:** Steven R. Hill 32451 (NY), E. L. Spriggs 460 (YU), E. L. Spriggs 510 (YU), E. L. Spriggs 520 (YU), E. L. Spriggs 521 (YU), E. L. Spriggs 476 (YU), E. L. Spriggs 490 (YU), E. L. Spriggs 475 (YU), E. L. Spriggs 478 (YU), Mark J. Loeschke 2593 (NY), Caleb A. Morse 11060 (NY), E. L. Spriggs 155 (YU), E. L. Spriggs 158 (YU), M. Nee 27324 (NY), E. L. Spriggs 500 (YU), E. L. Spriggs 491 (YU), E. L. Spriggs 498 (YU), E. L. Spriggs 484 (YU), E. L. Spriggs 514 (YU), E. L. Spriggs 525 (YU), E. L. Spriggs 455 (YU), E. L. Spriggs 363 (YU), E. L. Spriggs 361 (YU), E. L. Spriggs 367 (YU), E. L. Spriggs 360 (YU), E. L. Spriggs 347 (YU), E. L. Spriggs 57 (YU), E. L. Spriggs 341 (YU), E. L. Spriggs 332 (YU), E. L. Spriggs 445 (YU), E. L. Spriggs 632 (YU), E. L. Spriggs 430 (YU), E. L. Spriggs 283 (YU), E. L. Spriggs 286 (YU). ***Viburnum rufidulum*:** E. L. Spriggs 488 (YU), E. L. Spriggs 483 (YU), E. L. Spriggs 486 (YU), E. L. Spriggs 519 (YU), E. L. Spriggs 489 (YU), E. L. Spriggs 518 (YU), E. L. Spriggs 511 (YU), E. L. Spriggs 505 (YU), E. L. Spriggs 508 (YU), E. L. Spriggs 25 (YU), E. L. Spriggs 579 (YU), E. L. Spriggs 190 (YU), E. L. Spriggs 209 (YU), E. L. Spriggs 17 (YU), E. L. Spriggs 221 (YU), E. L. Spriggs 11 (YU), E. L. Spriggs 16 (YU), E. L. Spriggs 1 (YU), E. L. Spriggs 237 (YU), E. L. Spriggs 249 (YU), E. L. Spriggs 576 (YU), E. L. Spriggs 608 (YU), E. L. Spriggs 187 (YU), R. Dale Thomas 124,654 (NY), R. Dale Thomas 149,149 (NY), E. L. Spriggs 180 (YU), E. L. Spriggs 171 (YU), E. L. Spriggs 166 (YU). ***Viburnum obovatum*:** E. L. Spriggs 600 (YU), E. L. Spriggs 595 (YU), E. L. Spriggs 257 (YU), E. L. Spriggs 258 (YU), E. L. Spriggs 243 (YU), Paul Fortsch & Nancy Edmonson 119 (NY), John R. MacDonald 10052 (NY). ***Viburnum elatum*:** M. J. Donoghue 2 (A), M. J. Donoghue 30 (A), P. W. Sweeney 3063 (YU), P. W. Sweeney 3084 (YU), M. J. Donoghue 277 (A), M. J. Donoghue 75 (A). Rzedowski 31881 (LL). ***Viburnum lentago*:** Peter Leseca & Joe Elliott 1113 (NY), E. L. Spriggs 112 (YU), E. L. Spriggs 133 (YU), E. L. Spriggs 124 (YU), D. Sutherland 2894 (NY), E. L. Spriggs 145 (YU), E. L. Spriggs 104 (YU), E. L. Spriggs 139 (YU), E. L. Spriggs 149 (YU), E. L. Spriggs 101 (YU), E. L. Spriggs 647 (YU), E. L. Spriggs 85 (YU), E. L. Spriggs 649 (YU), E. L. Spriggs 432 (YU), E. L. Spriggs 428 (YU), E. L. Spriggs 73 (YU), E. L. Spriggs 401 (YU), E. L. Spriggs 412 (YU). ***Viburnum nitidum*:** E. L. Spriggs 252 (YU), E. L. Spriggs 2 (YU). ***Viburnum cassinoides*:** E. L. Spriggs 309 (YU), E. L. Spriggs 542 (YU). ***Viburnum nudum*:** E. L. Spriggs 607 (YU), E. L. Spriggs 621 (YU). ***Viburnum prunifolium* × *Viburnum rufidulum hybrids*:** E. L. Spriggs 523 (YU), E. L. Spriggs 524 (YU).