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Evolutionary dynamics of genome size in a radiation of woody plants

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PREMISE: Plant genome size ranges widely, providing many opportunities to examine how genome size variation affects plant form and function. We analyzed trends in chromosome number, genome size, and leaf traits for the woody angiosperm clade *Viburnum* to examine the evolutionary associations, functional implications, and possible drivers of genome size.

METHODS: Chromosome counts and genome size estimates were mapped onto a *Viburnum* phylogeny to infer the location and frequency of polyploidization events and trends in genome size evolution. Genome size was analyzed with leaf anatomical and physiological data to evaluate the influence of genome size on plant function.

RESULTS: We discovered nine independent polyploidization events, two reductions in base chromosome number, and substantial variation in genome size with a slight trend toward genome size reduction in polyploids. We did not find strong relationships between genome size and the functional and morphological traits that have been highlighted at broader phylogenetic scales.

CONCLUSIONS: Polyploidization events were sometimes associated with rapid radiations, demonstrating that polyploid lineages can be highly successful. Relationships between genome size and plant physiological function observed at broad phylogenetic scales may be largely irrelevant to the evolutionary dynamics of genome size at smaller scales. The view that plants readily tolerate changes in ploidy and genome size, and often do so, appears to apply to *Viburnum*.

KEY WORDS Adoxaceae; cell size; chromosome number evolution; genome size; leaf anatomy; polyploidy; *Viburnum*.

Plants exhibit wide variation in genome size, with almost 150 giga base pairs (Gpb) of genome size variation across land plants (Pellicer et al., 2018). This genome size variation is at least partially related to changes in chromosome number over the course of plant evolution. Variation in chromosome number through whole genome duplication, hybridization, chromosome loss, chromosome fusion, and chromosome fission is widely tolerated in plants and widespread in many clades (Cui et al., 2006; Estep et al., 2014; Mandáková et al., 2015; Hou et al., 2016; Lightfoot et al., 2017). Because changes in chromosome number and genome size have arisen repeatedly in distantly related lineages, there are many opportunities to study the mechanisms and implications of genome size variation, chromosome number variation, and the interplay between the two.

Changes in total chromosome number often show a characteristic multiplication of base chromosome number associated with polyploidy. This can be either autopolyploidy, resulting from the fusion of unreduced gametes from plants of the same species, or allopolyploidy, resulting from the fusion of unreduced gametes from separate species (Kihara and Ono, 1926; Ramsey and Schemske, 1998). Polyploidy increases chromosome number with respect to at least one of the parents and leads to genome size increase, at least in the short term. Aneuploidy—the loss or gain of whole chromosomes (De Storme and Mason, 2014; Lightfoot et al., 2017)—can also lead to genome size increases or decreases. Chromosome number change can also arise through rearrangement of chromosomes (Hou et al., 2016), with fission leading to an increase in chromosome number and fusion leading to a decrease. Although both of these mechanisms change the base chromosome count by one, they do not necessarily lead to changes in genome size because the original chromosomes are not gained or lost, but only modified.

While changes in chromosome number can change genome size, genome size can also vary independently of chromosome number (Price et al., 2005; Fleischmann et al., 2014). Nonpolyploidy related genome size increases occur largely through transposon

amplification (Hawkins et al., 2006), though smaller increases could also occur through tandem gene duplication and insertions. Genome size decreases can result from deletions, unequal homologous recombination, and illegitimate recombination, particularly in repetitive regions of the genome (Devos et al., 2002; Vitte and Bennetzen, 2006; Ren et al., 2018). Previous work shows a trend towards genome downsizing following polyploidy events (Leitch and Bennett, 2004; Vu et al., 2015), suggesting that there may be disadvantages to having a large genome, and promoting mechanisms to scale down genome size while chromosome number remains constant.

How might having a small or large genome influence basic organismal function? At the molecular scale, increases in ploidy can lead to increases in mRNA transcript abundance, although the relationship between expression and ploidy is not necessarily linear or consistent between genes (Coate and Doyle, 2010, 2015). The physical packing of more DNA into a nucleus could also affect gene regulation because of changes in proximity between chromatin and its interacting proteins in a more crowded nucleus (Almassalha et al., 2017; Sugawara and Kimura, 2017). Larger genomes are also linked to larger cell sizes (Müntzing, 1936; Mirsky and Ris, 1951; Cavalier-Smith, 1978; Beaulieu et al., 2008), although the exact mechanism is unclear and effects can differ between cell types (Marshall et al., 2012; Doyle and Coate, 2019). Increases in nuclear DNA content associated with endoreduplication, in which cells double their DNA without undergoing division, can be associated with larger cells and different cell morphologies (Melaragno et al., 1993), showing another mechanism linking an increase in nuclear DNA and cellular properties.

Given the cellular implications of genome size variation, more recent work has explored how cellular changes could influence emergent physiological and ecological properties. Polyploids can achieve faster climatic niche differentiation than their diploid progenitors, possibly facilitating polyploid speciation (Baniaga et al., 2020). In comparisons of diploids and chemically induced polyploids of the same species, polyploids showed changes in physiologically-relevant cellular traits, such as vein density, but did not exhibit concomitant changes in ecologically relevant responses, such as heat stress response or growth rate (Wei et al., 2020). In previous studies, increases in genome size have been correlated with slower growth rate (Cavalier-Smith, 1978; Sharpe et al., 2012; Müller et al., 2019), which could in turn limit plants with large genomes from colonizing stressful or seasonal environments with short growing seasons where fast growth is required (Knight et al., 2005; Qiu et al., 2019). Other work extrapolates further, suggesting that if increases in genome size lead to increases in cell size, then large cell size may constrain stomatal and leaf venation densities, which would present a hydraulic limitation to photosynthesis (Simonin and Roddy, 2018). A relationship between genome size and photosynthetic rate was proposed to place limits on the evolutionary success of nonflowering seed plants and ferns, which tend to have larger genomes than angiosperms; conversely, the ecological success of angiosperms has been attributed to their ability to achieve small genome sizes (Simonin and Roddy, 2018).

Polyploidy can have other evolutionary implications. It can be a source of evolutionary novelty, with new gene copies available to evolve independently and gain new functions (Ohno, 1970; Roose and Gottlieb, 1976; Wendel, 2000). The process of diploidization following a polyploidy event can lead to chromosome rearrangements and gene silencing, thereby influencing regulation and function (Soltis et al., 2015; Hu and Wendel, 2019). All of this suggests that whole genome duplications could provide opportunities for new evolutionary innovations in a polyploid lineage (Levin, 1983; Soltis and Soltis, 2016). Polyploidy may also influence speciation rates on a large scale, with some authors suggesting that polyploidy hinders lineage diversification because on average, diploids diversify at higher rates than polyploids (Mayrose et al., 2011, 2015). Others have countered that polyploidy is positively associated with lineage diversification rates (Tank et al., 2015; Landis et al., 2018) and that polyploidy and diversification must be compatible, because all living seed plants have radiated in spite of ancient polyploidy events, and inferred paleopolyploidy events are scattered broadly across the plant tree of life (Soltis et al., 2009, 2014; Jiao et al., 2011).

While recent studies linking genome size with various biological phenomena have produced some intriguing correlations, more work needs to be done to understand the universality of these relationships. Here we analyze the evolution of genome size and chromosome number across *Viburnum* (Adoxaceae), a clade of around 165 species of shrubs and small trees. We have been developing *Viburnum* as a model clade (Donoghue and Edwards, 2019), and we now know a great deal about phylogenetic relationships, biogeographic and ecological history, diversification, and functional trait evolution within this lineage (Schmerler et al., 2012; Chatelet et al., 2013; Edwards et al., 2014, 2017; Scoffoni et al., 2016; Spriggs et al., 2018; Landis et al., 2020). Additionally, an extraordinary set of studies by Egolf (1956, 1962), produced multiple chromosome counts for 69 species of *Viburnum*, indicating extensive variation in chromosome number, potentially multiple polyploidy events, and at least one shift in base chromosome number. In addition to analyzing the existing counts in a phylogenetic context for the first time, we add previously unreported counts for nine species from Mexico and 48 new genome size estimates, allowing us to analyze the dynamics of genome size and chromosome number evolution across the group. Understanding genome size evolution in *Viburnum* is also interesting from the standpoint of plant growth form. Earlier broad-scale studies suggested a decoupling of genome size–leaf trait relationships in woody plants (Beaulieu et al., 2008), but this has not been examined in detail within a single clade. By comparing our genome size estimates with measurements of guard cell length, stomatal density, vein density, and photosynthetic rates, we are able to interrogate how previously suggested genome size–trait relationships are maintained during a radiation of woody plants.

MATERIALS AND METHODS

Chromosome counts

We include chromosome counts for 78 species, 66 of which were obtained from Egolf (1956, 1962), three from Zhang et al. (2016), and nine that are new to this study. Twelve species included in Zhang et al. (2016) agreed with earlier counts from Egolf (1956, 1962), increasing our confidence in the original Egolf counts. We included all Egolf counts provided that they are currently recognized species and not hybrids, with a few exceptions. We did not include the Egolf (1956, 1962) counts for *V. microphyllum, V. hartwegii,* and *V. ellipticum* because (1) we doubted the provenance of the accessions, (2) there was only one example of each, and (3) the counts seemed unlikely given the counts of closely related species. We did not include

the count of $2n = 18$ for *V. setigerum* from Egolf (1956) because the author doubted it and later omitted it (Egolf, 1962).

The chromosome counts for nine additional species were prepared using methods similar to those detailed by Egolf (1956). Root tips were collected from plants growing in the greenhouse on a sunny day. Tips were fixed in dichlorobenzene for about two hours at room temperature, then placed in a watch glass containing 1 drop 1N HCl and 9 drops of staining solution (1% orecin/45% acetic acid/54% water). The watch glass was passed over a Bunsen burner three times so the solution was hot, but not boiling. The heated watch glass was placed on the bench, another watch glass was placed on top of the first, and the roots were allowed to cool in the watch glass for at least two minutes. One root tip was squashed individually on each slide. A drop of staining solution was placed in the middle of the slide; a root tip was placed in the staining solution and covered with a cover slip. The slide was then placed on the floor, covered with a 5 cm × 5 cm square of eraser, and stood upon by M. Moeglein for 5 minutes in order to flatten the root cells and chromosomes for imaging. The slide was then sealed with clear nail polish and imaged at 100× oil magnification on a Nikon Eclipse 90i compound microscope (Nikon, Tokyo, Japan). Each image was taken as a z-stack to ensure all chromosomes were in focus and all images from the stack showing chromosomes in focus were combined in Photoshop (Adobe Systems, San Jose, California, USA) to make the final image. Chromosomes from at least three cells from each species were counted.

Genome size estimates

Genome size estimates were generated using flow cytometry. Fresh leaf tissue from 48 species (Appendix S1) was collected into wet paper towels and placed in a cooler with ice packs in order to keep the leaves cool but not frozen. Leaves were processed for flow cytometry as quickly as possible to minimize degradation of nuclei, spending three days or less in the cooler before processing. Tissue was then co-chopped with *Glycine max*, *Zea mays*, and/or *Pisum sativum* as internal genome size standards (Doležel et al., 1994, 1998; Lysak and Doležel, 1998) and stained with propidium iodide using the Partec Sysmex Plant Precise P kit standard kit protocol (Sysmex, Kobe, Japan). Samples were run on a BD Biosciences LSRII (BD Biosciences, San Jose, California, USA) at low speed. At least five samples were run per individual, with varied proportions of *Viburnum* tissue and internal standard tissue to ensure the correct identification of peak order. The three samples showing the most similar peak heights between the *Viburnum* and standard nuclei were used to calculate nuclear DNA content. Picograms of DNA per somatic nucleus were calculated using

$$\frac{\text{Mode fluorescence (Viburnum)}}{\text{Mode fluorescence (Internal Standard)}} = \frac{\text{Picograms DNA per nucleus (Viburnum)}}{\text{Picograms DNA per nucleus (Internal Standard)}}$$

Picograms (pg) of DNA per somatic nucleus were converted to genome sizes using 1 pg DNA = 0.978 Gbp (Doležel et al., 2003), yielding a $2n$ Gbp genome size estimate.

Basic genome size (Leitch and Bennett, 2004), or Cx (Greilhuber et al., 2005), was calculated using Genome size ($2n$ Gbp) / Ploidy, where Ploidy = 2 for diploids ($2n$ = 16 or 18), 4 for tetraploids ($2n$ = 32 or 36), or 8 for octoploids ($2n$ = 72). When the chromosome count reported by Egolf (1956, 1962) was uncertain, we used the count in which our genome size estimate matched the Egolf (1956, 1962) accession (when available) or the most frequently observed count. Clade names are based on the classification and phylogenetic definitions of Clement et al. (2014).

Ancestral state reconstruction for chromosome number and genome size

Ancestral state reconstruction of chromosome number and genome size were inferred using the *Viburnum* phylogeny from Landis et al. (2020). For chromosome number evolution, we employed the analytical package chromEvol 2.0 (Glick and Mayrose, 2014). We used counts for 78 species from Egolf (1956, 1962), Zhang et al. (2016), and this study. We used the ancestral chromosome number estimates produced after 10000 simulations of the CONST_RATE model, assuming a constant rate of evolution and allowing for chromosome number transitions resulting from ascending dysploidy, descending dysploidy, and whole genome duplications. Where more than one chromosome number value per species was reported, we calculated the percentage frequency of each count and included this in our input data file. Variation within species is incorporated by chromEvol during ancestral state reconstruction. Uncertainty around node values was calculated as the posterior probability of the most likely chromosome number (Appendix S2).

We tested Brownian Motion (BM) versus Ornstein-Uhlenbeck (OU) as models of trait evolution for genome size and basic genome size using the R package 'geiger' (Pennell et al., 2014). Ancestral state reconstructions for genome size and basic genome size under BM and OU were calculated using the anc.ML function in phytools (Revell, 2012). We investigated relationships between genome size and chromosome number using phylogenetic linear models (PLM) under BM and OU using the R package 'phylolm' (Ho and Ané, 2014). All model tests were used in conjunction with the *Viburnum* phylogeny from Landis et al. (2020) pruned to include the species for which we have genome size estimates and chromosome counts.

Leaf trait measurement

Leaf tissue was collected fresh from either field-grown material or from living specimens in the Arnold Arboretum in Boston, Massachusetts, or the Washington Park Arboretum in Seattle, Washington (see Appendix S3 for voucher specimen information), and stored in formalin-acetic acid-alcohol (FAA) until further processing. Guard cell length and stomatal density were measured either from epidermal peels or from squares of leaf blade that had been immersed in 1 part glacial acetic acid: 4 parts 95% ethanol: 5 parts DI water. After treatment, leaf sections were baked at 40–50°C until translucent. Images were taken using a Nikon Eclipse E600 compound microscope (Nikon, Tokyo, Japan). Stomatal counts and guard cell measurements were made in ImageJ2 (Rueden et al., 2017). Vein densities were measured using methods similar to those described in (Scoffoni et al., 2016). Briefly, fresh leaf pieces were treated with sodium hydroxide followed by sodium hypochlorite on a hot plate. Leaves were then stained with Safranin O, imaged on a Nikon Eclipse E600 compound microscope (Nikon, Tokyo, Japan), and total vein length per area (mm/mm^2) measured using ImageJ2 (Rueden

et al., 2017). Measured and modeled values for light-saturated leaf photosynthetic rate per unit leaf area (A_{max}, μmol CO_2 m^{-2} s^{-1}) were taken from (Chatelet et al., 2013).

Leaf trait/genome size comparisons

We investigated relationships between genome size and leaf traits using PLM under BM and OU using the R package 'phylolm' (Ho and Ané, 2014). We also compared our genome size and trait measurements in *Viburnum* to a plant-wide genome size–trait database published in Simonin and Roddy (2018). All statistics comparing leaf traits and genome sizes were performed in R (R Core Team, 2018).

RESULTS

Chromosome number evolution

Haploid chromosome number ranges broadly in *Viburnum*, from $1n = 8$ to $1n = 36$. There are five $1n = 8$ species and 50 $1n = 9$ species in our sample. These species are our diploids. We also sampled four $1n = 16$ species and 13 $1n = 18$ species, which are likely tetraploids, and four $1n = 36$ species, which are likely octoploids (Fig. 1). Our nine new counts indicate that all sampled members of the Neotropical *Oreinotinus* clade are $1n=18$ tetraploids (Fig. 2). This contradicts previous counts by Egolf (1956, 1962), who reported that the two *Oreinotinus* species he sampled were $1n = 9$.

ChromEvol ancestral state reconstructions of chromosome number favor an ancestral chromosome number for *Viburnum* of $1n = 9$, with two later decreases in chromosome number from $1n = 9$ to $1n = 8$ (Fig. 1, Appendix S2). One of these decreases occurred in the ancestor of our samples of the Asian *Solenotinus* clade. The other reduction is in *V. plicatum*, which is closely related to *Solenotinus*, and includes individual plants that have been counted as both $1n = 9$ and $1n = 8$ (Janaki-Ammal, 1953; Egolf, 1956, 1962). ChromEvol inferred 9 polyploidization events within *Viburnum*, with three shifts from $1n = 9$ to $1n = 18$, four changes from $1n = 18$ to $1n = 36$, and two from $1n = 8$ to $1n = 16$. Five of these events are linked to single species in our sample, while two are at the base of small clades containing two or three species. The remaining two polyploidization events mark larger clades: *Porphyrotinus,* and a subclade within the *Solenotinus*, containing 43 and 8 species, respectively.

Genome size evolution

Genome size varied over 5-fold across *Viburnum*, with 2C values ranging from 4.29–24.23 Gbp (4.20–23.70 pg) (Fig. 3). These genome sizes are classified as small to intermediate when compared to plants in general (Leitch et al., 2005). Genome size and basic genome size evolution were all better fit by an OU model than BM (AIC 277.94 versus AIC 300.94, and AIC 111.77 versus AIC 115.42, respectively). However, all PLM under OU or BM showed the same relationships and significance between variables (Appendix S4). Genome size was significantly positively correlated with chromosome number ($r^2 = 0.617$, $P < 0.001$, OU PLM $r^2 = 0.740$, $P < 0.001$) (Fig. 4). When 2C genome size was divided by ploidy to calculate basic genome size, the basic genome size (Cx) ranged from 1.36–5.68 Gbp and the overall average was 3.23 Gbp (Fig. 5). The average basic genome size for diploids ($1n = 8$ or 9) was 3.34 Gbp, while the average basic genome size for polyploids ($1n = 16$, 18, or 36) was 3.06 Gbp, although the difference was not statistically significant (Welch two sample t-test, $P = 0.27$). Basic genome size was negatively correlated with chromosome number, but again the relationship was not significant ($r^2 = 0.033$, $P = 0.113$, OU PLM $r^2 = 0.007$, $P = 0.568$) (Fig. 4).

Genome size and leaf trait correlations

All leaf trait PLM were again slightly better fit by OU than BM, but correlations and significance between leaf traits and genome size showed the same trends regardless of the model used (Appendix S4). Genome size was significantly positively correlated with guard cell length ($r^2 = 0.164$, $P = 0.014$, OU PLM $r^2 = 0.177$, $P = 0.019$), and was weakly (although not significantly) negatively correlated with stomatal density ($r^2 = 0.067$, $P = 0.087$, OU PLM $r^2 = 0.010$, $P = 0.083$). Genome size was not significantly correlated with leaf vein density ($r^2 = -0.042$, $P = 0.881$, OU PLM $r^2 = 0.001$, $P = 0.860$) (Fig. 6). When we combined our leaf trait and genome size data set with the plant-wide data set from Simonin and Roddy (2018), all correlations between genome size and guard cell length, stomatal density, and vein density remained significant ($r^2 = 0.404$, $P < 0.001$, $r^2 = 0.296$, $P < 0.001$, and $r^2 = 0.457$, $P < 0.001$, respectively). However, the addition of our data did weaken the strength of the correlations reported by Simonin and Roddy (2018). Measured A_{max} values from Chatelet et al. (2013) were positively correlated with genome size ($r^2 = 0.179$, $P = 0.023$, OU PLM $r^2 = 0.225$, $P = 0.019$), and this positive correlation between genome size and photosynthetic rate was maintained when we included additional values of modeled A_{max} ($r^2 = 0.116$, $P = 0.016$, OU PLM $r^2 = 0.118$, $P = 0.026$) (Fig. 7).

DISCUSSION

Chromosome number evolution in *Viburnum*

Chromosome number is highly labile across *Viburnum*, there being at least five classes of chromosome numbers, two inferred decreases in base chromosome number, and at least nine instances of polyploidization. Based on our ancestral state reconstructions we unambiguously infer that the first viburnums were $1n = 9$ (i.e., diploids with $2n = 18$). This contradicts the conclusion of Egolf (1956, 1962), who favored the view that $1n = 8$ was ancestral. His assessment was based on the finding of $1n = 8$ in *V. sieboldii* and related species of *Solenotinus* (then section *Thyrsosma*), which were judged by Wilkinson (1948) to be the most "primitive" of the species that she sampled for flower anatomy. This is consistent with the idea of Hara (1983) that in *Viburnum* the panicle-like inflorescence (characteristic of *Solenotinus*) is ancestral to the more common umbel-like inflorescence. All phylogenetic analyses of *Viburnum* to date have refuted these assumptions. Instead, it is now clear that the panicle-like inflorescence is the derived state in *Solenotinus* (Clement et al., 2014), as is the $1n = 8$ condition.

While we are confident that $1n = 9$ is ancestral based on our current sample of $1n = 9$ species (Fig. 1, Appendix S2), it is important to note that counts have not yet been obtained from two deeply diverging, species-poor lineages: *V. clemensiae* and *V. amplificatum*, rare species found in the tropical forests of northern Borneo. We also note that placement of *V. clemensiae* is equivocal (Landis et al.,

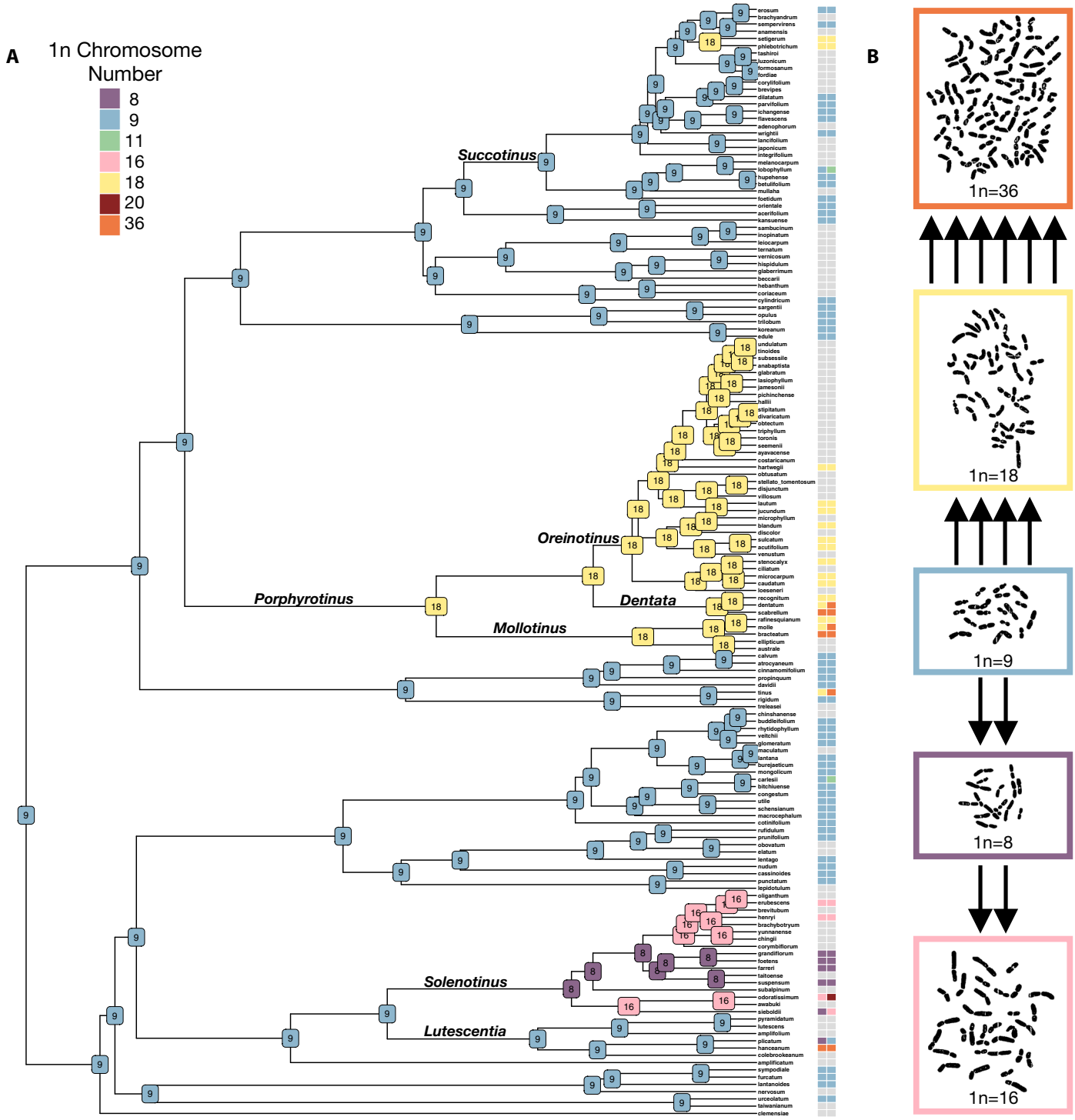

FIGURE 1. (A) Ancestral state reconstruction of 1n chromosome number for all *Viburnum* with colors indicating chromosome number and names on branches specifying clade names. Boxes at tips indicate chromosome numbers for extant species, while gray boxes represent missing counts. Box color in the left column represents the lowest count obtained for a given species, while box color in the right column represents the highest count obtained. (B) Diagram showing number and direction of chromosome number transitions. Number and direction of arrows indicates the number of transitions between chromosome numbers as determined by ancestral state reconstruction. Chromosome drawings are from Egolf (1956) and include *V. bracteatum* (1*n* = 36), *V. tinus* (1*n* = 18), *V. bitchiuense* (1*n* = 9), *V. foetens* (1*n* = 8), and *V. sieboldii* (1*n* = 16).

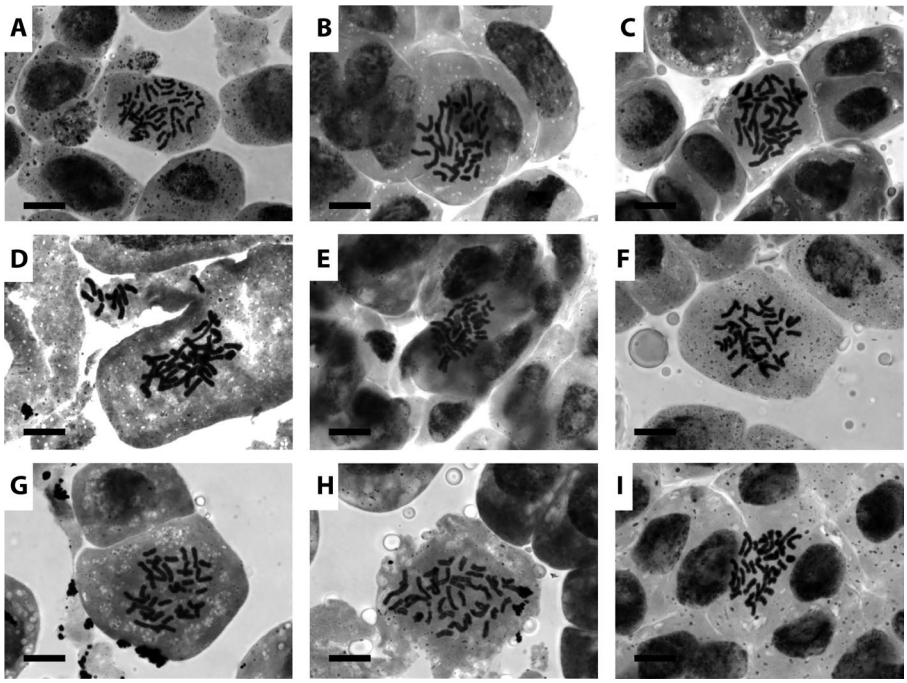

FIGURE 2. Chromosome squashes for (A) *V. acutifolium*, (B) *V. blandum*, (C) *V. caudatum*, (D) *V. hartwegii*, (E) *V. jucundum*, (F) *V. lautum*, (G) *V. microcarpum*, (H) *V. stenocalyx*, and (I) *V. sulcatum*. Scale bars represent a distance of 10 µm. All chromosome squashes were counted as 2n = 36 chromosomes.

2020): it is either in the position shown in Fig. 1, or it is sister to all of the rest of *Viburnum* as in Clement et al. (2014) and Spriggs et al. (2015). In either case, chromosome number and genome size data for this species could potentially alter support for our ancestral state reconstruction. On the other hand, an ancestral $x = 9$ inference is also strongly supported by outgroup comparison, because most lineages within Dipsacales are characterized by a base chromosome number of $x = 9$ (Sax and Kribs, 1930; Ourecky, 1970; Eriksson and Donoghue, 1997; Benko-Iseppon and Morawetz, 2000).

These considerations leave little doubt that $1n = 8$ was derived from $1n = 9$, and that aneuploidy occurred at least twice in *Viburnum*: once along the branch leading to the Asian *Solenotinus* clade, perhaps ~20 Mya (Landis et al., 2020), and once within *V. plicatum*, in which some plants are $1n = 9$, while others are $1n = 8$ (Janaki-Ammal, 1953; Egolf, 1956, 1962). We are unsure whether these shifts from $1n = 9$ to $1n = 8$ resulted from chromosome loss or chromosome fusion, but we are hopeful that an analysis of the widespread and exceptionally variable *V. plicatum* could shed light on the mechanism of anueploid reduction. Curiously, *V. hanceaum*, the sister of *V. plicatum*, has been counted as an octoploid ($1n = 36$) (Egolf, 1956, 1962). This favors a base number of $1n = 9$ in the *V. plicatum*–*V. hanceanum* clade, and hence a reduction to $1n = 8$ (one or more times) within *V. plicatum*.

There are several fairly deep polyploidization events in *Viburnum*, two of which mark significant clades. Within the $1n = 8$ *Solenotinus*, there were two shifts to $1n = 16$, and one of these subtends a clade of at least eight species that is nested within an eastern Himalayan radiation during the past 8-5 Ma (Spriggs et al., 2015). We have also unambiguously identified polyploidization subtending the entire New World *Porphyrotinus* clade, with some 44 species (about one-quarter of all *Viburnum* species). This had been suggested by Donoghue (1982) based on preliminary chromosome counts of $1n = 18$ for *V. lautum* and *V. blandum* of southern Mexico. Winkworth and Donoghue (2004) also hinted at this interpretation based on the phylogenetic distribution of multiple copies of the granule-bound starch synthase (GBSSI) locus. Here, for the first time, we provide definitive chromosome counts showing $1n = 18$ ($2n = 36$) for nine Mexican species (Fig. 2). These results, together with counts of $1n = 18$ for species of the closely related *Mollotinus* and *Dentata* clades, suggest that all species of *Porphyrotinus* are polyploid.

The *Porphyrotinus* event is the most ancient of any of the polyploidy events within *Viburnum*. This clade appears to have originated and entered North America from Eastern Asia in the Paleocene or early Eocene (60–50 Mya [Landis et al., 2020]). Much later, beginning perhaps 12 Mya, the *Oreinotinus* subclade shifted into cloud forests in Mexico and moved progressively southward and into the Andes of South America some 5 Mya (Landis et al., 2020). Spriggs et al. (2015) identified this *Oreinotinus* clade as the most rapid radiation within *Viburnum*. Within *Porphyrotinus* we note that there have been at least two fairly recent (~1–3 Mya) shifts from tetraploidy ($1n = 18$) to octoploidy ($1n = 36$) in Eastern North America: once in the uncommon *V. bracteatum* of the southeastern United States, and once or more within the widespread, highly variable, and taxonomically controversial *V. dentatum* species complex (represented in Fig. 1 by *V. dentatum*, *V. scabrellum*, and *V. recognitum*).

Our results relate to ongoing arguments concerning the long-term evolutionary consequences of polyploidy. On the one hand, it is thought that changes in chromosome number could foster evolutionary innovation and diversification (Cui et al., 2006; Soltis et al., 2014; Soltis and Soltis, 2016). Others have suggested that whole genome duplication is likely to be an evolutionary dead end, with polyploids more likely to go extinct than they are to diversify (Mayrose et al., 2011). In *Viburnum*, Spriggs et al. (2015) identified three rapid radiations. Two of these are associated with polyploidy: the New World *Porphyrotinus* clade and the eastern Himalaya element of the Asian *Solenotinus* clade. The third significant radiation—the Asian *Succotinus* clade with some 27 species—is not connected with polyploidy, although it does include one instance of tetraploidy (*V. setigerum* +*V. phlebotrichum*). Thus, polyploidy is not necessary for radiation, but neither is it a hindrance, i.e., polyploid clades are capable of diversifying rapidly, even in a group of woody plants. In *Viburnum* we doubt that polyploidy itself was a direct driver of increased diversification. Rather, both the *Porphyrotinus* and *Solenotinus* radiations are associated with movements into highly heterogeneous mountain regions that appear to have promoted divergence via geographical isolation (Spriggs et al., 2015). We also note that the rapid radiation of *Porphyrotinus* appears to have started long after the initial polyploidy event; i.e. it began with the shift into

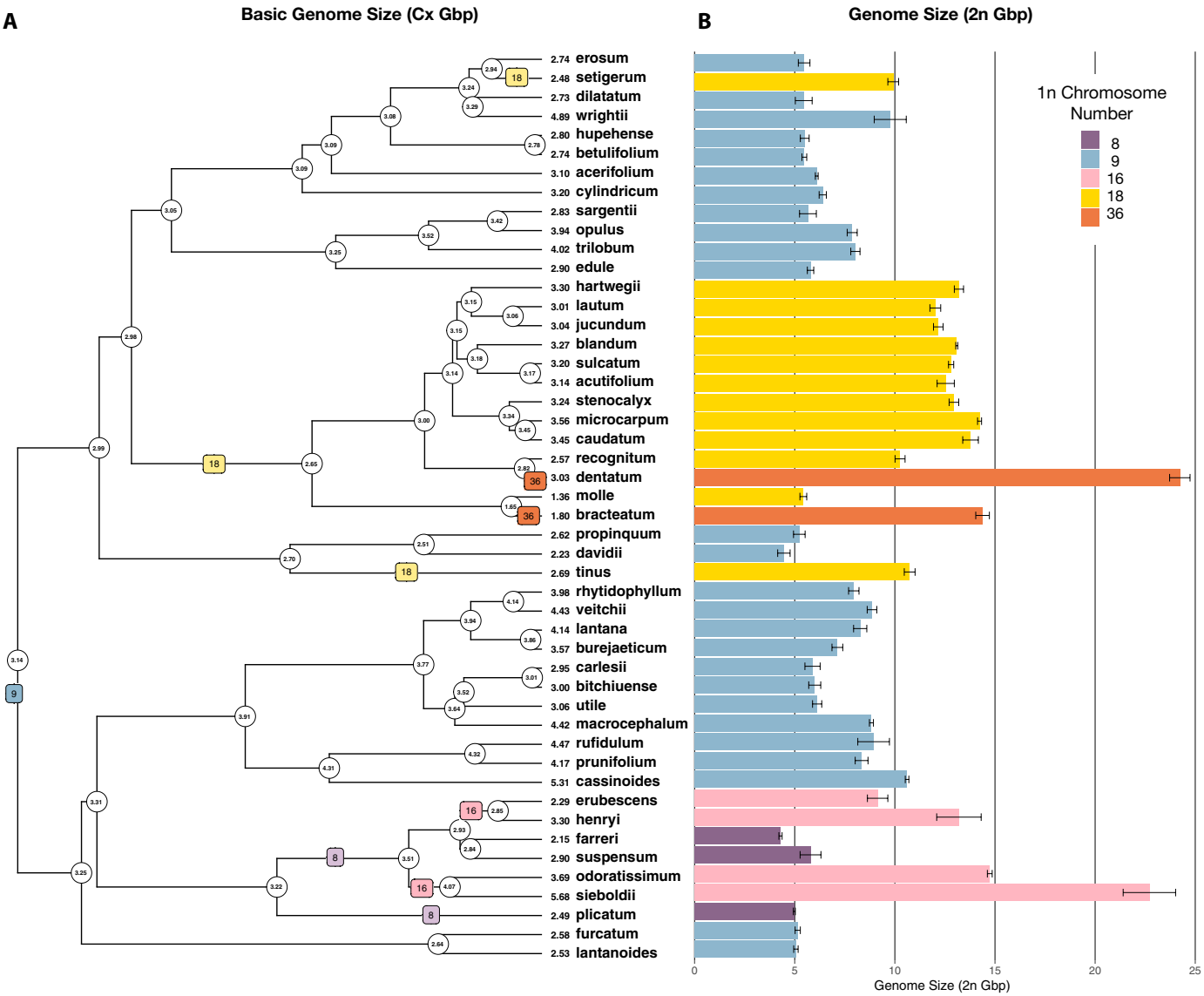

FIGURE 3. Genome size evolution in *Viburnum*. (A) Pruned phylogenetic tree including only species with chromosome number and genome size estimates. Basic genome sizes (Cx Gbp) are listed at tips, reconstructed basic genome sizes are placed at nodes, colored boxes along branches indicate polyploidization events. (B) Genome size estimates (2*n* Gbp) for each species indicated by bar height with color indicating the likely chromosome number of the measured individual. Error bars represent one standard deviation.

neotropical cloud forests, not with the much earlier movement into North America (Fig. 1).

Polyploidy and genome size evolution

Genome size is strongly correlated with chromosome number in *Viburnum* (Fig. 4), with polyploid individuals accounting for 14 of the largest genomes in our study (Fig. 3). When we controlled for chromosome number and looked at basic genome size evolution (Cx) we found weak evidence for genome downsizing. While basic genome sizes for polyploids were 0.28 Gbp smaller than diploids on average, the difference was not significant. Comparing sister diploid and polyploid lineages across the tree, it is obvious that basic genome size is sometimes reduced following polyploidy, and sometimes increased. For example, the diploid *V. erosum* has a larger basic genome size than its closest measured relative, the tetraploid *V. setigerum*, while the diploids *V. davidii* and *V. propinquum* have smaller basic genome sizes than their closest measured relative, the tetraploid *V. tinus*. More targeted genome size measurements in smaller clades within *Viburnum* could provide more power to resolve the effects of genome downsizing going forward. Although genome downsizing does not seem to be a significant force in shaping genome size dynamics across this lineage, it is worth noting that while the fraction of genome reduction in our tetraploids and octoploids does not appear large, they are still sometimes losing large amounts of DNA—on average a Cx genome size decrease of 0.56 Gbp or 1.12 Gbp for tetraploids and octoploids respectively. For

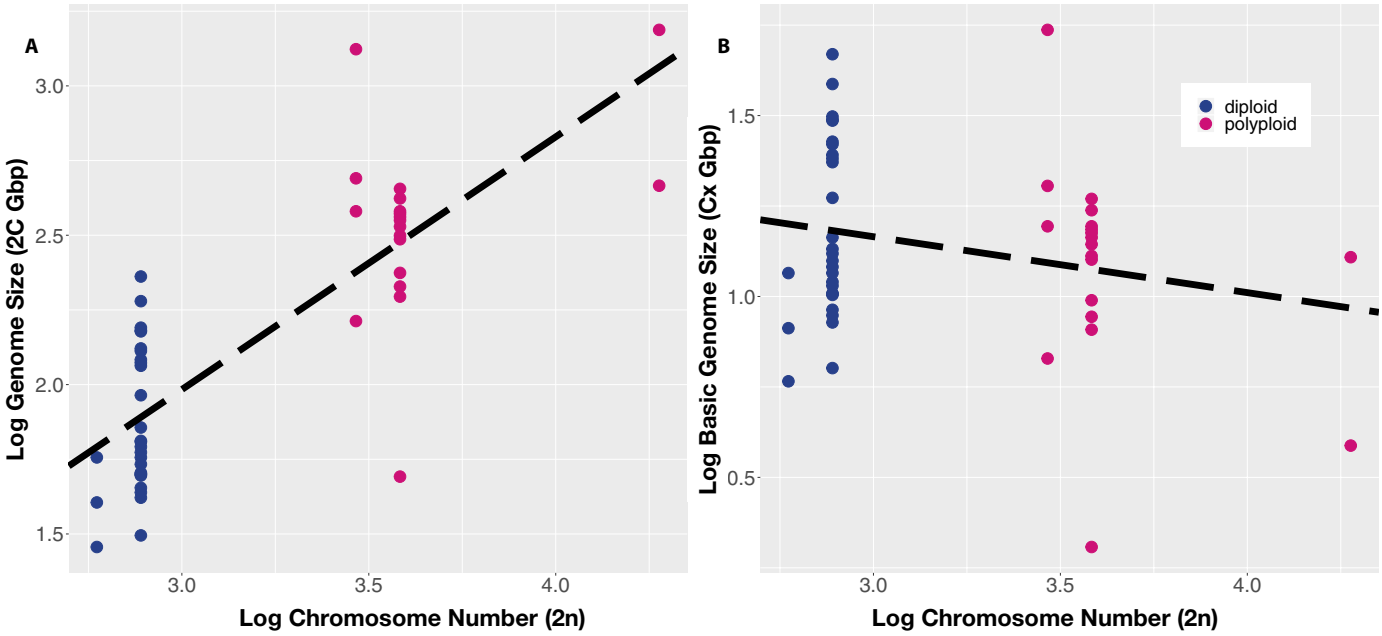

FIGURE 4. Logarithmically transformed 2n chromosome number plotted by (A) logarithmically transformed genome size (2*n* Gbp) and (B) logarithmically transformed basic genome size (Cx Gbp). Dotted lines represent line of best fit and colors indicate ploidy.

perspective, the entire genome of *Arabidopsis thaliana* is 0.136 Gbp (Arabidopsis Genome Initiative, 2000).

One clade (*Mollotinus*) appears especially interesting with respect to a potential genome downsizing event, though we are not yet confident in its placement. *Viburnum molle* was counted repeatedly as a polyploid (1n = 18) by (Egolf, 1956, 1962), yet it is among our smallest genome size measurements (2n = 5.43 Gbp). It appears, then, that *V. molle* has the smallest basic genome size (Cx = 1.36 Gbp) observed in this study. Our genome size estimate was obtained from one of the same individuals counted by Egolf (Arnold Arboretum of Harvard University, Boston, Massachusetts; *V. molle* 18294-A). If Egolf's count and our measurements are both correct, then *V. molle* would be a case of relatively extreme genome size miniaturization within *Viburnum*. Interestingly, the closely related *V. bracteatum* (1*n* = 36) had the second smallest base genome size in our study, and provides an equally impressive example of downsizing. Another pair of polyploids showing noteworthy genome size dynamics are the tetraploids *V. sieboldii* and *V. odoratissimum* (both 1*n* = 16); while they have some of the largest genomes in *Viburnum*, *V. odoratissimum* is close to half the size of *V. sieboldii*, suggesting that genome downsizing mechanisms have affected these two species unequally and to a lesser extent than the rest of *Viburnum*. Future studies focused on these two regions of the tree could be especially fruitful.

Our results stand in contrast to many examples showing no relationship or a negative correlation between chromosome number and genome size (Leitch and Bennett, 2004; Vu et al., 2015). Although a doubling in genome size after polyploidy is expected, the rate and tempo of genome size change afterward is not well understood. When is genome downsizing triggered and why would it proceed more or less rapidly in different contexts? Is the rate of downsizing the same across genome sizes or could it be proportional to chromosome number or genome size? One hypothesis is the "genome downsizing and threshold" model from Zenil-Ferguson et al., (2016), which suggests that large genome sizes or large monoploid numbers could promote genome downsizing. From this idea, it follows that genome size and chromosome number could be constrained (i.e., whole genome duplications selected against) in some clades but not in others. With fairly average monoploid numbers of 8 and 9, and genome sizes classified as small to medium, *Viburnum* may fall in the range in which its genome size or chromosome number are not strongly limiting.

Anatomical and physiological correlates of genome size

Even with an almost 20 Gbp range in the amount of DNA in somatic *Viburnum* nuclei, there was little support for the postulated anatomical correlates of genome size. The positive correlation between genome size and guard cell length was the only significant anatomical relationship we found that corresponded with earlier findings (Fig. 6). *Viburnum* stomata appear to be relatively small when compared to a broader plant-wide sampling of guard cell lengths. This pattern may reflect something unique about *Viburnum*, or perhaps differences in measurement methods; either way, it does not seem to have affected the correlation. The genome size-guard cell correlation has been accepted for some time (Masterson, 1994; Beaulieu et al., 2008). Recent work has suggested a more general relationship between genome size and plant cell size, with smaller genomes allowing plants to produce smaller cells, which could allow for higher leaf venation densities, photosynthetic rates, and overall growth rates (Simonin and Roddy, 2018). Yet there is little evidence that the major evolutionary changes in genome size across *Viburnum* have exerted much influence on these important ecophysiological traits. In fact, we actually found a positive correlation between genome size and photosynthetic rate (Fig. 7), the opposite of what we would expect if genome size limited maximum photosynthetic rate per area as suggested in (Simonin and Roddy, 2018; Roddy et al., 2020).

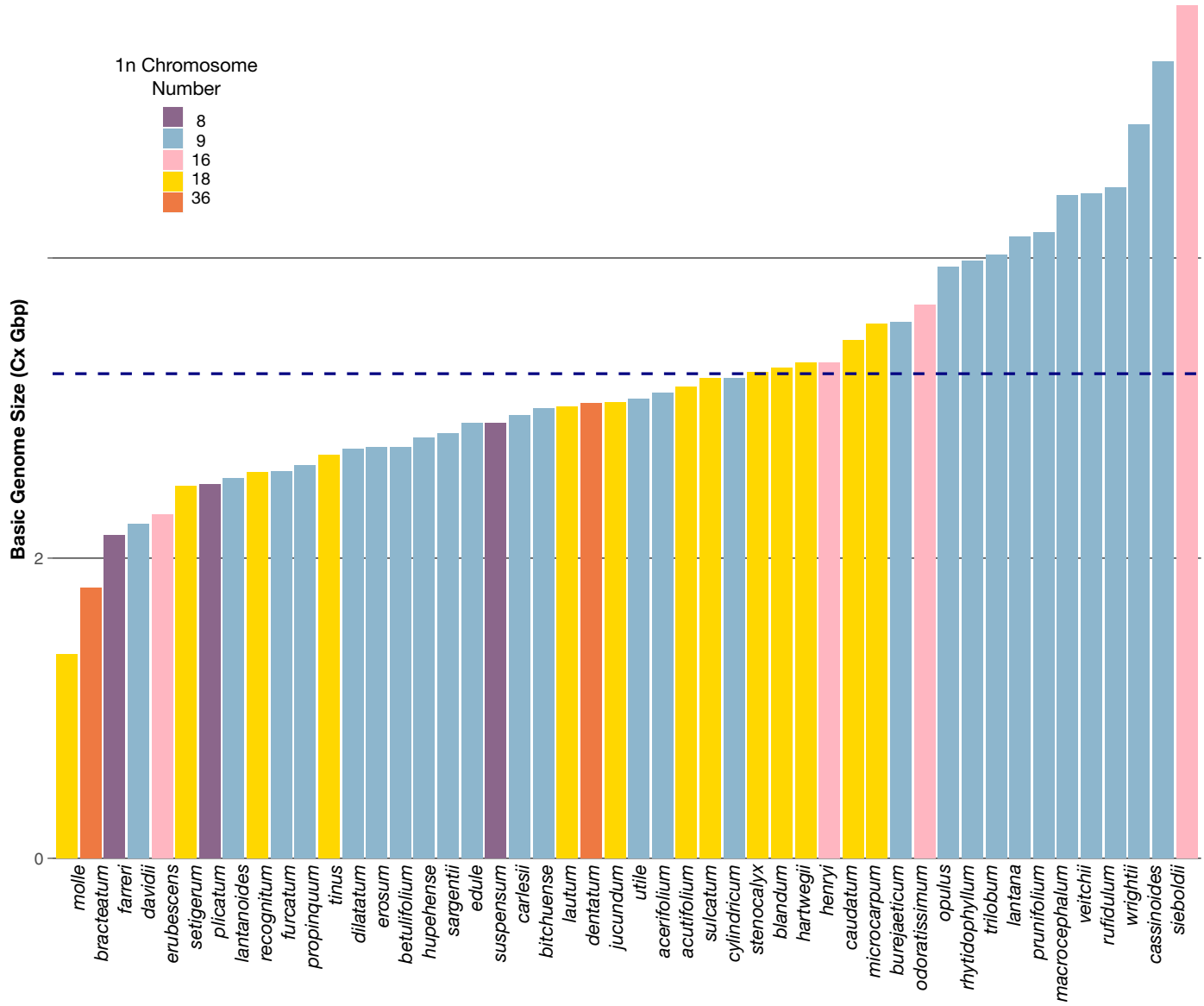

FIGURE 5. Bars representing basic genome size (Cx Gbp) across species with bars colored by likely chromosome number of the individual measured and dotted line showing average basic genome size (3.229 Gbp).

There are a variety of potential explanations for why genome size–trait relationships do not hold in *Viburnum*. For instance, previous work has shown that correlations between genome size and leaf traits tend to break down in trees and shrubs (Beaulieu et al., 2008) and it has been suggested that woody plants are less likely to undergo polyploidy in the first place (Zenil-Ferguson et al., 2017). Neither of these seem relevant here, because in theory, the Simonin and Roddy hypothesis should apply to all organisms, and we have clearly identified multiple polyploidy events associated with genome size changes in *Viburnum*. A third possibility is that the variation we see in *Viburnum* genome size may not be large enough to drive significant changes in these traits. Simonin and Roddy (2018) argued that because on the whole they have larger genomes, ferns and acrogymnosperms are more limited in how they can vary these physiologically important traits than can angiosperms. Absolute genome sizes do vary greatly in *Viburnum*, but the range of variation may not be large enough to expose any limitations on physiological function. Consequently, *Viburnum* may be free to explore anatomical and physiological trait space more or less unconstrained by genome size variation. This suggests to us that changes in genome size would then be predicted to be important for these functional traits only in very special cases, such as when genomes become exceptionally large. This recalls the finding of Edwards et al. (2014) in which leaf functional and anatomical traits in *Viburnum* did not follow the trends or tradeoffs described for global patterns underlying the leaf economics spectrum (Wright et al., 2004). Broad phylogenetic comparisons may place bounds on the phenotypic space that is possible to occupy. In reality, lineages may occupy many odd combinations within that space, suggesting that these broadly delineated boundaries provide a limited constraint on the evolutionary dynamics of the traits in question.

Finally, we suggest that the dissociation between vein density and genome size is *not necessarily* because genome size variation

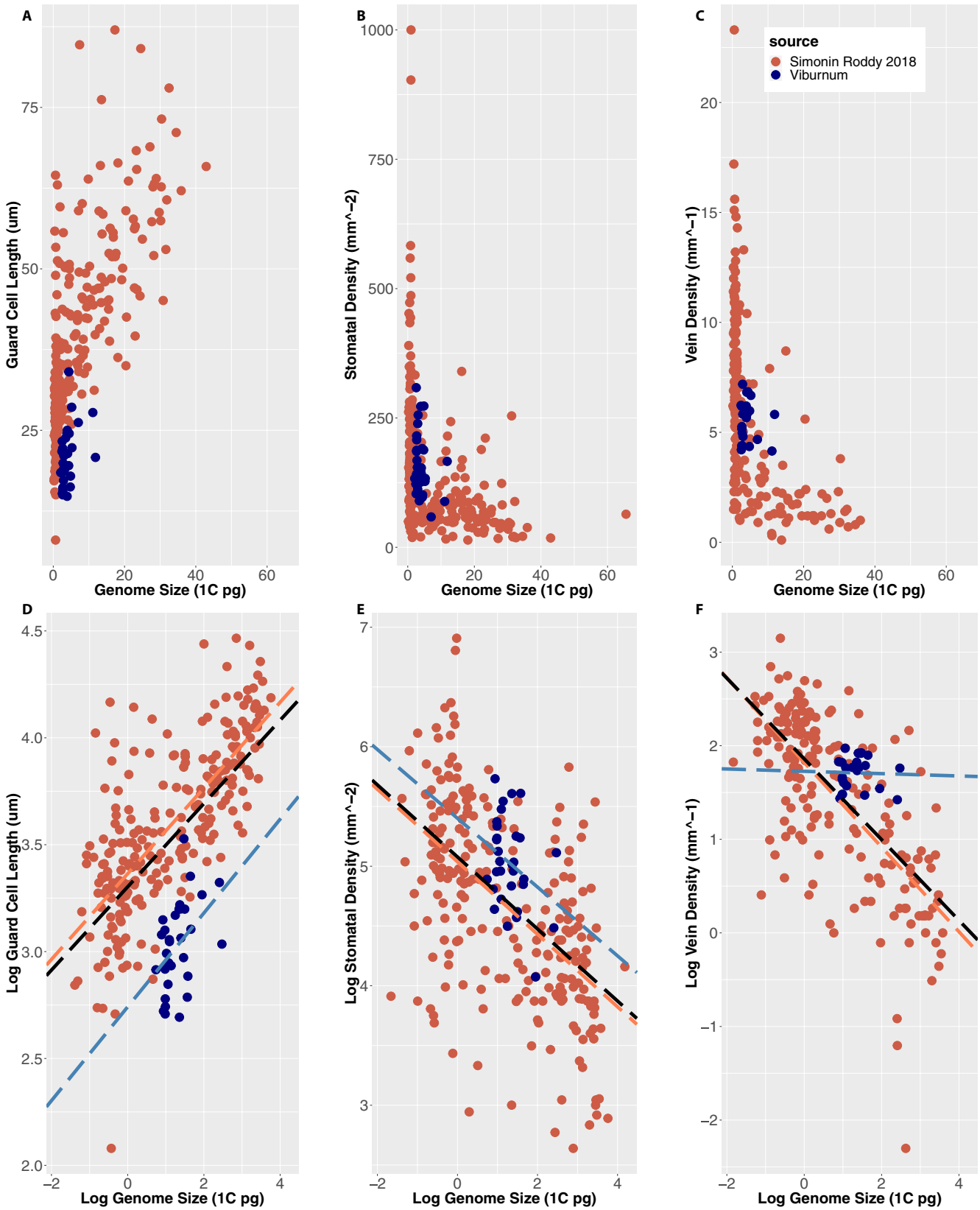

FIGURE 6. Relationships between genome size and guard cell length (A, D), stomatal density (B, E), and vein density (C, F) using untransformed (A, B, C) and logarithmically transformed values (D, E, F) for *Viburnum* (blue) combined with measurements from Simonin and Roddy 2018 (orange). Dotted lines in (D, E, F) depict lines of best fit for data from Simonin and Roddy 2018 (orange), data from this study (blue), and both data sets combined (black).

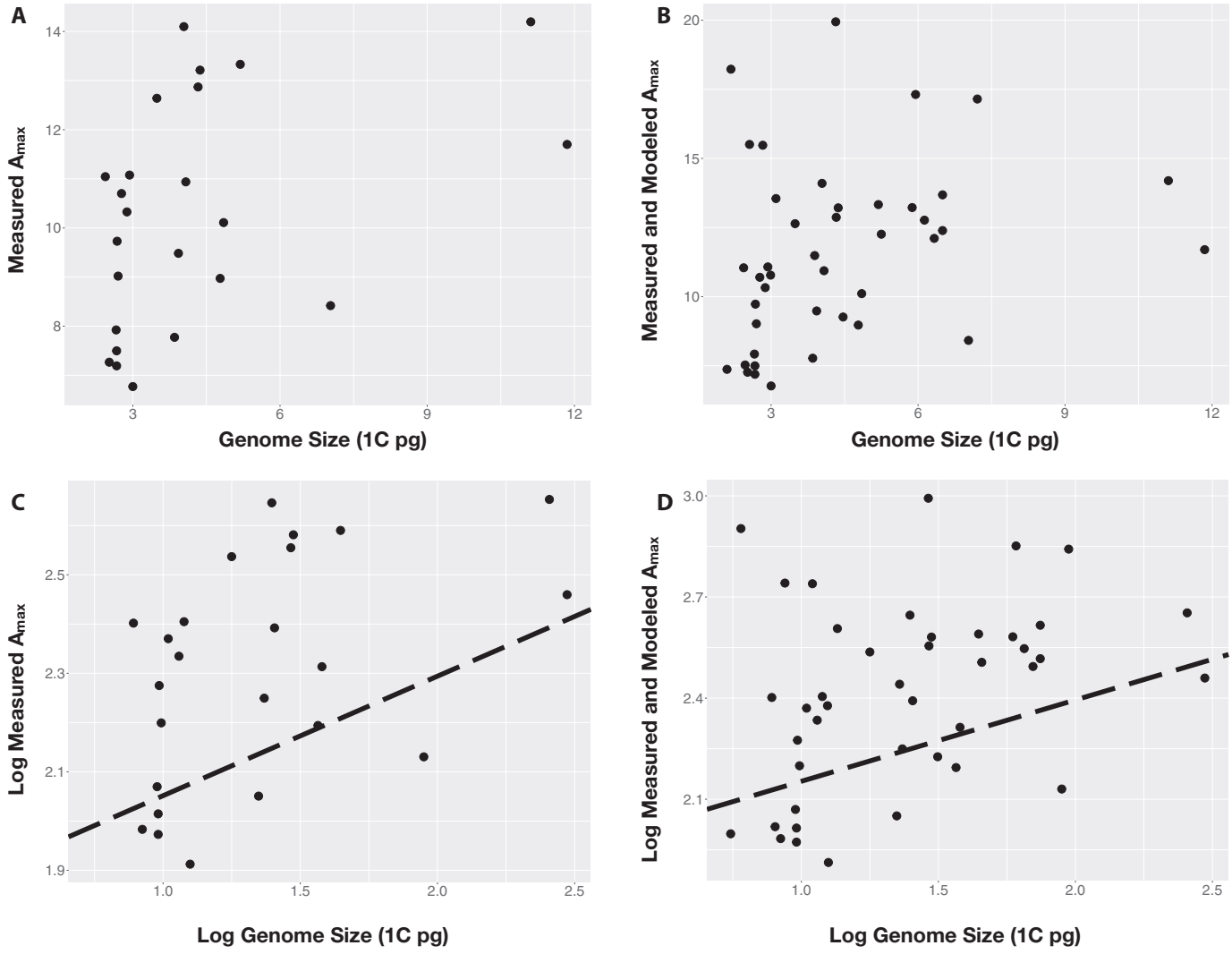

FIGURE 7. Relationships between genome size (1C pg) and A_{max} (light-saturated leaf photosynthetic rate per unit leaf area, umolCO_2 m^{-2} s^{-1}), for measured (A, C) and measured plus modeled (B, D) values.

at this scale never limits minimal cell size, but because cells within the leaf mesophyll are rarely built at the minimum cell size, and they can and do vary quite independently of vein density. The assumption of a causal link between minimum cell size and venation density seems unjustified to us—at the very least, many other mechanisms are available to increase vein density even if cell sizes are large (e.g., fewer cells could be made, or veins could proliferate into multiple layers, sensu Ogburn and Edwards (2013). We have not yet measured meristematic cell sizes in *Viburnum*, but this could be insightful. At the moment, our data suggest that whatever influence genome size might be having on cell size for certain cell types (e.g., guard cells) can be easily overcome by other cell types to produce a variety of leaf anatomical configurations.

CONCLUSIONS

We have uncovered extensive chromosome number variation and multiple polyploidization events within *Viburnum*. Contrary to previous interpretations, we infer that the first viburnums were $1n = 9$ and that $1n = 8$ evolved later. Our new chromosome counts and genome size estimates for a number of Mexican species show, for the first time, that polyploidy is connected with the most impressive radiation within *Viburnum* in the mountains of the neotropics. A second radiation in the Himalayas is also connected with polyploidy. Although we doubt that polyploidy drove these two radiations, our results demonstrate that polyploid lineages can be highly successful. One polyploidization event (at the base of *Porphyrotinus*) is inferred to be ancient, while the rest are much more recent. Variation in ploidy within species appears to be associated with broad geographic ranges and elevated levels of morphological variation (e.g., *V. dentatum*, *V. tinus*, *V. odoratissimum*, and *V. plicatum*).

We found that genome size is correlated with chromosome number and with guard cell length. However, even with 5-fold variation in genome size, we did not find consistent support for recently proposed links between genome size and other key functional traits. Overall, we conclude that genome size and chromosome number are not strong drivers of ecophysiological evolution

in *Viburnum*, and suspect that similar results will emerge in other lineages. As comparative analyses continue to increase in size and scope, patterns that emerge from these broadly sampled studies are often interpreted to be relevant at all scales, and often are referred to as the "major drivers" of evolution (Wright et al., 2004; Simonin and Roddy, 2018; Roddy et al., 2020). However, we have documented multiple cases in which global trait correlations do not hold up within individual smaller clades (Edwards, 2006; Edwards et al., 2014, 2015, 2017), and have highlighted the need to address the connection, or the lack of connection, across the different scales at which we engage with comparative data (Donoghue and Edwards, 2019). In the case of *Viburnum* and genome size, newly identified large-scale patterns appear to have little relevance to the evolutionary dynamics that we infer. Rather, our analyses of *Viburnum* evolution seem consistent with the view that plants can readily accommodate genomic doubling (Levin, 1983, Leitch and Leitch, 2008, Pellicer et al, 2018) and that this need not impose a major constraint on diversification or trait evolution. In contrast to the recent emphasis placed on the importance of genome reduction in the evolutionary success of angiosperms (Simonin and Roddy, 2018), one could easily argue the opposite: plants demonstrate a remarkable tolerance to drastic changes in ploidy and genome size, which must have played a critical role in their success, allowing for the generation of new species, novel genetic material, and subsequent evolutionary innovation.

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AUTHOR CONTRIBUTIONS

M.M., M.J.D., and E.J.E. designed this research, interpreted the data, and wrote this manuscript. M.M., D.C., M.J.D., and E.J.E. collected plant materials. M.M. generated genome size estimates and chromosome counts, while D.C. provided leaf anatomical data. M.M. analyzed the data.

DATA AVAILABILITY

Measured and modeled values for A_{max} were obtained from Supplemental Table 1 (Chatelet et al., 2013).

SUPPORTING INFORMATION

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

APPENDIX S1. *Viburnum* genome sizes, genome size accessions, chromosome counts, and base genome sizes.

APPENDIX S2. Posterior probabilities of node values from chromEvol ancestral state reconstruction of chromosome number.

APPENDIX S3. *Viburnum* leaf trait data including vein density, stomatal density, guard cell length, and accessions.

APPENDIX S4. Table listing phylogenetic correlations between traits under different evolutionary models.

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APPENDIX 1. Accession information for genome size measurements. (Species, location, accession/collection number.)

V. hartwegii , Yale Greenhouse, MKM45. ***V. jucundum***, Yale Greenhouse, MKM46. ***V. lautum***, Yale Greenhouse, MKM47. ***V. acutifolium***, Yale Greenhouse, MKM48. ***V. sulcatum***, Yale Greenhouse, MKM49. ***V. blandum***, Yale Greenhouse, MKM50. ***V. caudatum***, Yale Greenhouse, MKM051. ***V. microcarpum***, Yale Greenhouse, MKM52. ***V. stenocalyx***, Yale Greenhouse, MKM53. ***V. dentatum***, Arnold Arboretum, 5070-1-A. ***V. recognitum***, Yale Grounds, MKM44. ***V. molle***, Arnold Arboretum, 18294-A. ***V. bracteatum***, Arnold Arboretum, 1067-87-B. ***V. dilatatum***, Yale Grounds, MKM43. ***V. wrightii***, Arnold Arboretum, 825-63-A. ***V. erosum***, Arnold Arboretum, 963-85-A. ***V. setigerum***, Arnold Arboretum, 305-2002-A. ***V. betulifolium***, Arnold Arboretum, 255-2001-B. ***V. hupehense***, Arnold Arboretum, 362-95-B. ***V. melanocarpum***, Arnold Arboretum, 386-81-D. ***V. acerifolium***, Arnold Arboretum, 584-2008-D. ***V. cylindricum***, Berkeley, 93.1371. ***V. opulus***, Arnold Arboretum, 352-78-A. ***V. sargentii***, Arnold Arboretum, 1922-80-C. ***V. trilobum***, Arnold Arboretum, 361-2006-C. ***V. edule***, Yale Greenhouse, MKM42. ***V. propinquum***, US National Arboretum, USNA_49604-J. ***V. davidii***, Berkeley, 2012.0211. ***V. tinus***, Arizona, MJD_2018-AZ-1. ***V. erubescens***, Arnold Arboretum, 798-65-A. ***V. henryi***, US National Arboretum, USNA_67754-T. ***V. farreri***, Arnold Arboretum, 293-2003-C. ***V. suspensum***, Arizona, MJD_2018-AZ-2. ***V. odoratissimum***, Arizona, MJD_2018-AZ-3. ***V. sieboldii***, Arnold Arboretum, 616-6-B. ***V. plicatum***, Arnold Arboretum, 933-4-A. ***V. bitchiuense***, Arnold Arboretum, 1797-77-A. ***V. carlesii***, Arnold Arboretum, 2163-65-A. ***V. utile***, US National Arboretum, USNA_64876-J. ***V. macrocephalum***, US National Arboretum, USNA_sn. ***V. rhytidophyllum***, Arnold Arboretum, 1386-82-B. ***V. veitchii***, Arnold Arboretum, 457-94-A. ***V. lantana***, Arnold Arboretum, 206-96-B. ***V. burejaeticum***, Arnold Arboretum, 375-97-A. ***V. prunifolium***, Arnold Arboretum, 237-2006-A. ***V. rufidulum***, Arnold Arboretum, 21418-A. ***V. cassinoides***, Arnold Arboretum, 109-79-B. ***V. furcatum***, Arnold Arboretum, 17988-A. ***V. lantanoides***, Arnold Arboretum, 599-2008-B.

APPENDIX 2. Accession information for leaf anatomy measurements. (Species, location, accession(s).)

V. acerifolium , Arnold Arboretum, NA. ***V. betulifolium***, Arnold Arboretum, 255_2001A. ***V. bracteatum***, Arnold Arboretum, 1067_87, 6119A. ***V. cylindricum***, Washington Park Arboretum, 75_91. ***V. dentatum***, Arnold Arboretum, 352_95, 5070_1B, 5070_1C, 101_38A, 293_85A, 268_85A, 269_32A, 1800MASS. ***V. dilatatum***, Arnold Arboretum, 138_52A,

821_85, 20449A ***V. erubescens***, Arnold Arboretum, 798_65_A. ***V. farreri***, Washington Park Arboretum, 1190_49. ***V. furcatum***, Arnold Arboretum, 17988A, 17988B, 17988C ***V. hupehense***, Arnold Arboretum, 80_81B, 1748_80B, 1985_80C. ***V. molle***, Arnold Arboretum, 67_2000A, 67_2000B, 4643_1A. ***V. opulus***, Arnold Arboretum, 352_78C, 362_78E, 873_85A. ***V. rufidulum***, Arnold Arboretum, 3943_1D, 21418A. ***V. sieboldii***, Arnold Arboretum, 616_6A, 616_6B. ***V. trilobum***, Arnold Arboretum, 1097_60A, 1097_60B, 22900A. ***V. wrightii***, Arnold Arboretum, 1825_77A, 1825_77B, 1825_77C. ***V. bitchiuense***, Arnold Arboretum, 1097_77A, 2047_77A, 2047_77B. ***V. burejaeticum***, Arnold Arboretum, 375_97A, 397_97C. ***V. carlesii***, Arnold Arboretum, 892_61A, 17981_2A. ***V. cassinoides***, Arnold Arboretum, 18_79A, 109_79A, 874_85A. ***V. erosum***, Arnold Arboretum, 619_88_A, 79_80. ***V. lantana***, Arnold Arboretum, 206_96A, 206_96B. ***V. plicatum***, Arnold Arboretum, 933_4A, 18061A. ***V. propinquum***, Washington Park Arboretum, 136_67. ***V. prunifolium***, Arnold Arboretum, 1189_85B, 1910_81A, 22586A. ***V. rhytidophyllum***, Arnold Arboretum, 57_81B, 1386_82A. ***V. sargentii***, Arnold Arboretum, 398_68B, 1922_80C. ***V. setigerum***, Arnold Arboretum, 305_2002A, 1635_80A. ***V. tinus***, Washington Park Arboretum, 242_63. ***V. utile***, Washington Park Arboretum, 656_49. ***V. veitchii***, Arnold Arboretum, 101_81B, 101_81C, 457_94A.