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Research

Multiple origins of lipid-based structural colors contribute to a gradient of fruit colors in *Viburnum* (Adoxaceae)

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Summary

- Structural color is poorly known in plants relative to animals. In fruits, only a handful of cases have been described, including in *Viburnum tinus* where the blue color results from a disordered multilayered reflector made of lipid droplets. Here, we examine the broader evolutionary context of fruit structural color across the genus *Viburnum*.
- We obtained fresh and herbarium fruit material from 30 *Viburnum* species spanning the phylogeny and used transmission electron microscopy, optical simulations, and ancestral state reconstruction to identify the presence/absence of photonic structures in each species, understand the mechanism producing structural color in newly identified species, relate the development of cell wall structure to reflectance in *Viburnum dentatum*, and describe the evolution of cell wall architecture across *Viburnum*.
- We identify at least two (possibly three) origins of blue fruit color in *Viburnum* in species which produce large photonic structures made of lipid droplets embedded in the cell wall and which reflect blue light.
- Examining the full spectrum of mechanisms producing color in pl, including structural color as well as pigments, will yield further insights into the diversity, ecology, and evolution of fruit color.

Introduction

Color in nature mediates a wide variety of biotic interactions, including mate selection and predation, as well as pollinator and seed disperser attraction (van der Pijl, 1969; Schaefer & Ruxton, 2011). Most plant colors are produced by pigments, chiefly anthocyanins and carotenoids (Glover & Whitney, 2010), but a growing number of plants are known to use structural color either instead of, or in addition to, pigments. In contrast to the broadband reflection of wavelengths of light by pigments, structural color results from a physical interaction between light and nanostructures on the surface of an object that selectively reflects a (typically) narrow band of wavelengths (Glover & Whitney, 2010; Vignolini *et al.*, 2013). Physical structures can create a diverse variety of optical phenomena in addition to color, including true iridescence (where the perceived color depends on the angle of observation), glossiness, enhanced color saturation, and polarized light reflection (Vignolini *et al.*, 2012b; Moyroud & Glover, 2017; Wilts *et al.*, 2018). Structural colors have been extensively investigated in birds, beetles, and butterflies (Prum *et al.*, 1999, 2006; Vukusic *et al.*, 1999; e.g. Seago *et al.*, 2009; Noh *et al.*, 2010; Stavenga *et al.*, 2015), with new mechanisms still being discovered regularly (e.g. McCoy *et al.*, 2018).

Plants, however, have been neglected in comparison with animals, and structural color in plants appears to be rarer with fewer species and fewer types of structures. In flowers, the most common type of photonic structure so far described is iridescence produced by diffraction gratings (quasi-ordered striations in the cuticle of epidermal cells). For example, the iridescent blue halo produced by diffraction gratings on the surface of the pigmented flower spot of *Hibiscus trionum* enhances flower salience (Whitney *et al.*, 2009; Vignolini *et al.*, 2015; Moyroud *et al.*, 2017). Structural effects that produce high directional reflectivity and enhance the color of the underlying pigments have also been described in flowers of the California poppy (*Eschscholzia californica*; Wilts *et al.*, 2018), buttercup (*Ranunculus repens*; Vignolini *et al.*, 2012c), and mirror

orchid (*Ophrys speculum*; Vignolini *et al.*, 2012a). In leaves, two main mechanisms have been described: multilayer reflectors (Thomas *et al.*, 2010; Strout *et al.*, 2013), and photonic crystals (Jacobs *et al.*, 2016).

In fruits and seeds, only six origins of structural color have been identified to date. The fruits of *Pollia condensata* (Vignolini *et al.*, 2012b) and the endocarp (hardened inner wall of the ovary enclosing the seed) of *Margaritaria nobilis* (Vignolini *et al.*, 2016) both produce blue color by helicoidal assembly of cellulose microfibrils in the cell walls. *Elaeocarpus angustifolius* (Lee, 1991) and *Delarbrea michieana* (Lee *et al.*, 2000) use iridosomes, although these structures are poorly understood. In *Viburnum tinus* (Middleton *et al.*, 2020) and *Lantana strigocamara* (Sinnott-Armstrong *et al.*, 2022b), a disordered multilayer reflector constructed of lipids has evolved convergently, creating the metallic blue color of the fruits of these species. The presence of lipids also makes the fruits of *V. tinus* nutritionally rich, suggesting that this photonic structure may serve as an honest signal of a lipid-rich food source (Sinnott-Armstrong *et al.*, 2020a).

Our limited knowledge of structural color in plants raises the question of whether structural colors are truly extremely rare in plants relative to animals, or whether the paucity of known structural colors in plants is due, at least in part, to a lack of research. To test whether structural color may be more widespread than previously appreciated, we selected one clade (*Viburnum*) in which blue fruit color has evolved multiple times (Sinnott-Armstrong *et al.*, 2020a). *Viburnum* contains *c.* 165 species of mostly temperate, Northern Hemisphere shrubs and small trees that produce drupe fruits with a single seed surrounded by a hardened endocarp (Jacobs *et al.*, 2008; Clement *et al.*, 2021). *Viburnum* has a number of advantages as a system for studying structural color evolution, including that comprehensive phylogenetic information exists (Clement *et al.*, 2014; Spriggs *et al.*, 2015; Landis *et al.*, 2020), species vary greatly in their fruit colors, and functional fruit traits have been studied extensively (Sinnott-Armstrong *et al.*, 2020a; Clement *et al.*, 2021).

Here, we test whether the independent origins of blue fruit color in *Viburnum* are structural colors or pigmentary colors, and, if structural, whether colors are produced by the same type of nano-architecture. Our goals are to: survey cell wall architecture across *Viburnum*; characterize the morphology and development of the epicarp structure in another blue-fruited species, *Viburnum dentatum*; conduct optical simulations of the structure found in *V. dentatum*; and finally trace the evolution of epicarp cell wall architectures across *Viburnum*.

Materials and Methods

Sampling

We obtained ripe fruit material from 24 species of *Viburnum* L. (Adoxaceae, Dipsacales), and supplemented these with herbarium samples of six additional species. Ripe fruits were obtained from the Royal Botanic Gardens, Kew, the Arnold Arboretum of Harvard University, the Cambridge University Botanic Garden, and the Berkeley Botanical Garden, as well as from the campuses of Yale University and the University of Cambridge (accession nos. in Table S1). Supplementing these fresh fruits with herbarium specimens from the Yale Herbarium enabled us to add key species, including one of the earliest diverging *Viburnum* species (*V. clemensiae*), the sole yellow-fruited species (*V. amplificatum*), and additional species from the poorly sampled Central and South American *Oreinotinus* radiation (*V. venustum*, *V. pichinchense*, and *V. jucundum*) (Fig. 1).

Color reflectance

We measured the reflectance of immature and mature *V. dentatum* fruits using a Jaz spectrometer (Ocean Optics, Dunedin, FL, USA) equipped with a deuterium–halogen lamp and a UV detector. To hold the probe at 45° consistently across samples, we used an anodized aluminum probe holder, which also eliminated background radiation. Reflectance measurements were calibrated against a Spectralon white standard (Labsphere, North Sutton, NH, USA). After obtaining measurements, we used the R package PAVO (Maia *et al.*, 2019) to smooth the reflectance curve to 5-nm bands. We modeled the color of the fruits in bird visual space (using a UV-sensitive model visual system, 'avg.uv' in the PAVO package) and calculated the sum of shortwave (400–500 nm) reflectance for each fruit. We chose shortwave reflectance rather than UV reflectance because in *V. dentatum*, the reflectance drops precipitously below *c.* 390 nm, so shortwave reflectance more accurately captures the change in blue color during development.

Transmission electron microscopy

Fruits were dissected into 2 × 2 × 1 mm pieces and then placed in Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M buffer). The material remained in fixative until embedding following the protocol in Vignolini *et al.* (2012b). In brief, fruits were stained with osmium tetroxide for 2 h and then taken through a graduated ethanol–resin series before embedding in 100% LR White resin. Resin blocks were polymerized for 24 h under vacuum at 60°C. Following polymerization, we used a Leica UCT or UC7 ultramicrotome to cut ultrathin sections (70–100 nm) with a diamond knife. Sections were imaged on a Hitachi H-7650 transmission electron microscope (Hitachi High-tech, Tokyo, Japan) and a JEM 2100 Plus (Jeol Ltd, Tokyo, Japan) equipped with an RIO 16 CMOS camera (Gatan Inc., Pleasanton, CA, USA). The developmental sequence of *V. dentatum* fruits followed the same protocol, except that fruits were embedded in Epon 814 resin, with DMP added as an accelerator before drying under room air pressure at 60°C. We imaged sections of these fruits on an FEI T12 transmission electron microscope with an FEI Eagle 4 k × 4 k CCD.

We created serial tomograms of *V. dentatum* fruits by cutting thick sections (300 nm) and imaging on an FEI F30 transmission electron microscope through a tilt series from −60° to 60°, imaging every 1°. We processed and combined the serial tomograms using the IMOD software (Kremer *et al.*, 1996) following the methods described in Middleton *et al.* (2020). Serial tomograms are here displayed using an isosurface reconstruction. The

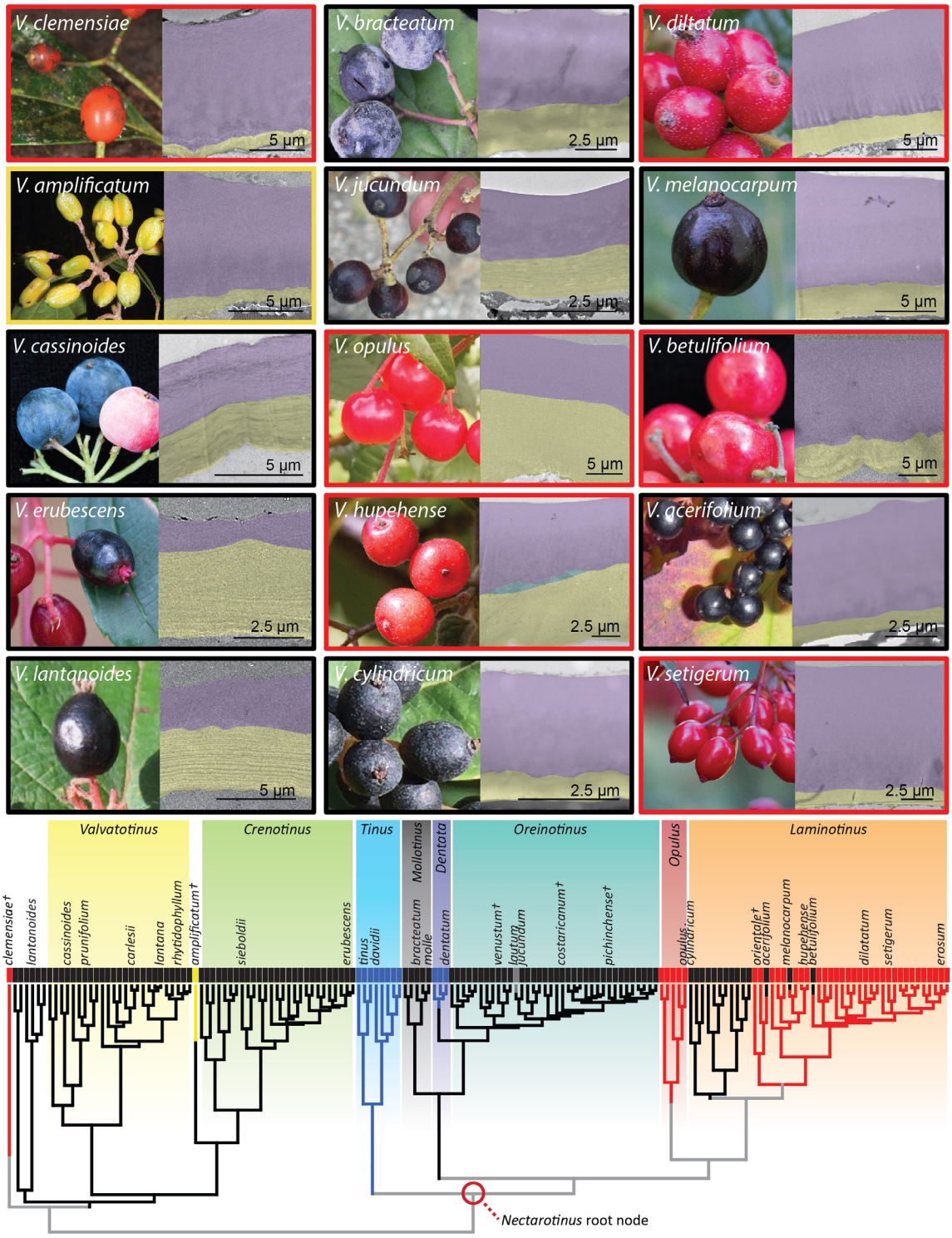

Fig. 1 Fruits of *Viburnum* species with typical epicarps. We used transmission electron microscopy (TEM) to characterize the cell wall structure of 30 species of *Viburnum*. Here, we illustrate a subset of those species with typical epicarps (cell wall and cuticle, no photonic structure) with photographs and TEM images of their cell wall architecture. Boxes around the images for each species correspond to the mature fruit color of that species. Several species (e.g. *V. cassinoides*) appear blue to humans due to a waxy bloom that causes increased reflectance in the UV region of the spectrum; thus, these fruits reflect both UV and blue light, which can be detected by UV-sensitive bird species (see Fig. S5; data from Sinnott-Armstrong *et al*., 2020a). TEM images have been given false color: purple, cuticle; yellow, typical cell wall; teal, globular region. The species studied are phylogenetically distributed across *Viburnum*. Species labeled on the phylogeny are those studied here (†, samples derived from herbarium specimens; all others are from fresh fruit material). Branches and tip labels show ancestral state reconstructions and mature fruit color (gray branches, equivocal reconstructions), respectively, using data from Sinnott-Armstrong *et al*. (2020a). Here, we have collapsed the two black-fruited categories from that study (black-sequentially developing and black-synchronously developing).

embedding and imaging process was the same for fresh and dried material, except that we allowed the dried fruits to rehydrate in water for 48 h before dissection and fixation.

Chemical characterization

To determine whether the structure in *V. dentatum* epicarp consisted of lipid globules, we followed the protocol in Sinnott-Armstrong *et al.* (2020b). In brief, we cryo-cut ultrathin sections of fresh *V. dentatum* fruit tissue and exposed the tissue to chloroform in a reflux extractor. We imaged the fruit before and after chloroform exposure in order to visualize the contrast change as a result of the extraction of materials due to exposure to an organic solvent.

Optical simulations

To characterize the structure in the cell walls of *V. dentatum* epicarp, we simulated the structure as though it were a highly disorganized version of the multilayered structure found in *V. tinus* (Jacucci *et al.*, 2019; Middleton *et al.*, 2020). As reported in Middleton *et al.* (2020), even highly disordered structures do reflect some blue light (see Fig. S1). Thus, we approximated the structure in *V. dentatum* as a highly disordered version of the structure in *V. tinus*. We took both a 2D and a 1D approach, both relying on the analysis of the amount of disorder.

In the 2D approach, we modeled the optical properties of the structure in *V. dentatum* by visualizing a 2D arrangement of spheres using high angular disorder. Angular disorder represents the angle between the center of one globule and that of adjacent globules. A low angular disorder results in globules forming a relatively flat line; high angular disorder results in more variation in the placement of globules such that they do not form a consistent flat layer. In *V. dentatum*, the globules generally do not form ordered layers, so we modeled the distribution of globules with a high angular disorder (where sigmaPhi (*Sp*) = 20). The sizes of the spheres for each species were obtained from transmission electron microscopy (TEM) images.

We then investigated the effect of globule density on reflectance, with densities ranging from 0.1% to 50%. We generated five structures of each density using code from Jacucci *et al.* (2019) and Middleton *et al.* (2020) and simulated reflectance from those structures using commercial software ('Lumerical FDTD Solutions'; see Jacucci *et al.*, 2019; Middleton *et al.*, 2020 for more details). At each globule density, we used the same size globules as we measured for *V. dentatum* (mean globule diameter = 129 nm).

In the 1D approach, we modeled the material as a multilayer with alternating cellulose (n = 1.55; Cranston & Gray, 2008) and lipid-refractive indices (dispersive refractive index; Kumar *et al.*, 2018). The distribution of layer thicknesses of both phases was measured from TEM profiles of *V. tinus* and *V. dentatum*, and this distribution of sizes was used for the 1D optical simulations. Monte Carlo simulations were produced using the layer distribution probabilities from the measured profiles (modeled as log-normal distributions) and scaled within 40% of measured values, assuming expansion in TEM preparation of *V. dentatum* due to osmotic pressure (King, 1991). The number of layers per species was approximated from TEM images (*V. tinus*, 120 layers, and *V. davidii*, 300 layers, with a compression of measured layer thickness by 10%, i.e. 90% of length measured). In *V. dentatum*, we used 60 layers and applied a compression of 30%, that is, 70% of measured layer thickness. Compression was applied equally to both phases, because it is unknown how the different materials are affected. The spectra were generated through averaging over sufficient randomly generated profiles to produce a smooth spectrum (*V. dentatum* 100 profiles, *V. tinus* 10 profiles, and *V. davidii* five profiles; these differ due to the much larger layer number in *V. davidii* than in *V. dentatum*). The same scaling factor (×90) was applied to all three models in order to better match the intensity of the modeled to the experimental data.

Developmental sequence

To characterize the development of cell walls in the epicarp of *V. dentatum*, we obtained fresh fruits at varying stages of fruit development and sampled four times between July and August 2021. For each fruit, we measured length and width using Mitutoyo calipers. We measured color reflectance for each fruit and selected a subset for embedding and TEM imaging, using the methods described in the previous section.

Ancestral state reconstructions

To quantify evolutionary patterns in cell wall architecture, we measured the thickness of the major regions of the epicarp cell wall from TEM images, across all sampled species and *V. dentatum* developmental stages, using the program Fiji (Schindelin *et al.*, 2012). We distinguished three main regions: the cell wall, the globular region (where present), and the cuticular region. We made three measurements per cell on at least three cells per species (except *V. jucundum* and *V. venustum*, where only two or one cell was measured, respectively) and averaged these measurements per species. We then performed stochastic character mapping, averaging across 1000 iterations, to infer trait evolution in *phytools* (Revell, 2012). We classified species into 'no globules', 'few globules, insufficient for color', and 'structural color' (where globular layer thickness = 0, 0–2, and > 2 μm, respectively).

Results

Cell wall architecture

Across *Viburnum*, we identify several distinct types of cell wall architectures in the epicarp tissue, which are associated with different fruit colors. Overall, *Viburnum* fruit cell walls vary along several axes: presence or absence of globules; degree of organization of those globules; density of the globules; and thickness of the globular region of the cell wall. The majority of *Viburnum* species sampled here have typical epicarp cells, with only a cuticle and a cell wall entirely lacking in globules (Fig. 1). Species with typical epicarp tissue are phylogenetically distributed across

Viburnum (Fig. 1) and display diverse mature fruit colors including black, red, and yellow. The fruit color of these species is solely due to pigments, as they lack globular regions (photonic structures) in the cell wall.

By contrast, blue-fruited *Viburnum* species have a modified cell wall architecture: they produce a thick globular region between the cell wall and the cuticle (Fig. 2). This globular region consists of tiny globules embedded in what appears to be a cell wall-like matrix. In *V. tinus* and *V. davidii*, the globular region contains a large, multilayered structure. This structure was previously described in *V. tinus* and shown to be made of lipids (Middleton *et al.*, 2020), but here we show that closely related species produce a similar structure, which can be even larger than that observed in *V. tinus* (*V. tinus* mean thickness = 16.7 ± 1.5 μm, *V. davidii* mean thickness = 31.8 ± 3.3 μm).

In the *Dentata* + *Oreinotinus* clade, some species have black fruits and display the typical cell wall plus cuticle architecture (e.g. *V. jucundum*; Fig. 1). Other species produce a globular structure of varying sizes (Fig. 2), from relatively thick (*V. dentatum*; mean = 7.1 ± 0.3 μm) to thin (*Viburnum lautum*; mean = 3.4 ± 1.7 μm) or very thin and sparse (*V. venustum*; mean = 1.8 ± 2.1 μm, Fig. S2). The globular structures produced in the *Dentata* + *Oreinotinus* clade are much more disordered than those produced by species in the *Tinus* clade (Fig. 2), and in some cases, the globular region is much thinner and/or does not produce densely packed globules resulting in less intense blue coloration in the fruit.

Chemical composition and 3D architecture of the photonic structure in *V. dentatum*

The globules in the cell wall of *V. dentatum* fruits are highly soluble in chloroform (Fig. 2e,f), indicating that they are lipids, although the exact type of lipids remains unknown. The photonic structure in *V. dentatum* does not appear to have any clear 3D order (Fig. 2g), further confirming the relatively disordered nature of the photonic structure.

Optical models and globule density

To test whether interspecies variations in the architecture of the photonic structure could account for differences in the appearance of the fruits, we investigated the effect of variable organization of lipidic globules on the optical properties of the epicarp.

We found that globule density strongly influences reflectance. Reflectance of blue light from the disordered structures simulated here is maximized at *c.* 30% globule density (Fig. 3). Lower globule densities rapidly reduced the amount of blue light reflected, while higher densities also reduced reflectance, although to a lesser extent. This variation corresponds with the observed variation in *Viburnum*: species with very low (e.g. *V. lantana*) and very high (e.g. *V. lautum*) globule densities have reduced reflection of blue light, while species with intermediate filling fractions (e.g. *V. dentatum*) have higher reflectance of blue light (Fig. 3).

Reflection microscopy from the fruit surface (Fig. S3) shows that intracell variation in reflectance (e.g. microdomains where some regions of the cell wall appear bluer and others greener) occurs within epicarp cells and contributes to overall reflectance from the fruit. Variations in layer density and order are also observed as prominent features of the TEM profiles, especially for *V. dentatum* (see Fig. 2). The 1D multilayer simulation used unweighted averages of Monte Carlo simulations from a measured distribution of profile elements with a compression factor. The models were generated to match the experimentally measured optical profiles using a reasonable principle that compression artifacts may be different for different species (because the chemical composition and local mechanical properties differ across species, compression introduced by embedding and sectioning may also differ across species). The inclusion of layered profiles with extremely high degrees of relative randomness, as observed experimentally, produces a broad and asymmetric optical reflectance peak, similar to the asymmetrical reflectance measured from the fruits (Fig. S3).

Overall, these simulations confirm that even very disordered structures reflect some blue wavelengths (Fig. S1) and demonstrate that the density of globules forming the photonic structure influences the amount of reflectance of blue light that occurs on the fruit surface (Fig. 3), and that variation within a cell wall contributes to the asymmetrical reflectance spectrum of the fruits (Fig. S3).

Development of the photonic structure in the epicarp of *V. dentatum* fruits

A detailed examination of cell wall organization throughout fruit development in *V. dentatum* demonstrates the accrual of lipid droplets embedded in the epicarp cell wall as fruits grow larger and rounder during maturation (Fig. 4). The increase in thickness of the globular layer over time is associated with a corresponding increase in blue reflectance (represented by shortwave reflectance, the sum of reflectance from 400 to 500 nm; Fig. 4a–d). Small fruits are oblong and exhibit little-to-no shortwave reflection, but over time they become larger and rounder (Fig. 4e). *Viburnum dentatum* fruits do not begin to accumulate globules until well into fruit development (Fig. 4c–e), when the fruits are already relatively large and round (length : width ratio of *c.* 1.3). At that point, a globular region becomes visible, and shortwave reflectance increases until fruit maturity. In *V. dentatum*, the cell wall (lacking globules) is thick even in young fruits (Fig. 4f), and the globular region is added on top of the cell wall such that the entire cell wall (cell wall lacking globules + globular region) thickens over time.

Evolution of epicarp cell wall architecture across *Viburnum*

We found that the evolution of cell wall architecture in the epicarp is tightly correlated with the degree of blue coloration of *Viburnum* fruits. Globular regions evolved at least four times in *Viburnum* (Fig. 5): (1) in the *Tinus* clade, represented by *V. tinus* and *V. davidii*; (2) in *Dentata* + *Oreinotinus*, represented by *V. dentatum*, *V. lautum*, and *V. venustum*; (3) in *V. lantana* and *V. rhytidophyllum* of the *Valvatotinus* clade; and (4) in *V. hupehense* of the

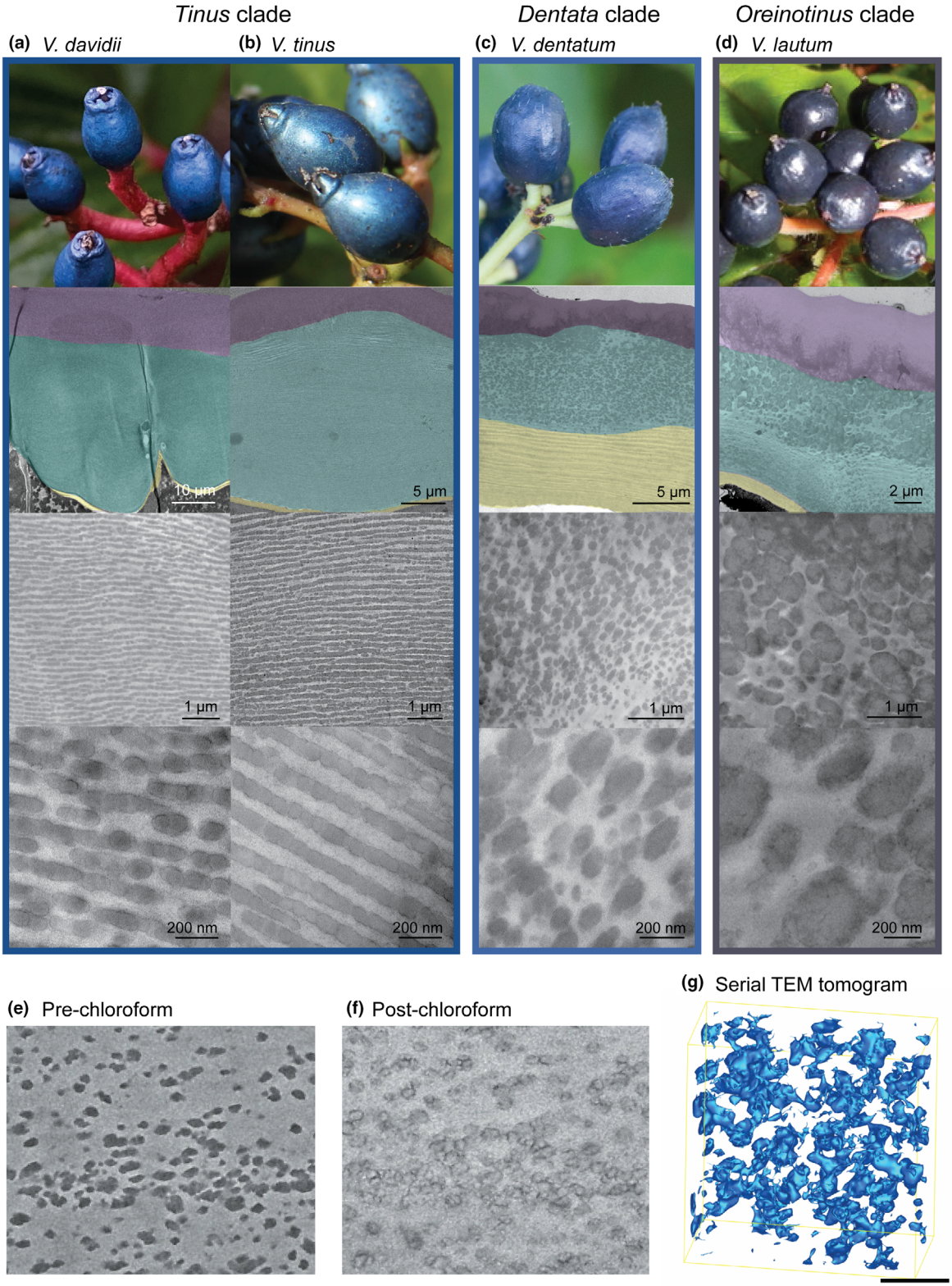

Fig. 2 *Viburnum* species with modified cell wall architectures. (a, b) *Viburnum davidii* and *V. tinus* (from the *Tinus* clade) exhibit similar large, multilayered structures, which produce metallic blue fruit color. (c) *Viburnum dentatum* and (d) *V. lautum* have modified cell wall architecture with large regions of nearly randomly distributed globules; these structures are much thinner on average than the multilayered reflectors in the *Tinus* clade. (e, f) The globules in *V. dentatum* are soluble in chloroform, indicating that they are lipidic: before chloroform treatment (e) dark, dense globules are visible, while after chloroform exposure (f), they have disappeared. (g) There is no 3D order to the structure of *V. dentatum*. Bar, 500 nm.

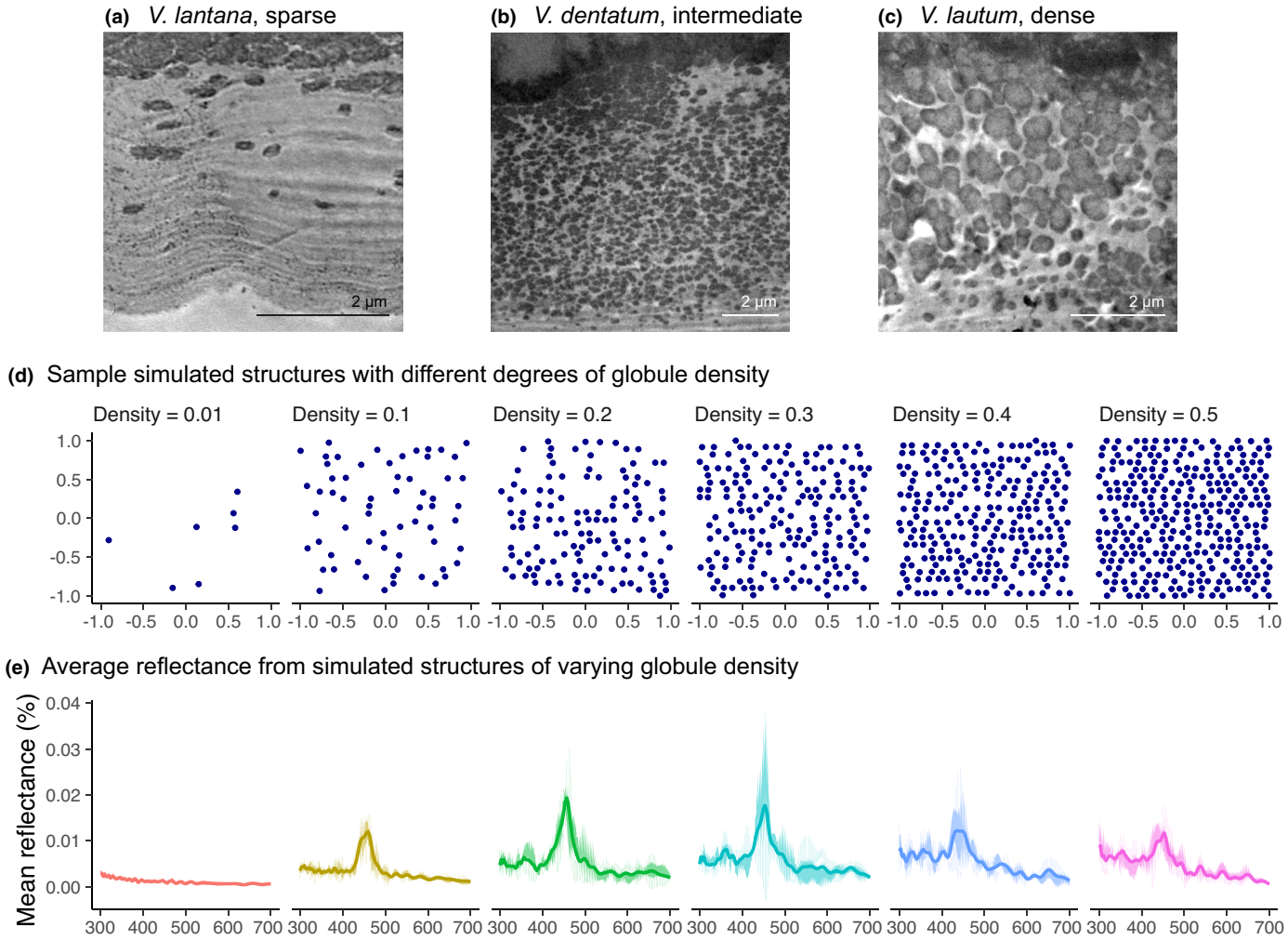

Fig. 3 Different *Viburnum* species, with modified cell wall architectures, exhibit variable globule densities: (a) some species produce very sparse globules (e.g. *V. lantana*), (b) some have intermediate density (e.g. *V. dentatum*), and (c) others have densely packed globules (e.g. *V. lautum*). (d) Simulations of structures with variable globule densities, where each panel represents a sample simulated structure with blue dots indicating the locations of simulated globules, and (e) the reflectances corresponding with simulated structures of varying densities (error bars represent one SD above and below the mean). Low densities, where the proportion of the structure that is filled by globules is low (a low filling fraction, where density = 0.01, 0.1), result in low reflectance of blue wavelengths. Intermediate densities (density = 0.2, 0.3) produce the highest reflectance of blue wavelengths, while high densities (density = 0.4, 0.5) again produce lower reflectance of blue wavelengths.

Laminotinus clade. Two origins of globular regions (3 and 4) have minimal globules that are sparsely distributed and insufficient to generate blue structural color, as shown by our optical simulations of structures with low globule density. The other two origins, in *Tinus* and *Dentata* + *Oreinotinus*, have much larger, thick globular regions and reflect blue light. Within *Dentata* + *Oreinotinus*, there are some species with well-developed globular regions (e.g. *V. dentatum* and, *V. lautum*) and others lacking globules (e.g. *V. jucundum* and *V. pichinchense*). It is uncertain how many times globular regions evolved in *Dentata* + *Oreinotinus*: they may have evolved once and been lost multiple times, or they may have evolved independently three times (in *Dentata*, *V. lautum*, and *V. venustum*). If globules evolved three times, then globular regions would have evolved at least six times across *Viburnum* although not all origins are sufficient to produce structural color.

Ancestral state reconstructions (Fig. 5) suggest that structural colors evolved at least three times in *Viburnum* (in the *Tinus* clade, in the *Dentata* clade, and in *V. lautum*) and that globular regions insufficient to influence color evolved three additional times (in *V. venustum*, *V. hupehense*, and *V. lantana* plus *V. rhytidophyllum*). These results depend on the method of reconstruction used, and alternative methods of ancestral state reconstruction suggest multiple losses of structural color in the *Oreinotinus* clade, rather than multiple independent origins (Fig. S4). Regardless, it is clear that the deposition of globules into the epicarp cell wall is a labile trait across *Viburnum* and has, in several instances, evolved sufficient density, order, and/or size to reflect blue light.

Discussion

We identify and describe here at least two, and possibly three, independent origins of structural color in *Viburnum* fruits, both using similar materials (lipid globules embedded in the cell wall)

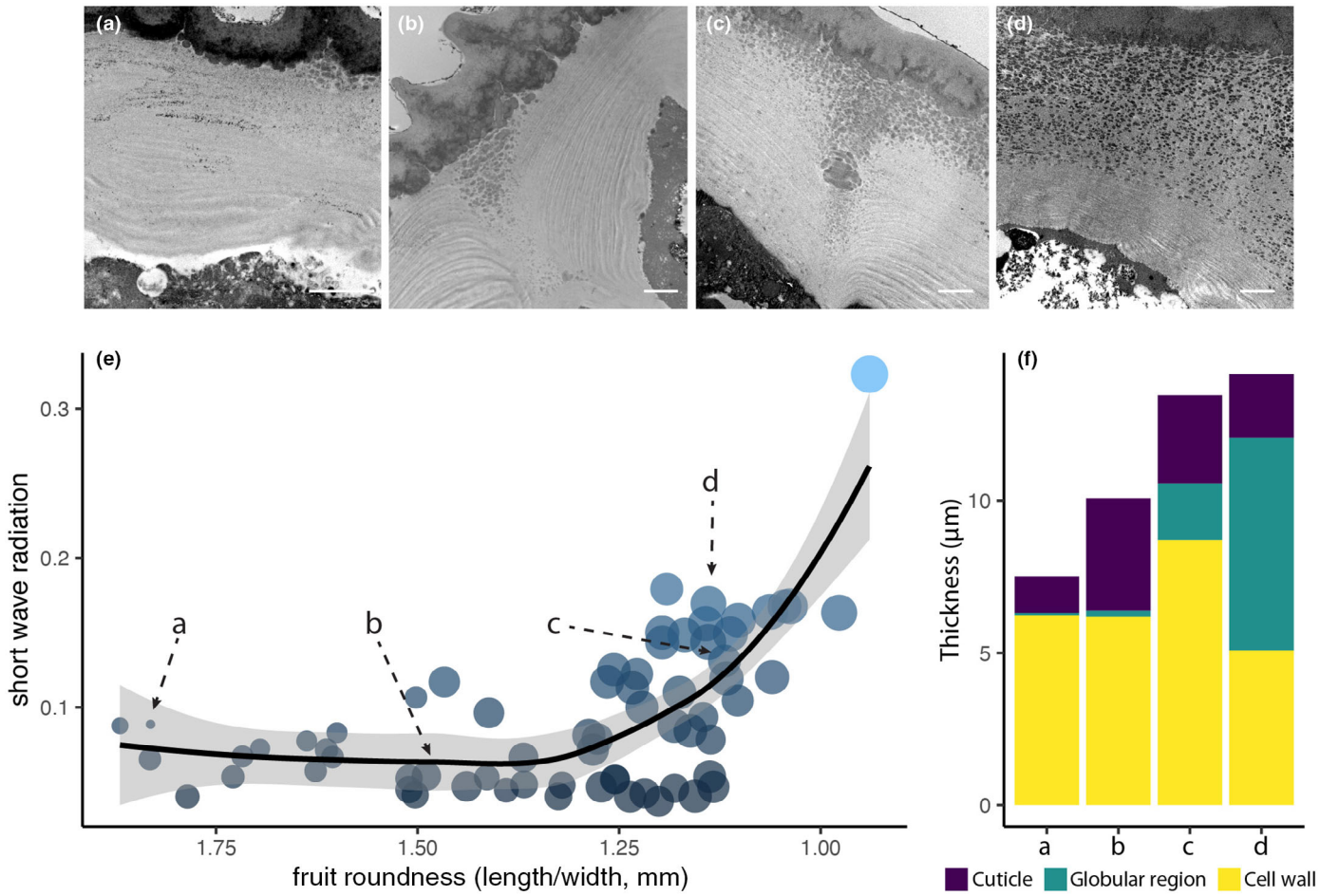

Fig. 4 Development of the disordered structure in *Viburnum dentatum* begins before fruit maturation. We characterized the architecture of epicarp cell walls during the development of *V. dentatum* fruits using transmission electron microscopy (TEM), as well as measurements of fruit size and color. (a–d) TEM images of epicarp cells at various fruit developmental stages from youngest (a) to oldest (d) show that as fruits mature, globules accumulate in the cell wall, and the globular region thickens. (e) Measurements of fruit size and reflectance of 64 immature fruits indicate that the accumulation of globules is associated with other changes in fruit size and color. As fruits grow rounder (length : width ratio closer to 1.0) and larger, they reflect more in the 400–500 nm region of the spectrum. Point sizes indicate relative fruit width for each fruit, black line is a Loess trend line, and gray shading = 95% confidence interval. (f) Cell wall (lacking globules) is > 5 µm thick even in very young fruits, and as the fruit develops the globular region thickens over time while the cell wall remains relatively constant. Bars, 2 µm. The fruits sampled for TEM imaging in (a–d) are indicated with the same letters in each panel of the figure.

with very different degrees of disorder (moderately ordered in the case of *V. tinus* and its relatives and highly disordered in the case of *V. dentatum* and its relatives). Optically, the photonic structure in *V. dentatum* is best understood as a highly disordered version of a multilayer reflector, similar to that of *V. tinus* but with greater disorder. Chemically, the structure in *V. dentatum* also appears similar to that of *V. tinus*: *V. dentatum* uses lipids, which are readily soluble in chloroform, as in *V. tinus* (Middleton *et al.*, 2020). Developmentally, we know little about the accumulation of lipid droplets in cell walls or about the mechanisms that produce the photonic structure previously uncovered in *V. tinus*. However, in *V. dentatum*, fruits grow larger and rounder with no accumulation of globules in the epicarp cell walls until partway through their development, at which point globules begin to accumulate corresponding with the increase in blue reflectance from the fruit. As maturation progresses, the photonic structures become larger and the fruits reflect blue wavelengths of light more strongly.

These results are similar in certain respects to the structure recently reported in *Lantana strigocamara* (syn. *L. camara*), but also differ in important ways. Chemically, the globules in *Viburnum* structural colors are soluble in chloroform, while those in *L. strigocamara* are not (Sinnott-Armstrong *et al.*, 2022b). Developmentally, the epicarp cell wall of *L. strigocamara* follows a different trajectory from that of *V. dentatum*: in *L. strigocamara*, the photonic structure begins to emerge more or less immediately after the cuticle has been deposited (Sinnott-Armstrong *et al.*, 2022b), while in *V. dentatum*, the photonic structure begins to form much later. Thus, although other structurally colored fruits also use lipidic compounds, the associated structures differ morphologically, chemically, and developmentally.

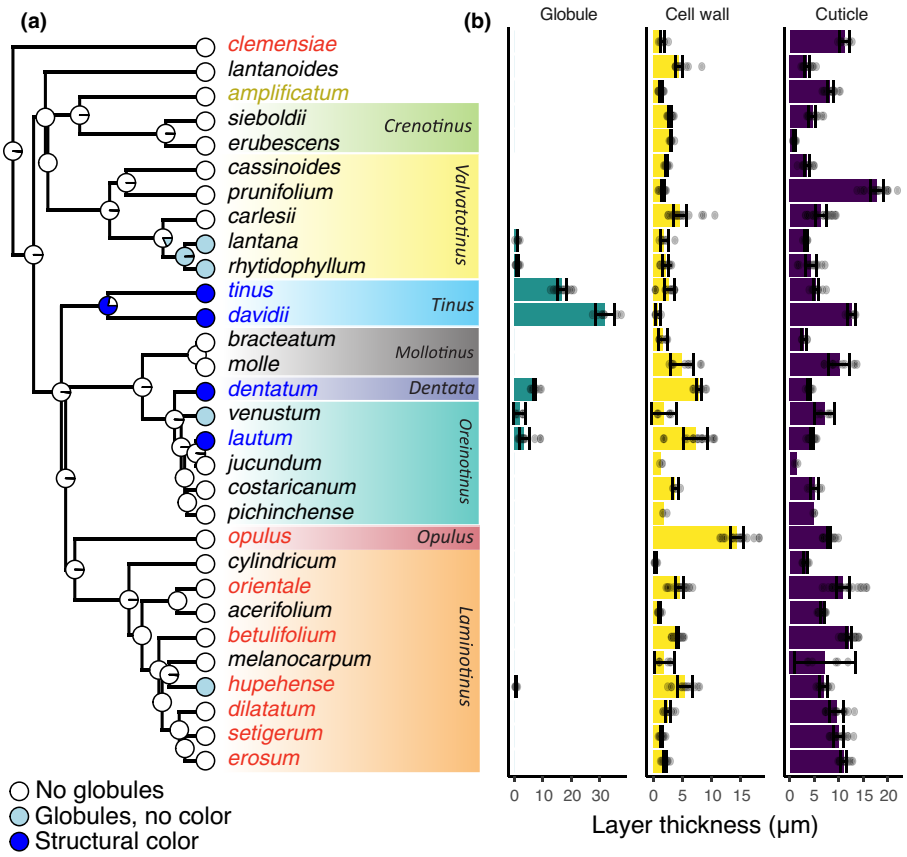

Fig. 5 Evolution of epicarp cell wall architectures across the 30 species of *Viburnum* sampled in this study. (a) Ancestral state reconstructions of globular region thickness indicate that globular regions evolved at least four times: (1) in the ancestor to *V. lantana* and *V. rhytidophyllum* where globules are not sufficiently dense to produce blue color; (2) in the *Tinus* clade, with multilayered reflectors; (3) in *Dentata* + *Oreinotinus*, where there may have been multiple origins and/or losses of structural color and globular regions; and (4) in *V. hupehense*, which has a very thin (< 1 μm) globular region which is neither sufficiently thick nor organized to produce blue color. (b) Globular region thickness co-varies with blue fruit coloration, with the species with the bluest fruit color having the largest globular regions. Cell wall and cuticle thickness also vary across species, with some species displaying very thick cell walls (e.g. *V. opulus*) or cuticle (e.g. *V. prunifolium*). Error bars are ±1 SD around the mean, and dots indicate measurements of each cell wall component.

The evolution of structural colors across *Viburnum*

Based on our knowledge of the *Viburnum* phylogeny, it appears that lipid globules embedded in the cell wall evolved at least four times across the genus. In two instances, globules are produced in a structure that is sufficiently thick, with densely packed globules, to produce structural color (Figs 1, 5). All members of the *Tinus* clade have a similar metallic blue fruit color, and the additional species of the *Tinus* clade that we examined here (*V. davidii*) displays a similar multilayered lipid structure to that observed in *V. tinus*. We recognize eight species in the *Tinus* clade. The three species in Europe, including *V. tinus*, form a clade that is sister to the five species in Eastern Asia, including *V. davidii* (Fig. 1; Landis *et al.*, 2020). From this, we hypothesize that they all inherited the *V. tinus*/*V. davidii* multilayer reflector from the common ancestor of the *Tinus* clade. This supposition is supported by additional observations, including that fruits of species in the *Tinus* clade display a similar syndrome of other traits, including high lipid content, a large round endocarp, and very little pulp that is low in moisture (Sinnott-Armstrong *et al.*, 2020a, 2022a; Clement *et al.*, 2021). We infer that this entire suite of fruit traits originated along the branch subtending the *Tinus* clade before its diversification.

The evolutionary history of the second origin of structural color, in *V. dentatum* and its relatives in the *Oreinotinus* clade, is less clear. *Viburnum dentatum* evolved a moderately thick photonic structure (*c.* 7 μm). The remaining species of the eastern North American *Dentata* clade (including *V. recognitum*, *V. scabrellum*, and *V. venosum*) have similar blue to blue-gray fruit color. We presume that they have a similar structure to that in *V. dentatum*, and that this condition evolved along the branch leading to *Dentata*. *Dentata* is sister to the *Oreinotinus* clade, which contains *c.* 42 species in the cloud forests of Mexico, Central America, and South America (Donoghue *et al.*, 2022). The majority of *Oreinotinus* species have black fruits, but a few have fruits that appear gray or slightly blue. Here, we sampled one of those species, *V. lautum*, and found that it has a structure resembling that observed in *V. dentatum*, but thinner and with a denser packing of globules. The thinner structure and greater density of packing of globules explains the reduced peak of reflectance of blue wavelengths.

It is currently unclear whether a structure like that seen in *V. dentatum* and *V. lautum* evolved along the branch leading to the entire clade that includes *Dentata* and *Oreinotinus* and was subsequently lost several times, or whether it evolved independently multiple times. Additional species, such as *V. venustum*, have epicarp cell walls that resemble that of *V. lautum*, but with a much sparser distribution of globules, which suggests that some features of the *V. dentatum*-like cell wall architecture are likely ancestral. Other species of *Oreinotinus* appear to lack globules entirely (e.g. *V. pichinchense* and *V. jucundum*). The existence of these other species lacking globules requires that the globular structure either evolved independently multiple times (e.g. separately in *V. dentatum* and *V. lautum*, and possibly in other species not

sampled here), or was lost multiple times (e.g. in *V. jucundum* and *V. pichinchense* + *V. costaricanum*).

Ancestral state reconstructions suggest that structural color evolved independently in *V. dentatum* and *V. lautum*, although different methods of reconstruction suggest that the structure may instead have been inherited and lost several times. Determining which scenario occurred will require much more extensive sampling of the *Oreinotinus* + *Dentata* clades and/or additional lines of evidence (such as genetic and/or chemical data). Regardless of the ancestral state, these results suggest that the globular components of the cell wall can be rather easily modified during evolution, thus producing the gradation of structures, density of globules, and macroscopic colors observed in *Viburnum* fruits, ranging from the bright metallic blues of the *Tinus* clade to the dull blue of *Dentata* to the blue-gray color of *V. lautum*.

Why structural colors evolved in *Viburnum* remains an open question. We think it is unlikely to be the result of selection by a single disperser species, or of selection by a class of dispersers with unusual visual systems. Structurally colored *Viburnum* fruits primarily reflect blue light with only a small amount of reflectance of UV light, meaning that the vast majority of dispersers can perceive the colors of these fruits without issue (Cuthill *et al.*, 2000). No special visual apparatus is required, suggesting that selection based on visual systems, as has been implicated in the evolution of fruit colors in general (McKey, 1975; Howe & Estabrook, 1977; Janson, 1983; Valenta & Nevo, 2020), is an unlikely explanation for why and how these structural colors evolved.

Viburnum fruits/seeds are almost entirely dispersed by frugivorous birds, though mammals are implicated in some cases. In general, *Viburnum* plants are visited by more than one bird species. Although European robins (*Erithacus rubecula*) and Eurasian blackcaps (*Sylvia atricapilla*) particularly rely on *V. tinus* fruits, especially during the winter (Herrera, 1981; Jordano & Herrera, 1981), a variety of dispersers consume *V. tinus* fruits (Thebaud & Debussche, 1992). Furthermore, our phylogenetic analyses indicate that the photonic structure evolved long before the immediate ancestor of *V. tinus* moved into the Mediterranean region (Sinnott-Armstrong *et al.*, 2020a). Thus, the modern-day associations of *V. tinus* with certain frugivorous birds do not account for the initial evolution of structural color at the base of the *Tinus* clade, which occurred much earlier in time and likely in Asia.

Viburnum dentatum is also dispersed by a number of bird species (Whelan *et al.*, 1988), but, as with the dispersers of *V. tinus*, these are all common dispersers in the forests of eastern North America (e.g. *Turdus migratorius*, the American robin; *Bombycilla cedrorum*, the cedar waxwing; Sargent, 1990). It seems unlikely that the visual systems of these dispersers played much of a role in selecting for fruit coloration. It is interesting to note, though, that *V. tinus* and *V. dentatum* (and their close relatives) do mature their blue fruits synchronously and provide a pulse of resources for migratory birds in particular (Sinnott-Armstrong *et al.*, 2020a). We suspect that the production of an honest signal to attract the services of such birds (e.g. Middleton *et al.*, 2020; Sinnott-Armstrong *et al.*, 2020a) could well have played a role in the evolution of their distinctive colors.

Signaling function of lipidic structural colors

A remarkable concordance between these results and other recent work is that blue-fruited *Viburnum* species, which use lipid globules embedded in their cell walls to produce their blue color, also have high nutritional lipid content (Sinnott-Armstrong *et al.*, 2020a). This correlation suggests a hypothesis as to the origins of structural color in *Viburnum* fruits: if the synthesis of lipids in the epicarp cell wall is governed by the same genetic mechanism as synthesis of other lipids of the same type (e.g. fatty acids or cuticle), then dispersers may select for either a high lipid nutritional content *or* a blue fruit color, either of which would necessarily result in an increase in the other trait. For instance, if birds preferred lipid-rich nutritional content (Smith *et al.*, 2013, 2015), selection for nutritional lipids could concurrently select for increased lipid accumulation in the cell wall and correspondingly increase the reflectance of blue wavelengths in cases where those lipids are arranged into globules. Alternatively, birds may learn to associate blue color with nutritional lipids and preferentially consume bluer fruits, which in *Viburnum* contain lipid globules in the epicarp cell wall. Blue fruit color would thus become an honest signal of a nutritional reward. Fruits reflecting in the blue bandwidths seen in these fruits, over the range 400–450 nm, should appear blue not only to human visual systems but also in avian visual systems (both with and without UV sensitivity) (Cuthill *et al.*, 2000), and in insects (Chittka & Menzel, 1992).

One curious feature of *Viburnum* structural color and nutrition is the negative correlation between the brightness of the color and how much pulp the fruit produces. The brightest, bluest species (*V. davidii*) has little pulp, while the least blue species (*V. dentatum*) has much more pulp surrounding the seed (Sinnott-Armstrong *et al.*, 2020a; note that although *V. lautum* was classified as black in that study, it is also lipid-rich and, as we show here, has a high-density photonic structure resulting in a blue-gray color). This negative correlation suggests an interaction between color, nutrition, and pulp volume relative to seed size: a brighter color might compensate (by attracting more animal dispersers) for a lower volume of pulp and/or for a less lipid-rich pulp. Once the connection was established between color and lipid content in these plants, there may have been further selection to reduce the amount of pulp.

Although the exact type of lipid used to produce these structural colors is unknown, based on their solubility in chloroform, we suspect that both the *V. tinus* and *V. dentatum* structures utilize fatty acids that could be digestible by animal dispersers. This differs from the condition in *L. strigocamara*, where the structure is likely produced by cuticle-related lipidic polymers, although the exact identity of the lipids remains unknown in that species as well (Sinnott-Armstrong *et al.*, 2022b). The type of lipid used is important, however, because the honest signaling hypothesis requires that the lipids be digestible by animals; thus, identifying the type of lipid and its digestibility will be necessary to eventually understand the potential signaling function of structural colors in *Viburnum* fruits.

Honesty and deceit in structurally colored fruits

Two other structurally colored diaspores display an extreme version of this negative correlation between color intensity and amount of pulp: *Pollia condensata* and *Margaritaria nobilis* both lack nutritional value but have highly modified cell walls that produce, in the case of *P. condensata*, a color that has been described as one of the brightest in nature (Cazetta *et al.*, 2008; Vignolini *et al.*, 2012b). These colors are highly distinctive in the context of global fruit color diversity (Stournaras *et al.*, 2013) and can persist, appearing ripe, for long periods of time due to the use of cellulose to build the photonic structure (Galetti, 2002; Cazetta *et al.*, 2008; Stournaras *et al.*, 2015).

Fruits that make an attractive color but lack nutritional content have been called 'mimetic fruits' (e.g. Cazetta *et al.*, 2008), but these species might be better termed 'deceitful'. If the scenario proposed here is correct, it would be evolutionarily difficult for the structurally colored *Viburnum* species to completely lose their nutritional reward, simply due to the mechanism underlying their structural colors: losing lipid content in the pulp might result in loss of lipids from the cell wall and consequently their fruit color. This places a constraint on the possible evolutionary path that these species could take: species that use nutritional materials to produce their structural colors are capable of evolving an honest signal, while species that use an indigestible material (cellulose) are able to evolve a deceitful signal.

Other traits, such as the ability of the fruit to remain on the plant, correspond with these different strategies. Structurally colored *Viburnum* fruits do not remain on the plant for long and generally are consumed rapidly, while the deceitful structurally colored *Pollia* and *Margaritaria* tend to be consumed only rarely and remain on the plant for long periods of time (Cazetta *et al.*, 2008; Vignolini *et al.*, 2012b).

How rare is structural color in fruits?

Our findings highlight two important factors that have limited research on structural color in fruits. First, the initial search for structural color in fruits largely focused on species for which pigments cannot be extracted (e.g. in *E. angustifolius*; Lee, 1991). In *Viburnum*, however (as in most structurally colored plant species identified to date), structural colors co-occur with pigments. The only anthocyanins identified so far in *Viburnum* are cyanidins, which are also common in the related *Sambucus* and *Lonicera* (Lawrence *et al.*, 1939; Ishikura, 1975; Jordheim *et al.*, 2007). The co-occurrence of structural color with pigments in fruits should not be surprising, because pigments play a variety of functional roles in the fruit, including defense against fungal pathogens (Cipollini & Levey, 1997; Schaefer *et al.*, 2008; Schaefer, 2011) and creating contrasting color between immature and mature fruits that enhances dispersal (Willson & Thompson, 1982; Willson & Whelan, 1990; Schmidt *et al.*, 2004). All structurally colored species in *Viburnum* have pigments in their fruits. If we had limited our search for structural colors to species without pigments, we would have missed the unusual architecture that we have described here in *Viburnum* fruits.

Second, not all structural colors appear extreme or distinctive to the human eye. Although *V. tinus* and *V. davidii* have distinctive fruit colors (Stournaras *et al.*, 2013) that visually appear metallic, the dull blue color of *V. dentatum* fruits, and the blue-gray of *V. lautum*, are much less striking. It is the evolutionary context of other blue-fruited *Viburnum* species using structural color that makes *V. dentatum* interesting. In examining fruit color evolution across *Viburnum*, as we did here, we find that photonic structures contribute to the diversity of fruit colors across the clade, and especially to the gradation between black, gray, and blue colors. These less extreme structural colors may be more widespread than those currently recognized. We also observe that the mechanism used to produce the color (e.g. lipids in the cell wall combined with pigments) suggests potential signaling functions of these colors, a topic of great interest in seed dispersal ecology.

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Author contributions

MAS-A and MJD designed the research question, and MAS-A, MJD, BJG, PJR, SV and EM planned the project. MAS-A, RM, YO and GJ performed data collection, data analysis, and simulations. MAS-A wrote the first version of the manuscript, and all co-authors contributed to revisions.

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Data availability

All data are publicly available on Data Dryad at doi: 10.5061/dryad.3bk3j9knw.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Simulations of order and disorder in layered structures.

Fig. S2 Transmission electron micrograph of *Viburnum venustum*.

Fig. S3 1D simulations of disorder.

Fig. S4 Alternative ancestral state reconstructions.

Fig. S5 Reflectance spectra from select *Viburnum* species.

Table S1 Voucher and collection numbers.

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