Latent developmental and evolutionary shapes embedded within the grapevine leaf

Daniel H. Chitwood[1,4], Laura L. Klein[2], Regan O'Hanlon[2],
Steven Chacko[2], Matthew Greg[2], Cassandra Kitchen[2],
Allison J. Miller[2], Jason P. Londo[3]

[1]Donald Danforth Plant Science Center, St. Louis, MO 63132 USA
[2]Department of Biology, Saint Louis University, St. Louis, MO 63103 USA
[3]United States Department of Agriculture, Agriculture Research Service, Grape Genetics Research Unit, Geneva, NY 14456 USA

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[4]To whom correspondence should be addressed

Daniel H. Chitwood
Donald Danforth Plant Science Center
975 North Warson Road
St. Louis, MO 63132
Phone: (314) 587-1609
Fax: (314) 587-1709
e-mail: dchitwood @ danforthcenter.org

Summary

•Across plants, leaves exhibit profound diversity in shape. As a single leaf expands, its shape is in constant flux. Plants may also produce leaves with different shapes at successive nodes. Additionally, leaf shape varies among individuals, populations, and species due to evolutionary processes and environmental influences.

•Because leaf shape can vary in many different ways, theoretically the effects of distinct developmental and evolutionary processes are separable, even within the shape of a single leaf. Here, we measure the shapes of >3,200 leaves representing >270 vines from wild relatives of domesticated grape (*Vitis* spp.) to determine if leaf shapes attributable to genetics and development are separable from each other.

•We isolate latent shapes (multivariate signatures that vary independently from each other) embedded within the overall shape of leaves that can predict developmental stages independent from species identity and vice versa. Shapes predictive of development are then used to stage leaves from 1,200 varieties of domesticated grape (*Vitis vinifera*), revealing that changes in timing underlie leaf shape diversity.

•Our results indicate distinct latent shapes combine to produce a composite morphology in leaves, and that developmental and evolutionary contributions to shape vary independently from each other.

Introduction

Leaf morphology represents a beautiful and tangible example of the infinite phenotypic possibilities in nature. Underlying leaf shape diversity is a quantitative genetic (Langlade *et al.*, 2005; Kimura *et al.*, 2008; Tian *et al.*, 2011; Chitwood *et al.*, 2013) and developmental genetic (Bharathan *et al.*, 2002; Kim *et al.*, 2003; Blein *et al.*, 2008) framework. It is possible that aspects of leaf shape are functionally neutral and reflect developmental constraint (Chitwood *et al.*, 2012a; 2012b), but numerous hypotheses about the function of different leaf shapes exist, including how shape impacts thermal regulation, hydraulic constraints, light interception, biomechanics, and herbivory (Parkhurst and Loucks, 1972; Nicotra *et al.*, 2011; Ogburn and Edwards, 2013). Fossil leaf size and dissection are correlated with the paleoclimate (Bailey and Sinnott, 1915; Wolfe, 1971; Greenwood, 1992; Wilf *et al.*, 1998), a relationship that persists in extant taxa (Peppe *et al.*, 2011), and with implications for the chemical, structural, and physiological economics of leaves (Wright *et al.*, 2004). Correspondingly, functional traits related to leaf shape display phylogenetic signal in some clades (Cornwell *et al.*, 2014). Understanding the spatial and temporal patterns of leaf shape variation is a central theme in studies focusing on plant biodiversity, impacts of global climate change, and agricultural efficiency.

Leaf shape varies not only across evolutionary timescales and within a functional ecological context, but during development as well. Two distinct temporal processes regulate leaf shape during development. 1) The shape of individual leaves is in constant flux as local regions within the leaf expand at different rates. This phenomenon, allometric expansion, was explored as early as Hales' *Vegetable Staticks* (1727). Using a grid of pins, regularly spaced puncture points in fig leaves were tracked to determine if their relative spacing changed during development. The same experiment can be microscopically studied using fluorescent particles today (Remmler and Rolland-Lagan, 2012; Rolland-Lagan *et al.*, 2014). 2) The leaves that emerge at successive nodes differ in their shape, as the shoot apical meristem from which they derive transitions from a juvenile to adult stage of development. This process, heteroblasty, can affect other features of leaves besides shape, such as

93 cuticle and trichome patterning (Goebel, 1900; Ashby, 1948; Poethig, 1990;
94 Kerstetter and Poethig, 1998; Poethig, 2010).
95
96 The developmental stage of a leaf and the position of the node from which it arises
97 (leaf number) are distinct temporal factors affecting leaf shape. Genetic changes in
98 the timing of either process (e.g., protracted development of individual leaves or
99 precociously adult leaf morphology in early nodes) between species can lead to
100 evolutionary differences in leaf shape, a process known as heterochrony. The timing
101 of these processes can be changed non-genetically as well, through responses to
102 environmental changes during the lifetime of a plant, known as plasticity (Allsopp,
103 1954; Diggle, 2002). Distinguishing effects of developmental stage from leaf number
104 provides mechanistic insights into how genetic changes during evolution or plastic
105 changes in response to the environment are achieved (Jones, 1993; 1995). For
106 example, recent work has linked molecular pathways regulating the timing of shape
107 changes throughout the shoot (miR156/172 and their targets) with leaf morphology
108 (CUC-induced serrations) through a mediator (TCPs) (Rubio-Somoza *et al.*, 2014).
109
110 If distinct molecular pathways affect different traits within the infinite features
111 defining the architecture of a leaf, then the outline and venation topology of leaves
112 should theoretically be decomposable into latent (hidden) shapes; i.e., multivariate
113 morphological signatures that vary independently from each other (Chitwood and
114 Topp, 2015). For example, shape differences defining species, as well as shape
115 differences defining developmental stage, may be detectable within each leaf and
116 subsequently isolated from each other. Shape differences that define species,
117 regardless of developmental context, may vary distinctly from those features that
118 define developmental context, regardless of species. These shapes are latent
119 because, while they are present in each leaf, they manifest within the context of
120 other factors that affect shape as well. Latent shapes globally affect leaf shape in
121 different ways, and each of the features comprising the latent shapes alone do not
122 necessarily discriminate the effects of genetics or development. From this
123 perspective, the shape of a given leaf—from any species, from any time point during

development or any position in the plant—would result from the confluence of latent shapes regulated by these processes. The single organ that we call a leaf would actually be a composite of latent features that vary by genetic versus developmental effects, independently from each other.

Grapevine (*Vitis* spp.) leaves exhibit a breathtaking range of variation in leaf shape, making this genus ideally suited to explore latent shapes resulting from evolutionary and developmental processes (**Fig. 1**). Taxonomists studying *Vitis* have used variation in leaf lobing and leaf margins to delimit the nearly 60 species in the genus (Moore, 1991; Ren and Wen, 2007). Leaf shape is also important in assessing intraspecific variation in the European grapevine (*Vitis vinifera* subsp. *vinifera*), which is grown around the world for wine-making and table grapes. Unique among crops, variation in grape leaf shape (together with other vine features) was once, and is still today, used by viticulturists to quantitatively classify grape varieties, a field known as ampelography (αμπελος, "vine" and γραφος, 'writing') (Galet, 1952). Grape leaves have several homologous points amenable to landmark-based analyses (Galet, 1979; Chitwood *et al.*, 2014), increasing the biological interpretation of morphometric data compared to species with stochastic venation topologies or limited homology (such as *Arabidopsis*, tomato, *Antirrhinum*, etc.). Like Hales' grid of pins (1727), naturally homologous points in grape leaves allow developmental stage and leaf number effects to be quantitatively tracked, potentially revealing separable latent processes contributing to the composite morphology known as a leaf.

Methods and Methods

Germplasm, sample collection, and scanning

Over 270 vines in the USDA *Vitis* germplasm collection in Geneva, NY were sampled in June, 2013. Using an automatic label maker, vine identification number and the identity of organs (or lack thereof) at each node, beginning with the first sampled leaf at the tip, were recorded in the field. Leaves, beginning with the first leaf at the

tip that could be flattened and scanned (~1 cm in length), were collected in order, shoot tip to base, as a stack and placed into a Ziploc bag to which the label was affixed. A single shoot was collected per vine, and bags were placed into a cooler until scanning. A description of the number of vines (genotypes) collected for each species and hybrid, as well as the number of leaves representing different developmental stages (Sn) and leaf numbers (Ln) can be found in supporting information (**Fig. S1**).

Leaves were arranged on a scanner (Mustek A3 1200S), in the collected order from the shoot, and next to each leaf was placed a small label indicating nodes (as measured by developmental stage) and organ identity opposite the node ("C", cluster; "T", tendril; "N", no organ). The abaxial side of leaves was imaged. The file name of the image indicates the vine ID, and the appended letter indicates which image in the series the file represents for each vine. The raw scans are publically available at the following link:
https://dataverse.harvard.edu/dataverse/vitis_leaves

Landmarking

For both wild *Vitis* species and reanalyzed *V. vinifera* subsp. *vinifera* data, 17 landmarks were placed, in order, for each leaf using the ImageJ (Abramoff *et al.*, 2004) point tool. Landmarks and their order were as follows: 1) petiolar junction, 2) midvein tip, 3) left distal sinus, 4) right distal sinus, 5) left distal lobe tip, 6) right distal lobe tip, 7) left proximal sinus, 8) right proximal sinus, 9) left proximal lobe tip, 10) right proximal lobe tip, 11) left terminus petiolar vein, 12) right terminus petiolar vein, 13) branch point midvein, 14) branch point left distal vein, 15) branch point right distal vein, 16) branch point left proximal vein, 17) branch point right proximal vein. Using ggplot2 (Wickham, 2009) in R (R Core Team, 2014), graphs for landmarks from each image were visually checked for errors. If errors were detected, the landmarking was redone for those particular samples.

186 *Morphometric analysis and visualization*

187

188 Once a quality landmarked dataset was created, a Generalized Procrustes Analysis
189 (GPA) was undertaken using the R package shapes (Dryden, 2013). For 17
190 landmarks in two dimensions (x,y coordinates) for 3,292 leaves of wild *Vitis* species
191 and 9,548 *V. vinifera* subsp. *vinifera* leaves, GPA was performed using the procGPA
192 function, reflect=TRUE. Separate Procrustes analyses were performed for 1) all wild
193 *Vitis* species leaves at all shoot positions and 2) *V. vinifera* subsp. *vinifera* and subsp.
194 *sylvestris* leaves and wild *Vitis* species leaves selected for the corresponding shoot
195 position as the domesticated grape dataset. Eigenleaves were visualized using the
196 shapepca function and PC scores, percent variance explained by each PC, and
197 Procrustes-adjusted coordinates were obtained from procGPA object values. To test
198 for correlation between PCs and developmental stage or leaf number, Spearman's
199 rho was calculated, whereas for variability of PC values across species a Kruskal-
200 Wallis test was used.

201

202 Linear Discriminant Analysis (LDA) on Procrustes-adjusted coordinates was
203 performed using the lda function from the MASS package (Venables and Ripley,
204 2002). Species, developmental stage, and leaf number were all analyzed
205 independent of each other. The predict function (stats package) and table function
206 (base package) were used (dependent on MASS) to reallocate leaves (whether by
207 species, developmental stage, or leaf number) using the linear discriminants. For the
208 case of predicting *V. vinifera* subsp. *vinifera* developmental stage and leaf number,
209 the wild *Vitis* species data was used as a training set to predict values for
210 domesticated grape leaves. For the wild *Vitis* species data, for each leaf there is
211 actual versus apparent species, developmental stage, and leaf number identities.
212 Relative developmental stage and relative leaf number are calculated as apparent
213 value – actual value. Mean relative developmental stage and relative leaf number
214 values for each vine were used to determine significant deviation of species relative
215 values from zero using a one sample, two tailed t-test. Correlation between relative
216 values and first tendril node was analyzed using Spearman's rho.

As previously described (Chitwood *et al.*, 2014), Germplasm Resources Information Network (GRIN) trait values, averaged on a per accession basis, for *V. vinifera* subsp. *vinifera* vines were correlated with each other and morphometric and predicted temporal data using the rcorr function from Hmisc (Harell, 2013) using Spearman's rho and false-discovery rate controlled using the Benjami-Hochberg procedure (Benjamini and Hochberg, 1995). Hierarchical clustering for *V. vinifera* subsp. *vinifera* traits, and for averaged Procrustes-adjusted coordinates for species throughout the genus *Vitis*, was carried out using the hclust function on a distance matrix calculated from correlation performed on complete, pairwise observations and visualized using the as.phylo function from the package ape (Paradis *et al.*, 2004).

Visualization was performed in ggplot2 (Wickham, 2009) using geom_bar, geom_boxplot, geom_point, geom_segment, geom_tile, and stat_smooth functions, among others, and color schemes derived from colorbrewer2.org.

Results

Developmental stage versus leaf number

To determine the effects of developmental stage, leaf number, and evolutionary lineage (taxonomic identity) on leaf shape we scanned >3,200 leaves from wild relatives of domesticated grape held by the USDA germplasm repository in Geneva, NY. Like many living collections of perennial crops, the Geneva repository houses multiple genotypes of different *Vitis* species in common conditions. This extensive collection includes wild-collected accessions of at least 19 North American and Asian *Vitis* species as well as assorted *V. vinifera* hybrids. From the Geneva repository we sampled >270 vines representing 12 *Vitis* species, 4 *V. vinifera* hybrids, and 3 species from the related genus *Ampelopsis.* Each sampled vine represents a unique genetic accession. The number of vines sampled for each

248 species varied widely (**Fig. S1**), but in this study we focus on 10 species for which ≥5
249 accessions were sampled: *Vitis riparia* (71 vines), *V. labrusca* (40 vines), *V. cinerea*
250 (37 vines), *V. rupestris* (28 vines), *V. acerifolia* (16 vines), *V. amurensis* (16 vines), *V.*
251 *vulpina* (13 vines), *V. aestivalis* (8 vines), *V. coignetiae* (5 vines), and *Vitis palmata* (5
252 vines). All *Vitis* leaves possess a midvein, distal and proximal veins, a petiolar vein,
253 as well as proximal and distal lobes and sinuses, and wide variation in the width of
254 the petiolar sinus (**Fig. 1a**). We leverage these homologous points and others to
255 measure 17 landmarks in all leaves (**Table S1**). Later, we compare the leaves from
256 the wild relatives of grape described above to previously published data on 1,200
257 varieties of domesticated grape (Chitwood *et al.*, 2014), which we describe in
258 subsequent sections.
259
260 For each vine accession, a representative shoot was selected and the shoot position
261 of each leaf recorded (**Fig. 1b-c**). Developmental stage is measured by counting
262 from the youngest, first measureable leaf at the shoot tip. The time between
263 successively initiated leaves is a plastochron, and the youngest initiated leaf
264 primordium at the shoot apical meristem is denoted P1 (plastochron 1) to indicate
265 this. But because we begin not with P1 (which is microns in size) but with the first
266 measureable leaf (~1 cm in size), we use S1 (for "stage") to denote the youngest
267 measured leaf at the shoot tip counting numerically upwards (S2 . . . Sn) towards the
268 shoot base. Contrastingly, leaf number begins with the first initiated leaf (L1) found
269 at the shoot base and counts numerically upward (L2 . . . Ln) towards the shoot tip
270 (**Fig. 1c**). These two metrics are used to differentiate the effects of developmental
271 stage (Sn) from leaf number (Ln) (**Fig. 1c**).
272
273 The effects of developmental stage are expected to be strongest in young leaves at
274 the shoot tip (with low S numbers and high L numbers; **Fig. 1c**), as their shape is in
275 flux during their expansion. Older leaves at the shoot base (with low L numbers and
276 high S numbers) are more influenced by leaf number, because they reflect changes
277 in mature leaf shape at successive nodes. The vast majority of the vines we sampled
278 possess leaves corresponding to S1-S10 and L1-L10 (86% represent developmental

stages up to P10 and 88% represent leaf numbers up to L10, **Fig. S1**) and we restrict our analyses to these positions (**Fig. 1c**).

Morphospace of leaves in the genus Vitis

Landmarks are aligned (accounting for translation, rotation, and scaling) using a Generalized Procrustes Analysis (GPA). A Principal Component Analysis (PCA) is then performed to visualize the major sources of shape variance among leaves. The first four PCs explain 73.2% of shape variance among leaves from *Vitis* species, developmental stages, and leaf numbers (**Fig. 2**). More than half of the shape variance, represented by PC1 and PC2, is influenced by lobing and the width of the petiolar sinus. Low PC1 and high PC2 values readily distinguish highly dissected *Vitis* species and some members of the related genus *Ampelopsis* (**Fig. 2b**).

In order to examine the variability of developmental stage and leaf number in morphospace, we visualize each PC as a locally weighted scatterplot smoothing (LOWESS) curve plotted against stage and leaf number. Developmental stage and leaf number vary less by PC1 than by PC2 and PC3 (i.e., PC1 values are relatively developmentally-invariant compared to PC2 and PC3 values) (**Fig. 2c-d**). PC2 and PC3 values are largely indistinguishable between species across developmental stages (**Fig. 2c**) and leaf number (**Fig. 2d**), suggesting strongly conserved morphological features in developing leaves across species for these shape attributes, particularly for early developmental stages (**Fig. 2c**). Because PCs are orthogonal (i.e., uncorrelated), the fact that species, developmental stage, and leaf number correlate differentially with PCs is suggestive that each might be represented by independent shape attributes present within leaves. Re-visualizing the morphospace by developmental stage (**Fig. 2e**) and leaf number (**Fig. 2f**) demonstrates these factors mostly vary by PC2 and PC3, whereas species shape differences traverse along morphospace paths defined by PC1, PC2, and PC3 (**Fig. 2b**).

310 To help qualitatively understand the different ways grape leaves differ among
311 species and developmental contexts, we compared average shapes (**Fig. 3**). Related
312 members of Moore's Series Ripariae (*V. acerifolia*, *V. riparia*, and *V. rupestris*)
313 (Moore, 1991; Miller *et al.*, 2013) are defined by a shallow petiolar sinus, especially
314 *V. rupestris* (**Fig. 3a**). Outside of the Ripariae, *V. vulpina* and *V. cinerea* also exhibit
315 shallow petiolar sinuses but to a lesser extent, while the petiolar sinus of the
316 remaining species (*V. aestivalis, V. labrusca, V. palmata, V. coignetiae, V. amurensis*) is
317 more acute. *Vitis palmata* exhibits especially deep distal lobing relative to other
318 species. Each species' leaves also vary by developmental stage (**Fig. 3b**) and leaf
319 number (**Fig. 3c**), usually by the length of the leaf tip and the shallowness of the
320 petiolar sinus. In the next section, we determine the extent that shape attributes
321 varying by species, developmental stage, and leaf number are separable from each
322 other.
323
324 *Latent shapes independently predict species, developmental stage, and leaf number*
325
326 We used a Linear Discriminant Analysis (LDA) to maximize the separation of leaf
327 attributes (whether species, developmental stage, or leaf number) from each other
328 using all measured shape information. The resulting linear discriminants can be
329 used to predict the apparent class of a leaf and are useful for comparing actual
330 versus apparent leaf identities as confusion matrices (**Fig. 4**).
331
332 Linear discriminants separating species, without regard to developmental stage or
333 leaf number, can be used to predict species identity (**Fig. 4a-c**). For most taxa, the
334 largest proportion of predicted leaves corresponds to the taxonomic identity
335 assigned to that species (**Fig. 4b**, the diagonal indicates the proportion of correctly
336 assigned leaves). Incorrectly assigned leaves are most often confused for other
337 species that are phylogenetically related to the assigned species. For example, a
338 majority of *V. acerifolia* leaves are confused for its close relative *V. riparia*; similarly,
339 the largest group of *V. aestivalis* leaves are confused for its relative *V. labrusca*. In
340 contrast, the Asian *V. coigenetiae* leaves are confused for more distant relatives

native to North America, including *V. labrusca* and *V. cinerea* and sometimes even *V. riparia,* perhaps indicating convergent evolution, germplasm misidentification, or segregating leaf shape differences among these accessions. Interestingly*, Vitis* x*andersonii,* described as a hybrid of *V. coignetiae* and either *V. vulpina* or *V. riparia*, is strongly mistaken for *V. riparia*, providing circumstantial evidence of parentage. As has been suggested by ampelographers and taxonomists (Moore, 1991; Galet, 1952), our results demonstrate that leaf shape is often sufficient to identify species, even in the absence of developmental information. Instances where leaves of one species are assigned to another offer a valuable opportunity to develop hypotheses about phylogenetic history, hybridization, and convergent evolution that can be tested with additional evolutionary and ecological analyses.

Reciprocally, we wondered whether developmental stage and leaf number, regardless of species identity, might be similarly informative about developmental context. Indeed, linear discriminants trained on developmental stage or leaf number can predict the developmental context of a leaf for either measure (**Fig. 4d-4i**). Developmental stage can be predicted with a Spearman's correlation coefficient of rho = 0.70 (**Fig. 4d**), and leaf number with rho = 0.61 (**Fig. 4g**). Prediction of both developmental stage and leaf number is more accurate at the beginning of their respective series, where the effects of each are anticipated to be the strongest (**Fig. 1c**). Based on these observations we conclude that developmental context, measured by either developmental stage or leaf number, can be predicted independently of genotypic information. These results suggest that the effects of development on leaf shape are conserved across genotypes and species, and have implications for isolating distinct molecular pathways that affect leaf shape over evolutionary versus developmental time scales.

The ability to predict developmental context separate from species identity provides a method to quantify differences between species attributable to changes in developmental timing, also known as heterochrony. For each leaf, we calculate relative developmental stage and relative leaf number as the apparent value minus

372 the actual value (e.g., an S5 leaf predicted to be S7 would have a relative stage value
373 of +S2, and an L4 leaf predicted to be L2 would have a relative value of –L2).
374 Relative values indicate, for a given leaf, how many nodes ahead or behind
375 (developmentally speaking) that leaf appears to be. Averaging the relative
376 developmental stage and leaf number values for each vine, we can detect species
377 that are precociously ahead or lagging behind from expected developmental stage
378 (**Fig. 4e**) and leaf number values (**Fig. 4h**). For example, the mean relative
379 developmental stage of *V. rupestris* vines is about +1S ahead of their actual
380 developmental stage (**Fig. 4e**) and about -2L behind actual leaf number (**Fig. 4h**).
381 This accelerated development (Sn) and protracted display of juvenile leaf types
382 typically found at the base of the shoot (Ln) contributes towards the unique shape of
383 *V. rupestris* leaves compared to other *Vitis* spp. (**Fig. 3**). *Vitis rupestris* leaves (**Fig.**
384 **3a**), older leaves (high Sn values, **Fig. 3b**), and juvenile leaves (low Ln values, **Fig. 3c**)
385 all share a characteristic wideness conferred by a diminished leaf tip.
386
387 Within *Vitis*, the identity of inflorescences, which appear opposite leaves, transform
388 from clusters at the shoot base to tendrils at the shoot tip (Srinivasan and Mullins,
389 1981; Gerrath, 1988; Gerrath, 1993; Boss and Thomas, 2002) (**Fig. 1c**). Both
390 changes in leaf shape at successive nodes and the transformation of inflorescence
391 identity indicate temporal changes in the development of the shoot apical meristem,
392 known as heteroblasty. The cluster-to-tendril transformation serves as a discrete,
393 binary indication of the heteroblastic transition. If heteroblasty regulates both the
394 latent shapes predicting leaf number and the cluster-to-tendril transition, we would
395 assume they would be correlated. Indeed, there is a significant negative correlation
396 between relative leaf number and the first node a tendril is observed: that is, in
397 vines with precocious, adult leaves (i.e., higher relative leaf numbers, typical of
398 leaves found more towards the shoot tip), tendrils appear at earlier nodes closer to
399 the base of the shoot, linking two different measures of premature heteroblastic
400 change (**Fig. 4i**). Moreover, this correlation is not observed for relative
401 developmental stage (**Fig. 4f**). Taken together, these results indicate that latent
402 shapes predictive of developmental stage and leaf number are functionally distinct,

403 reflecting the developmental progression of leaves (Sn) and their heteroblastic
404 transitions across successive nodes of the shoot (Ln) separately.
405
406 Average leaf shapes indicate that developmental stage and leaf number vary by the
407 prominence of the leaf tip and the shallowness of the petiolar sinus (**Fig. 4j-l**). The
408 shape changes for these two factors are inversely related, but obviously
409 distinguishable from each other, given their differential correlation with tendril
410 position (**Fig. 4f**, **4i**). Upon closer inspection, the distal lobe tips and the branch
411 point of the petiolar vein distinguish shape changes attributable to developmental
412 stage and leaf number (**Fig. 4l**), isolating the shape attributes unique to these
413 functionally distinct processes.
414
415 *Morphological differences between domesticated grape and wild relatives*
416
417 Previously, we measured >9,500 leaves from *V. vinifera* subsp. *vinifera*
418 (domesticated grape) and its wild progenitor *V. vinifera* subsp. *sylvestris*. These
419 leaves, collected from the USDA germplasm repository in Winters, CA, represent
420 over 2,300 vines and 1,200 varieties (Chitwood *et al.*, 2014). These leaves were
421 sampled to measure purely genetic effects and minimize the influence of
422 development by collecting four successive leaves from the midpoint of the shoot.
423 That species identity can be predicted independently from developmental context in
424 wild *Vitis* spp. (**Fig. 4a-c**) with disparate leaf shapes lends credence to the
425 assumption that developmental context can be ignored, which is almost always
426 invoked when mapping traits for quantitative genetic purposes or taxonomically
427 defining species.
428
429 We wanted to compare leaves from domesticated grape and its wild progenitor to
430 the other *Vitis* species described above. To do so, we restricted our analysis *in silico*
431 to leaves at the same shoot positions we had originally collected in domesticated
432 grape (the four leaves closest to the midpoint of the shoot). In the combined
433 morphospace, PC1 and PC2 describe nearly 60% of shape variance (**Fig. 5**). Similar

434 to the wild *Vitis* species-only morphospace (**Fig. 2a**), lobing and the petiolar sinus
435 define the first PCs. Petiolar veins in domesticated grape varieties can be so angled
436 they cross each other, and because of this the closure of the petiolar sinus
437 represented by high PC1 values is particularly strong (**Fig. 5a**). Leaf dissection
438 defined by high PC1 and high PC2 values clearly delineates less lobed wild *Vitis*
439 species from more acutely lobed domesticated grape varieties (**Fig. 5b**). Highly
440 dissected species such as *Ampelopsis acontifolia*, *V. piasezkii*, *V. thunbergii*, and *V.*
441 *vinifera* var. Ciotat form a distinct morphological grouping.
442
443 While there are clear patterns in leaf shape related to development and species
444 identity, correspondence between the morphospace of wild and domesticated *Vitis*
445 species and evolutionary relationships among *Vitis* species is complex. Some, but
446 not all, well-known phylogenetic relationships are reflected in leaf morphology.
447 (**Fig. 5c**) (Miller *et al.*, 2013; Zecca *et al.*, 2012). Notably, clustering averaged shapes
448 from different genotypes reveals that *V. vinifera* subsp. *vinifera* is morphologically
449 sister to *V. vinifera* subsp. *sylvestris*, the wild progenitor of the domesticated grape
450 (**Fig. 5c**, Group II) (Zecca *et al.*, 2012; Myles *et al.*, 2011). Also within Group II are
451 two Asian species, *V. amurensis* and *V. coignetiae*, which together with *V. vinifera*
452 subsp. *vinifera* and *V. vinifera* subsp. *sylvestris* represent members of a Eurasian
453 clade. Within Group III are found members of Series Ripariae (*V. acerifolia*, *V. riparia*,
454 *V. rupestris*). Leaf shapes of *Vitis* hybrids cluster based on parental lineages, with *V.*
455 x*champinii* and *V.* x*doaniana* clustering in Group III presumably because of their *V.*
456 *rupestris* and *V. acerifolia* heritages (respectively) and *V.* x*andersonii* and *V.* x*novae-*
457 *angliae* clustering closer with their *V. coignetiae* and *V. labrusca* parents
458 (respectively). These observations suggest similarities in leaf shape may reflect
459 recent evolutionary events, domestication, or contemporary interspecific gene flow.
460
461 At broader levels of phylogenetic scale, however, patterns of leaf shape similarity
462 do not appear to correspond with known evolutionary relationships. For example,
463 together with *V. vinifera* subsp. *vinifera*, *V. vinifera* subsp. *sylvestris*, and two Asian
464 species found Group II (discussed above) are other North American species (*V.*

465 *labrusca, V. palmata*), several hybrids, and *Ampelopsis brevipedunculata*, taxa that
466 are not known to be closely related to one another. A previously identified clade
467 based on molecular data (*V. aestivalis*, *V. cinerea*, *V. labrusca*, and *V. vulpina)* here is
468 divided between Groups II and III. Although *Vitis* is well known to be a monophyletic
469 genus (Wen *et al.*, 2007; 2013), based on leaf shape three species of *Ampelopsis*
470 cluster in three different groups, together with *Vitis* species. These data suggest that
471 while leaf shape may bear signatures of recent evolutionary events, leaf shape does
472 not appear to track with phylogeny at larger scales; distantly related species, even
473 from distinct genera, resemble each other in leaf shape through evolutionary
474 convergence.
475
476 *Changes in developmental timing underlie morphological diversity in domesticated*
477 *grape leaves*
478
479 Even though leaves from equivalent positions in the shoot clearly separate species
480 and varieties by genetic effects (**Fig. 5**), such effects may still be developmental in
481 nature, an example of heterochrony. If changes in the relative timing of
482 developmental stage (Sn) or leaf number (Ln) have occurred, they would contribute
483 to morphological differences between species, as described above for wild *Vitis* spp.
484 (**Fig. 4e, 4h**). The conserved latent shapes defining developmental stage and leaf
485 number in morphologically disparate *Vitis* species can be used predictively to detect
486 such changes in timing.
487
488 We used wild species leaves, from S1-S10 and L1-L10 (where developmental stage
489 and leaf number effects are the strongest, respectively) as a training set, to predict
490 the stage (Sn) and leaf number (Ln) values of domesticated grape leaves (**Fig. 6**).
491 When comparing leaf averages across predicted developmental stages (**Fig. 6a**) and
492 leaf numbers (**Fig. 6b**), it is apparent that variation in petiolar sinus depth is the
493 major predictor of developmental timing in domesticated grape leaves (**Fig. 6c**).
494 Variation in the petiolar sinus is a recurrent theme in morphology associated with
495 both developmental stage and leaf number (**Figs. 3b-c; 4j-l**). Indeed, the predicted

496 developmental stages and leaf numbers of domesticated grape leaves are
497 significantly correlated with PC1 and PC2 (**Fig. 6d-e, 6g-h**; **Table S2**), which
498 together explain near 60% of total shape variance for measured *Vitis* genotypes (**Fig.**
499 **5a**). From this, we conclude that latent shape attributes modulating developmental
500 stage and leaf number explain large amounts of shape variance in domesticated
501 grape, indicating changes in developmental timing (heterochrony) underlie the
502 morphological evolution of domesticated grape varieties.
503
504 *The relationship of leaf shape and timing to other traits in domesticated grape*
505
506 Previously, numerous other traits, from timing of bud burst and flowering to berry
507 ripening and titratable acids and sugar content, had been measured on the
508 domesticated grapevines described above (Chitwood *et al.*, 2014). We were curious
509 about the correlational context of overall leaf shape (represented by PCs) and
510 developmental timing (predicted developmental stage and leaf number) to these
511 other traits. One trait, "PETSINMALF 2009", is a qualitative, 0-8 rating of petiolar
512 sinus depth, and is tightly correlated to PC1, PC2, and predicted developmental
513 stage and leaf number, confirming that our quantitative measures reflect intuitive
514 perceptions of leaf shape. (**Fig. 6f, 6i-j**; **Table S2**). Leaf morphology traits, including
515 predicted developmental timing, are largely separate from bud burst and bloom
516 dates ("BUDBURST DATE", "BLOOMDATE", "LEAFDATE"), indicating that these
517 timing features are separate from the overall transition to reproductive
518 development in grapevine (**Fig. 6j**). Further, leaf shape is distinct from overall size
519 of leaves ("LEAF SIZE"), demonstrating that grape leaf shape is not merely a
520 developmental constraint correlated with overall size (i.e., allometry) (**Fig. 6j**). One
521 group of traits significantly correlated and clustering with leaf shape is hirsuteness,
522 both in leaves and the shoot ("LEAF HAIR", "SHOOT HAIR"), which we had observed
523 previously in domesticated grape (Chitwood *et al.*, 2014). Leaf hirsuteness varies by
524 both developmental stage, as trichomes become less dense as the leaf expands, and
525 also by leaf number. It is interesting to think about how thermoregulation, disease
526 resistance, or other trichome mediated functionality may vary as a developmental

527 constraint as developmental timing changed over the evolution and domestication
528 of grapevines.
529
530 **Discussion**
531
532 Many descriptions of complex traits, from faces to voices, and even the evolution of
533 cultural products, such as violins (Zhand *et al.*, 1997; Kuhn *et al.*, 2000; Claes *et al.*,
534 2014; Chitwood, 2014), potentially stand to benefit from isolating features uniquely
535 regulated by distinct pathways. Like a face, leaf shape is modulated in complicated
536 ways by genetics, development, and the environment. The latent shapes we describe
537 here, describing developmental stage and leaf number, embedded within the overall
538 shape of a leaf, and conserved across the genus *Vitis,* fit the definition of a
539 "cryptotype". Recently, we had suggested the term "cryptotype," borrowed from
540 linguistics (which uses both "phenotype" and "cryptotype" parallel to the biological
541 definitions) (Whorf, 1945), to describe latent, combinatorial features (e.g., 17
542 homologous landmarks) within a shared multivariate space that vary independently
543 from each other (Chitwood and Topp, 2015). Cryptotypes are not unreal or even
544 abstract; they are simply a set of features, that when combined, best discriminate
545 one biological process (e.g., evolutionary history) from others (e.g., developmental
546 stage). The shapes that arise through this combination of features are latent only
547 because they are not immediately recognizable when each of their component
548 landmarks is considered alone; the effects of genetics and development affect the
549 entirety of the leaf. The multivariate cryptotypes of genotype, developmental stage,
550 and leaf number stand in contrast to traditional univariate phenotypes, such as
551 height, biomass, or leaf length, width, or area.
552
553 Defining such combinative, latent shapes is critical to understand the mechanisms
554 by which morphological diversity manifests in plants. The unequal expansion of
555 leaves (allometry) and the different types of leaves displayed at nodes (resulting
556 from heteroblasty) have been studied for centuries (Hales, 1727; Goethe, 1817;
557 Goebel, 1900; Goethe (trans.), 1952), but the inability to distinguish the effects at a

558 morphological level, within the shared morphology of single leaves where these
559 processes act, has led to confusion. Early hypotheses that shade prolongs juvenility
560 through a heteroblastic mechanism were later refuted by carefully studying the
561 morphology of leaf primordia (Jones, 1995). Similar, the degree that changes in the
562 development of leaves or the heteroblastic series influences evolutionary changes—
563 and plasticity—remains an open question.
564
565 Recent studies have uncovered a transcriptomic basis underlying heteroblasty
566 (Efroni *et al.*, 2008), and similarly dramatic transitions to reproductive fates (Park *et*
567 *al.*, 2012), within meristems. An understanding of molecular clocks, regulating
568 phase change through small RNAs (Chuck *et al.*, 2007; Wu *et al.*, 2009), and their
569 signals, such as sugar (Yang *et al.*, 2013; Yu *et al.*, 2013), has come into focus. Of the
570 many gene regulatory networks regulating leaf shape, specific pathways have been
571 identified mediating heteroblastic shape changes (Rubio-Somoza *et al.*, 2014).
572 Similar molecular descriptions of leaf development exist (Ichihashi *et al.*, 2014) and
573 the crosstalk between these two processes is large. Strangely, even though
574 molecular correlates of leaf development and heteroblasty have been uncovered, a
575 phenotypic basis for the morphological effects of temporal patterning remains
576 obscure, except in a qualitative sense.
577
578 Decomposing complex morphologies into latent shapes regulated by distinct
579 pathways is required to precisely describe the mechanisms that, together, produce
580 the composite shape of a single leaf. By isolating latent shapes that are regulated by
581 evolution and development, we can focus on those attributes of the leaf most
582 relevant for producing the diversity of leaf shapes observed in nature, and consider
583 the functional contributions of leaf morphology to assessments of biodiversity, plant
584 responses to global climate change, and the genetic improvement of crop species.
585
586
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592

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Figure Legends

Figure 1: Morphological features defining the temporal development of *Vitis* leaves. (a) All *Vitis* leaves possess distal (yellow), proximal (orange), and petiolar (red) veins, as well as distal and proximal lobes and sinuses and a petiolar sinus. 17 homologous landmarks (black circles) were used in this study. Morphologically diverse species are shown. (b) Leaf series, scaled by the length between distal lobe tips, showing developmental stage and leaf number shape variance. (c) Unscaled *V. riparia* leaves showing changes in size at the shoot tip and base. Developmental stage (Sn) is measured starting from the shoot tip and leaf number is measured from the shoot base (Ln). Inflorescences, opposite leaves, skipping every third node, transform from clusters into tendrils from the shoot base to tip.

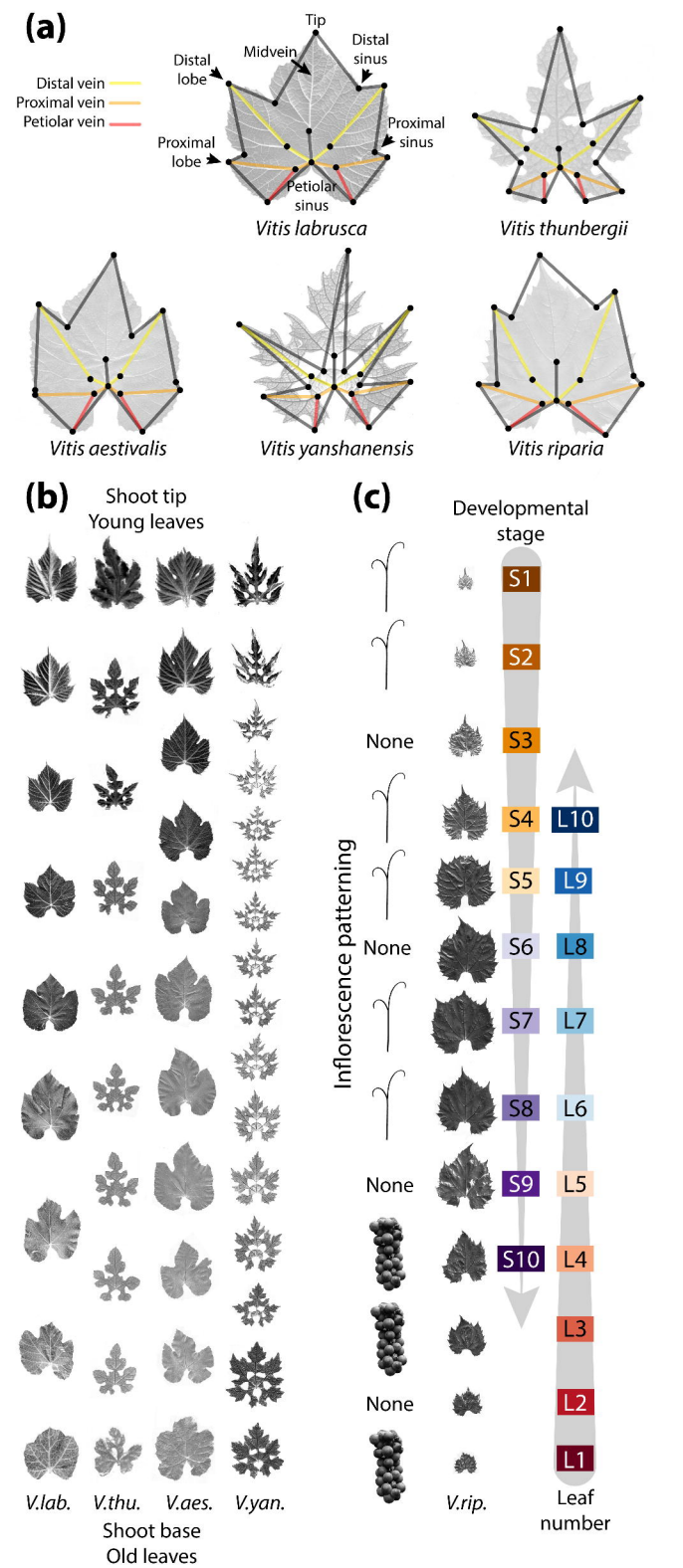
Figure 2: Morphospace of leaves in the genus *Vitis*. (a) "Eigenleaves" showing leaf morphs represented by Principal Components (PCs) at -/+ 3 standard deviations (s.d.) and shape variance explained by each. (b) Select species (indicated by color) representing morphological diversity in the dataset projected onto the morphospace. 95% confidence ellipses are drawn. (c and d) Locally Weighted

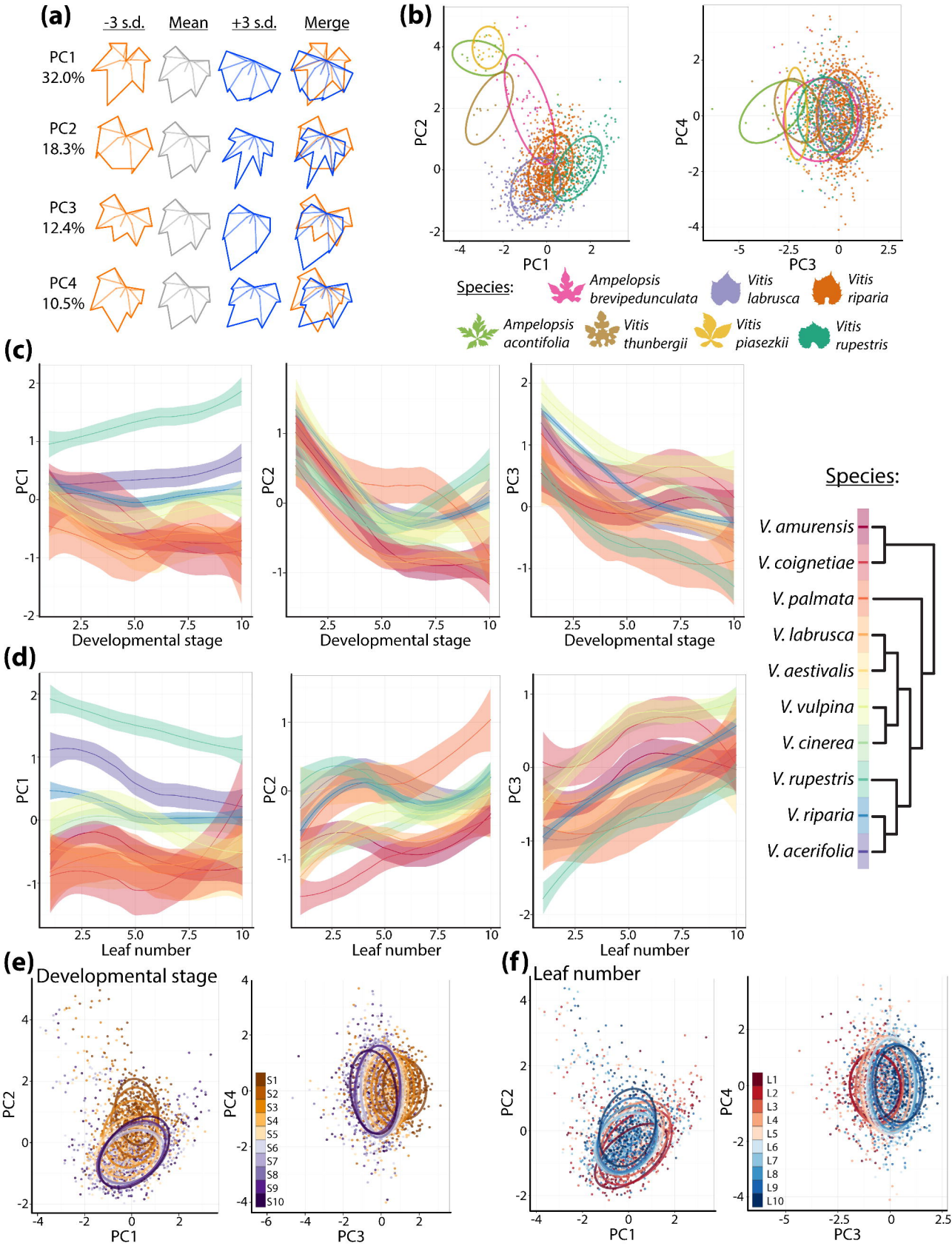
853 Scatterplot Smoothing (LOWESS) showing the relationship between PCs 1-3 and (c)
854 developmental stage and (d) leaf number. Phylogenetic relationships between
855 species are indicated by color. (e) Developmental stage and (f) leaf number
856 projected onto the morphospace.
857
858 **Figure 3: Shapes represented among *Vitis* species, developmental stage, and**
859 **leaf number.** (a) Outlines representing the average shape of each pairwise
860 comparison of species, indicated by magenta and green. Phylogenetic relationships
861 are indicated. (b and c) Comparison of average shapes across (b) developmental
862 stages and (c) leaf number for each species indicated by color.
863
864 **Figure 4: Species, developmental stage, and leaf number can be predicted**
865 **independently of each other.** (a) Confusion matrix resulting from prediction of
866 species identity using a Linear Discriminant Analysis (LDA). The panel should be
867 read left to right: for each species indicated on the left, leaves appeared to be
868 derived from which apparent species (indicated on bottom)? Developmental stage
869 and leaf number were not considered in the prediction. (b) Same as (a), except the
870 apparent species assigned to each actual species have been converted into a
871 proportion. Proportion totals equal one adding left to right. (c) The proportion of
872 correctly assigned leaves for each species. (d) Similar to (a), actual developmental
873 stage (left) and apparent developmental stage (bottom), predicted without species
874 or leaf number information. (e) Relative developmental stage values (apparent –
875 actual developmental stage) averaged for vines from each species. Significant
876 deviations from 0 indicated by * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$). (f)
877 Correlation between mean relative developmental stage of vines and first tendril
878 node. Fitted linear model is drawn. (g-i) same as (d-f) but for leaf number. Note the
879 significant correlation between first tendril node and relative leaf number (i) not
880 seen for developmental stage (f). (j and k) Latent shape attributes defining
881 developmental stage (j) and leaf number (k) indicated with average leaf shapes. (l)
882 Comparison of changes in latent shape attributes across developmental stage
883 (orange, outline S1; purple, arrow to S10) and leaf number (grey, outline L1; black,

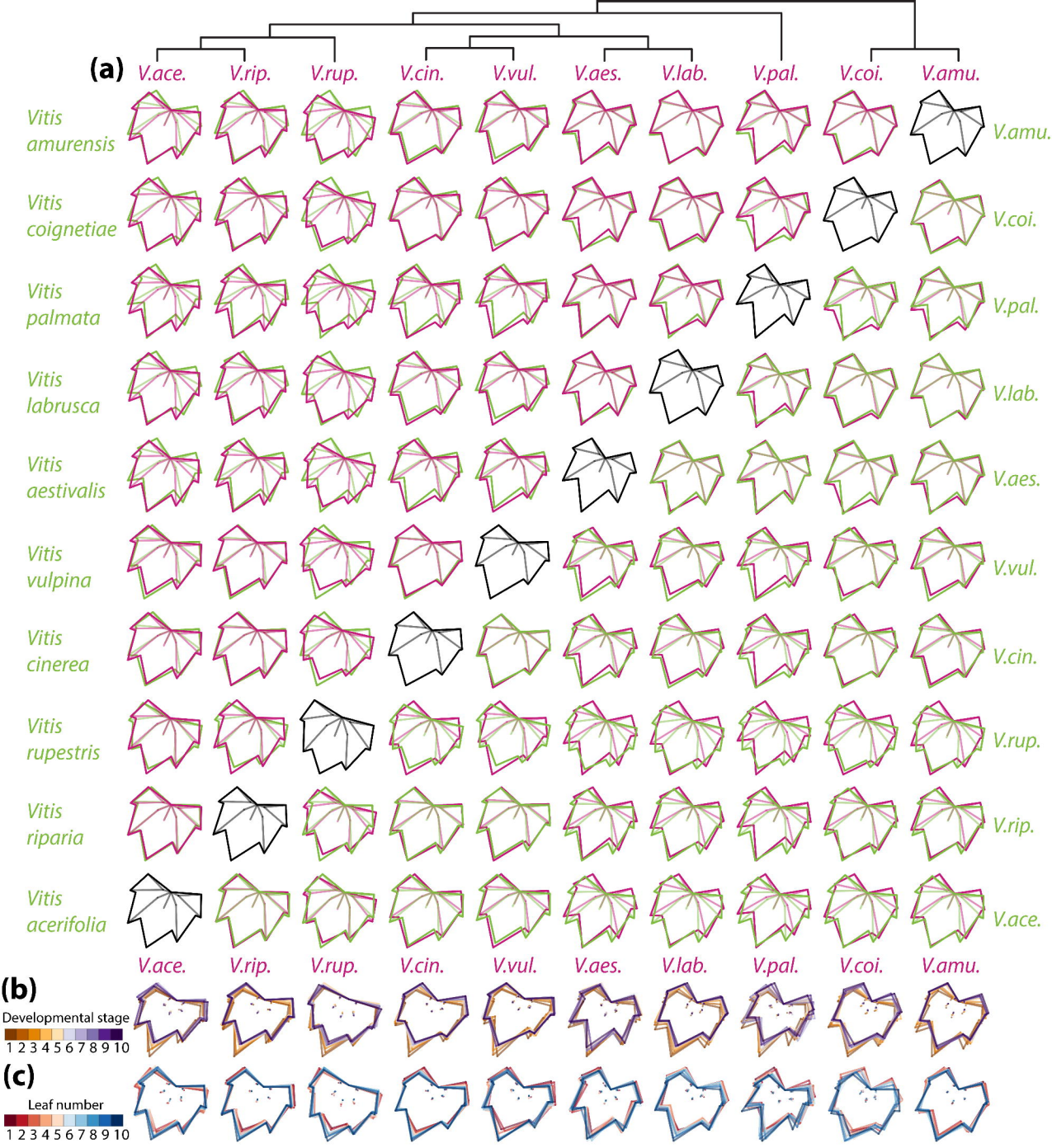
884 arrow to L10). Note non-inverse relationship between developmental stage and leaf
885 number changes in the distal lobe tips and petiolar vein branch points (indicated).
886
887 **Figure 5: Morphospace of wild *Vitis* species and domesticated grape.** (a)
888 "Eigenleaves" showing leaf morphs represented by PCs at -/+ 3 s.d. and shape
889 variance explained by each. Lobing and petiolar sinus variation indicated. (b)
890 Comparison of wild *Vitis* species leaf shape (red, black) with *V. vinifera* subsp.
891 *vinifera* and *V. vinifera* subsp. *sylvestris* (green, dark green, respectively), and highly-
892 lobed species (blue). Strong shape variation in lobing and petiolar sinus depth
893 indicated. (c) Hierarchical clustering based on morphology. Phylogenetic groupings
894 indicated in light gray. Groups mentioned in text indicated with Roman numerals.
895
896 **Figure 6: Evolution in developmental timing underlies leaf shape diversity in**
897 **domesticated grape.** (a and b) Latent shape attributes defining developmental
898 stage (a) and leaf number (b) in domesticated grape indicated with average leaf
899 shapes. (c) Comparison of changes in latent shape attributes across predicted
900 developmental stage (orange, outline S1; purple, arrow to S10) and predicted leaf
901 number (grey, outline L1; black, arrow to L10). (d and e) Predicted developmental
902 stage (d) and predicted leaf number (e) projected onto the *V. vinifera* subsp. *vinifera*
903 morphospace demonstrates changes in developmental timing (heterochrony)
904 underlie leaf shape diversity in domesticated grape. (g – i) Correlation between PC1
905 and PC2 with predicted developmental stage (i), predicted leaf number (h), and a
906 qualitative measure of petiolar sinus depth, PETSINMALF 2009 (i). (j) Hierarchical
907 clustering of measured traits in grape. Morphological and predicted temporal traits
908 (yellow), hirsuteness (green), reproductive transitions (blue), and overall leaf size
909 (grey) indicated.
910
911 **Supplemental Data**
912
913 **Figure S1:** Numbers of species, vines, leaves, and shoot positions sampled.
914

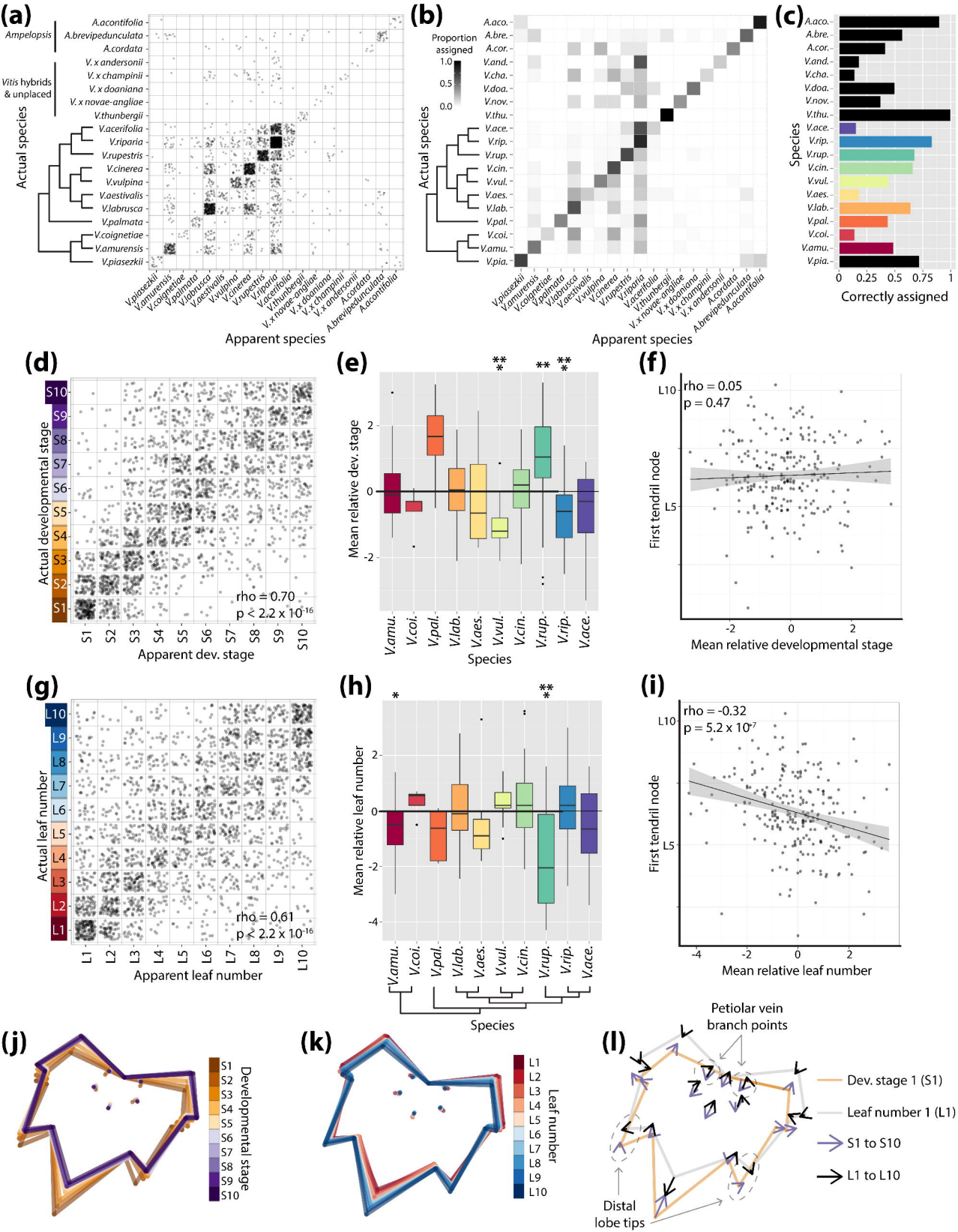
Table S1: Procrustes-aligned coordinates.

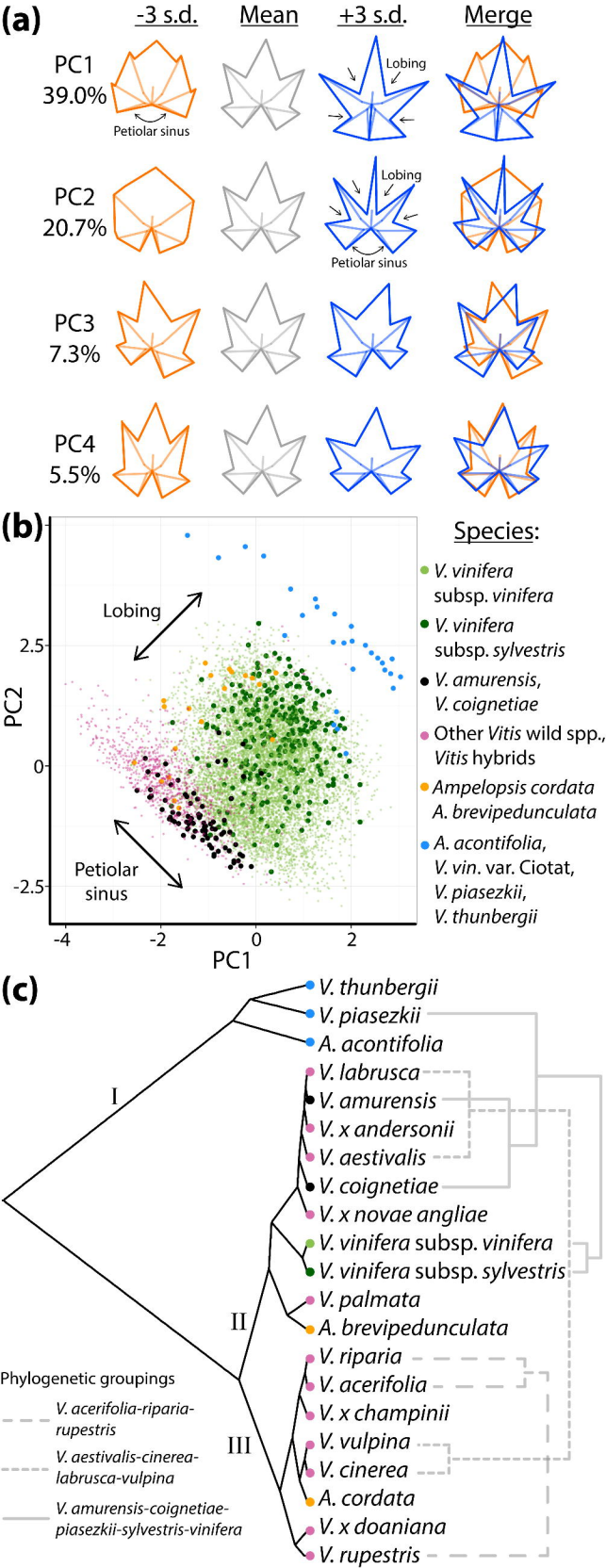
Table S2: Correlation between traits.

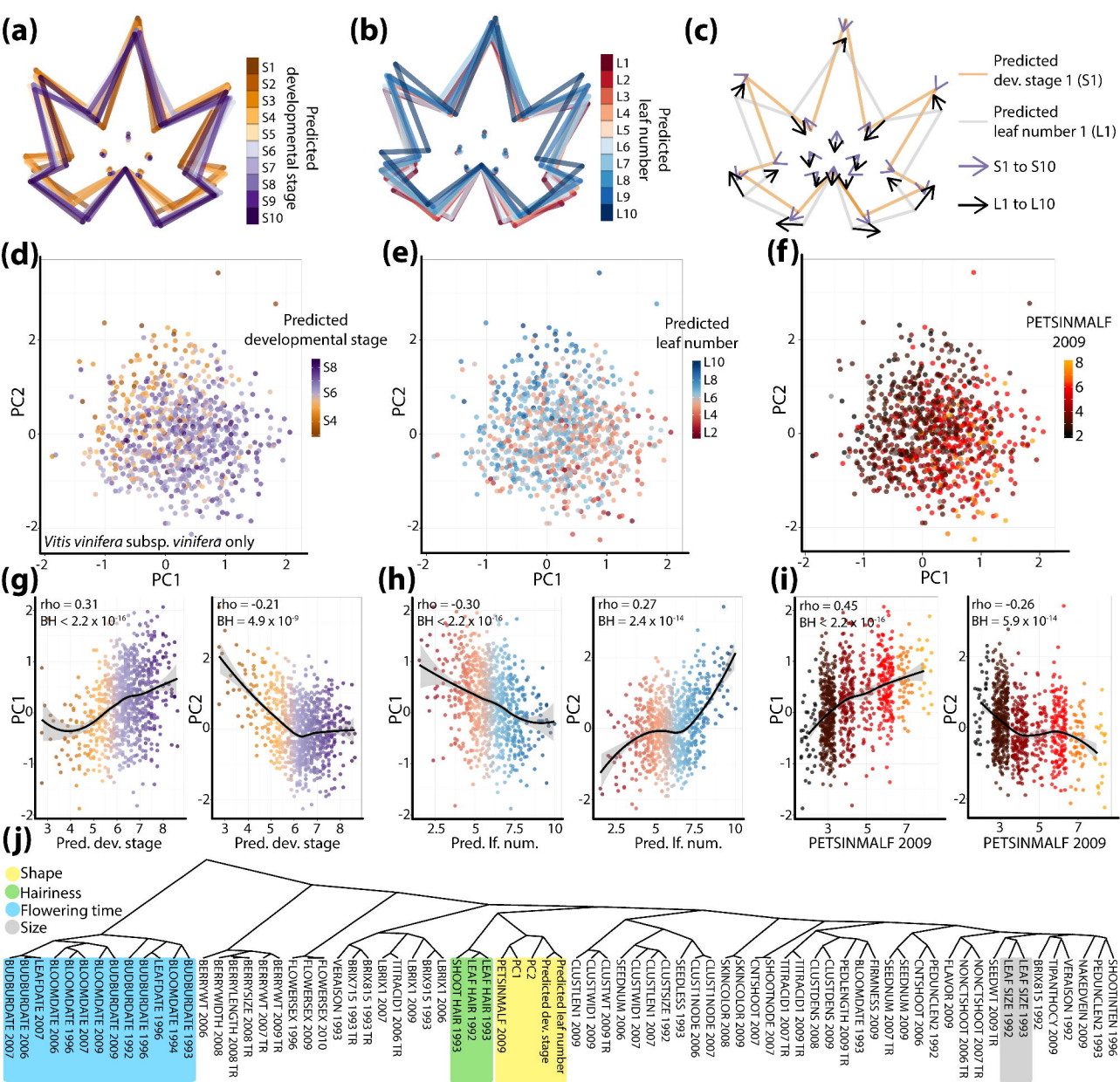

(a)
Tip
Midvein
Distal sinus
Distal lobe
Distal vein
Proximal vein
Petiolar vein
Proximal sinus
Proximal lobe
Petiolar sinus
Vitis labrusca
Vitis thunbergii
Vitis aestivalis
Vitis yanshanensis
Vitis riparia
(b)
Shoot tip
Young leaves
V.lab.
V.thu.
V.aes.
V.yan.
Shoot base
Old leaves
(c)
Developmental stage
S1
S2
None
S3
S4
S5
None
S6
S7
S8
None
S9
S10
None
Inflorescence patterning
L10
L9
L8
L7
L6
L5
L4
L3
L2
L1
V.rip.
Leaf number


(a)
-3 s.d.
Mean
+3 s.d.
Merge
PC1 32.0%
PC2 18.3%
PC3 12.4%
PC4 10.5%
(b)
PC1
PC2
PC3
PC4
Species:
Ampelopsis brevipedunculata
Vitis labrusca
Vitis riparia
Ampelopsis acontifolia
Vitis thunbergii
Vitis piasezkii
Vitis rupestris
(c)
PC1
PC2
PC3
Developmental stage
Species:
V. amurensis
V. coignetiae
V. palmata
V. labrusca
V. aestivalis
V. vulpina
V. cinerea
V. rupestris
V. riparia
V. acerifolia
(d)
Leaf number
(e)
Developmental stage
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
(f)
Leaf number
L1
L2
L3
L4
L5
L6
L7
L8
L9
L10


(a)
V.ace.
V.rip.
V.rup.
V.cin.
V.vul.
V.aes.
V.lab.
V.pal.
V.coi.
V.amu.
Vitis amurensis
Vitis coignetiae
Vitis palmata
Vitis labrusca
Vitis aestivalis
Vitis vulpina
Vitis cinerea
Vitis rupestris
Vitis riparia
Vitis acerifolia
(b)
Developmental stage
1 2 3 4 5 6 7 8 9 10
(c)
Leaf number
1 2 3 4 5 6 7 8 9 10


(a)
Ampelopsis
A.acontifolia
A.brevipedunculata
A.cordata
Vitis hybrids & unpiaced
V. x andersonii
V. x champinii
V. x doaniana
V. x novae-angliae
V.thunbergii
V.acerifolia
V.riparia
V.rupestris
V.cinerea
V.vulpina
V.aestivalis
V.labrusca
V.palmata
V.coignetiae
V.amurensis
V.piasezkii
Actual species
Apparent species
(b)
Proportion assigned
1.0
0.5
0.0
Actual species
Apparent species
(c)
Species
Correctly assigned
0
0.25
0.5
0.75
1
(d)
Actual developmental stage
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
rho = 0.70
p < 2.2 x 10-16
Apparent dev. stage
(e)
Mean relative dev. stage
Species
V.amu.
V.coi.
V.pal.
V.lab.
V.aes.
V.vul.
V.cin.
V.rup.
V.rip.
V.ace.
(f)
rho = 0.05
p = 0.47
L10
L5
First tendril node
Mean relative developmental stage
(g)
Actual leaf number
L1
L2
L3
L4
L5
L6
L7
L8
L9
L10
rho = 0.61
p < 2.2 x 10-16
Apparent leaf number
(h)
Mean relative leaf number
Species
(i)
rho = -0.32
p = 5.2 x 10-7
First tendril node
Mean relative leaf number
(j)
Developmental stage
(k)
Leaf number
(l)
Petiolar vein branch points
Dev. stage 1 (S1)
Leaf number 1 (L1)
S1 to S10
L1 to L10
Distal lobe tips


(a)
-3 s.d.
Mean
+3 s.d.
Merge
PC1
39.0%
Petiolar sinus
Lobing
PC2
20.7%
Lobing
Petiolar sinus
PC3
7.3%
PC4
5.5%
(b)
Lobing
Petiolar sinus
PC2
PC1
2.5
0
-2.5
-4
-2
0
2
Species:
V. vinifera subsp. vinifera
V. vinifera subsp. sylvestris
V. amurensis, V. coignetiae
Other Vitis wild spp., Vitis hybrids
Ampelopsis cordata A. brevipedunculata
A. acontifolia, V. vin. var. Ciotat, V. piasezkii, V. thunbergii
(c)
I
II
III
V. thunbergii
V. piasezkii
A. acontifolia
V. labrusca
V. amurensis
V. x andersonii
V. aestivalis
V. coignetiae
V. x novae angliae
V. vinifera subsp. vinifera
V. vinifera subsp. sylvestris
V. palmata
A. brevipedunculata
V. riparia
V. acerifolia
V. x champinii
V. vulpina
V. cinerea
A. cordata
V. x doaniana
V. rupestris
Phylogenetic groupings
V. acerifolia-riparia-rupestris
V. aestivalis-cinerea-labrusca-vulpina
V. amurensis-coignetiae-piasezkii-sylvestris-vinifera


(a)
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
Predicted developmental stage
(b)
L1
L2
L3
L4
L5
L6
L7
L8
L9
L10
Predicted leaf number
(c)
Predicted dev. stage 1 (S1)
Predicted leaf number 1 (L1)
S1 to S10
L1 to L10
(d)
Predicted developmental stage
S8
S6
S4
PC2
PC1
Vitis vinifera subsp. vinifera only
(e)
Predicted leaf number
L10
L8
L6
L4
L2
PC2
PC1
(f)
PETSINMALF 2009
8
6
4
2
PC2
PC1
(g)
rho = 0.31
BH < 2.2 x 10-16
PC1
Pred. dev. stage
rho = -0.21
BH = 4.9 x 10-9
PC2
Pred. dev. stage
(h)
rho = -0.30
BH < 2.2 x 10-16
PC1
Pred. lf. num.
rho = 0.27
BH = 2.4 x 10-14
PC2
Pred. lf. num.
(i)
rho = 0.45
BH < 2.2 x 10-16
PC1
PETSINMALF 2009
rho = -0.26
BH = 5.9 x 10-14
PC2
PETSINMALF 2009
(j)
Shape
Hairiness
Flowering time
Size
BUDBURDATE 2007
BUDBURDATE 2006
LEAFDATE 2007
BLOOMDATE 2006
BLOOMDATE 1996
BLOOMDATE 2007
BUDBURDATE 1992
BUDBURDATE 2009
BUDBURDATE 1996
LEAFDATE 1996
BLOOMDATE 1994
BUDBURDATE 1993
BERRYWT 2006
BERRYWIDTH 2008
BERRYLENGTH 2008 TR
BERRYSIZE 2008 TR
BERRYWT 2009 TR
BERRYWT 2007 TR
FLOWERSEX 1996
FLOWERSEX 2009
FLOWERSEX 2010
VERAISON 1993
BRIX715 1993 TR
BRIX815 1993 TR
LBRIX1 2007
TITRACID1 2006 TR
LBRIX1 2009
BRIX915 1993
LBRIX1 2006
LEAF HAIR 1993
LEAF HAIR 1992
SHOOT HAIR 1993
PETSINMALF 2009
PC1
PC2
Predicted dev. stage
Predicted leaf number
CLUSTLEN1 2009
CLUSTWID1 2009
CLUSTWT 2009 TR
CLUSTWID1 2007
CLUSTLEN1 2007
CLUSTSIZE 1992
SEEDLESS 1993
SEEDNUM 2006
CLUSTINODE 2006
CLUSTINODE 2007
SKINCOLOR 2009
SKINCOLOR 2008
CNTSHOOT 2007
SHOOTNODE 2007
TITRACID1 2007 TR
TITRACID1 2009 TR
CLUSTDENS 2008
CLUSTDENS 2009
PEDLENGTH 2009 TR
BLOOMDATE 1993
FIRMNESS 2009
SEEDNUM 2007 TR
SEEDNUM 2009
CNTSHOOT 2006
PEDUNCLEN2 1992
FLAVOR 2009
NONCTSHOOT 2006 TR
NONCTSHOOT 2007 TR
SEEDWT 2009 TR
LEAF SIZE 1992
LEAF SIZE 1993
BRIX815 1992
TIPANTHOCY 2009
VERAISON 1992
NAKEDVEIN 2009
PEDUNCLEN2 1993
SHOOTINTEN 1996