

## **Divergent leaf shapes among *Passiflora* species arise from a shared juvenile morphology**

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**Short title:** Heteroblasty and *Passiflora* leaf shape

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1 **Abstract**

2

3 PREMISE OF THE STUDY: Not only does leaf shape vary between *Passiflora* species,  
4 but between sequential nodes of the vine. The profound changes in leaf shape within  
5 *Passiflora* vines reflect the temporal development of the shoot apical meristem from  
6 which leaves are derived and patterned, a phenomenon known as heteroblasty.

7

8 METHODS: We perform a morphometric analysis of more than 3,300 leaves from 40  
9 different *Passiflora* species using two different methods: homologous landmarks and  
10 Elliptical Fourier Descriptors (EFDs).

11

12 KEY RESULTS: Changes in leaf shape across the vine are first quantified in allometric  
13 terms; that is, changes in the relative area of leaf sub-regions expressed in terms of  
14 overall leaf area. Shape is constrained to strict linear relationships as a function of size  
15 that vary between species. Statistical analysis of leaf shape, using landmarks and EFDs,  
16 reveals that species effects are the strongest, followed by interaction effects, and  
17 negligible heteroblasty effects. The ability of different nodes to predictively discriminate  
18 species and the variability of landmark and EFD traits at each node is then analyzed.  
19 Heteroblastic trajectories, the changes in leaf shape between the first and last measured  
20 leaves in a vine, are then compared between species in a multivariate space.

21

22 CONCLUSION: Leaf shape diversity among *Passiflora* species is expressed in a  
23 heteroblastic-dependent manner. Leaf shape is constrained by linear, allometric  
24 relationships related to leaf size that vary between species. There is a strong species x  
25 heteroblasty interaction effect for leaf shape, suggesting that different leaf shapes  
26 between species arise through changes in shape across nodes. The first leaves in the  
27 series are not only more like each other, but are also less variable across species. From  
28 this similar, shared leaf shape, subsequent leaves in the heteroblastic series follow  
29 divergent morphological trajectories. The disparate leaf shapes characteristic of  
30 *Passiflora* species arise from a shared, juvenile morphology.

31

## 32 INTRODUCTION

33

34 Heteroblasty is a phenomenon that results from the temporal development of the shoot  
35 apical meristem, creating successive changes in the traits of the lateral organs it produces  
36 at each node, including the shapes of leaves. Johann Wolfgang von Goethe described the  
37 transformation in leaf shape across a shoot as a “metamorphosis”, correctly suggesting  
38 that lateral organs are serially homologous structures, from the juvenile and adult leaf  
39 shapes a plant displays during vegetative development to reproductive organs (Goethe,  
40 1952; Friedman and Diggle, 2011; Chitwood and Sinha, 2014). There have been many  
41 hypotheses about the origins of heteroblastic changes in leaf shape. Inspired by Ernst  
42 Haeckel, it was hypothesized that juvenile leaf shapes represented the ancestral condition,  
43 and that adult leaves were derived leaf forms (Cushman, 1902; Cushman, 1903). In  
44 parallel, another school of thought led by Karl Goebel favored a more environmental  
45 explanation for heteroblasty: only supported by the photosynthesis of cotyledons and  
46 young leaves, the shape of juvenile leaves represented the aborted development of mature  
47 leaf morphology (Goebel, 1908). Careful morphological analysis of young leaf primordia  
48 refutes such an idea in Cucurbits (Jones, 1992; Jones, 1995), but classical (Allsopp,  
49 1953a; Allsopp, 1953b; Allsopp, 1954; Njoku, 1956; Njoku, 1971; Roebbelen, 1957;  
50 Feldman and Cutter, 1970) and molecular experiments (Yang et al., 2013; Yu et al.,  
51 2013) in other species suggest that sugar can serve as a signal hastening the heteroblastic  
52 progression of leaf forms.

53

54 *Passiflora* species exhibit dramatic heteroblastic changes in leaf shape (**Fig. 1**).  
55 *Passiflora edulis* begins with an ellipsoid leaf shape, that transitions to a tri-lobed leaf  
56 later in the series (**Fig. 1A**). Like *P. edulis*, *P. caerulea* leaves begin ellipsoid, but  
57 transition to a highly-dissected tri-lobe leaf morph and then to four- and five-lobed  
58 leaves, and even seven-lobed leaves, as previously reported (Allsopp, 1967) and observed  
59 by the authors. Sometimes the transition to lobed leaves is erratic, as in *P. racemosa*, in  
60 which the lobes manifest asymmetrically or can revert to the juvenile form with no lobes  
61 later in the leaf series (**Fig. 1A**). Other *Passiflora* species exhibit variable degrees of  
62 heteroblastic changes in leaf shape (**Fig. 1B**). Unlike the generalized theories of

63 heteroblastic changes in leaf shape inspired by Haeckel or put forth by Goebel, a specific  
64 hypothesis has been proposed for the dramatic changes in leaf shape observed in  
65 *Passiflora*. Just as diversity in leaf shape among *Passiflora* species is hypothesized to  
66 result from diversifying selective pressure from egg-laying *Heliconius* butterflies using  
67 leaf shape as a cue (Klucking, 1992; MacDougal, 1994; Gilbert, 1975; Dell'aglio, 2016)  
68 diverse leaf shapes in a single vine, resulting from heteroblasty, are thought to similarly  
69 deceive butterflies by mimicking non-host plants during critical stages of the vine  
70 lifecycle (Gilbert, 1982).

71

72 Morphometric approaches are critical to separating shape attributes that differentiate  
73 species (or genotypes, in genetic studies) regardless of developmental context (Chitwood  
74 et al., 2013; Chitwood et al., 2014a) versus purely heteroblastic changes in leaf shape that  
75 are shared among closely related species (Chitwood et al., 2012; Chitwood et al., 2016a).  
76 Most studies focus on genotype effects that alter leaf shape regardless of node position in  
77 the shoot, but the genetic basis of natural variation in heteroblastic shape change itself  
78 can also be measured (Chitwood et al., 2014b). The shape features that vary between  
79 species are often distinct from those that differentiate leaves arising from sequential  
80 nodes, such that using discriminant analyses, species identity can be predicted regardless  
81 of node position and vice versa (Chitwood et al., 2016a; Chitwood et al., 2016b). This  
82 result is also true among *Passiflora* species (although as later mentioned, it is restricted to  
83 certain nodes along the shoot; Chitwood and Otoni, 2016a), demonstrating distinct  
84 contributions of species identity and heteroblasty to the shape of each leaf.

85

86 Here, we analyze more than 3,300 leaves from 40 *Passiflora* species (Chitwood and  
87 Otoni, 2016a; 2016b) but focus on heteroblastic changes in leaf shape. Superimposing  
88 averaged leaves from across species, the first two leaves of the series are highly similar in  
89 their shape, and more deeply lobed leaves arise later in the the leaf series. Leaf shape  
90 changes across the heteroblastic series are allometrically constrained to linear  
91 relationships dependent on leaf size, but these allometric constraints vary between  
92 species. A statistical analysis of leaf shape shows that species effects are the largest  
93 followed by species x heteroblasty interaction effects; heteroblasty effects are negligible.

94 How such strong species x heteroblasty effects manifest among *Passiflora* species is  
95 investigated further. Discriminant analysis of shape features capable of predicting species  
96 identity for leaves at each node demonstrates that leaves arising from the first nodes at  
97 the base of the vine are more like each other and more easily confused between species.  
98 The similarity in shape between leaves arising from early nodes between species is  
99 supported by an observed lack of variability in juvenile compared to adult leaves.  
100 Analysis of changes in shape between the first and last leaves of a vine in a multivariate  
101 space suggest that juvenile leaves between species arise from a shared leaf shape. The  
102 diversity of leaf shapes observed among *Passiflora* species arises from divergent  
103 heteroblastic changes in leaf shape away from a shared, juvenile leaf form.

104

## 105 **MATERIALS AND METHODS**

106

### 107 *Data Description*

108

109 Portions of these Materials and Methods are recapitulated from Chitwood and Otoni  
110 (2016a). The two manuscripts analyze different biological problems arising from  
111 the same dataset (Chitwood and Otoni, 2016b). Recapitulating the methods here is  
112 meant to aid the reader and is in line with Committee on Publication Ethics (COPE)  
113 guidelines.

114

115 The 555 original scans used for analysis (Chitwood and Otoni, 2016b) represent 40  
116 different species of *Passiflora* in which the order of leaves arising from the vine is  
117 recorded (starting with “1” for the youngest leaf scanned from the growing tip of  
118 each vine). We importantly note: the numbering of nodes in the raw scans described  
119 above (Chitwood and Otoni, 2016b), starting at the tip of the shoot, is opposite from  
120 the numbering of nodes presented in the manuscript, in which numbering (starting  
121 with “1”) begins with the oldest leaf at the base of the shoot. The reason for this  
122 opposite numbering in the manuscript is that by beginning the counting of nodes  
123 with “1” at the shoot base the numbering aligns with the heteroblastic series (which  
124 begins with the first emerged leaf).

125

126 Data from more than 3,300 leaves, and the code to analyze the data and reproduce  
127 the figures in this manuscript, are provided, including both landmark data (found in  
128 the GitHub repository under  
129 `PassifloraLeaves/Paper2/Figure2/1.all_landmarks/0.refromated_Proc_coord.txt`),  
130 measuring the vasculature, lobes, and sinuses, and Elliptical Fourier Descriptor  
131 (EFD) data (found in the GitHub repository under  
132 `PassifloraLeaves/Paper2/Figure2/0.harmonics/0.passiflora_nef.txt`), which  
133 quantifies leaf outlines (Chitwood, 2016).

134

135 *Plant materials and growth conditions*

136

137 *Passiflora* germplasm was kindly provided by R. Silva (Viveiros Flora Brasil, Araguari,  
138 MG, Brazil), Dr. F.G. Faleiro (EMBRAPA Cerrados, Planaltina, DF, Brazil), Prof. M.M.  
139 Souza (Universidade Estadual de Santa Cruz - UESC, Ilhéus, BA, Brazil), M. Peixoto  
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141 Grosso, Tangará da Serra, MT, Brazil), and Prof. C.H. Bruckner (Universidade Federal  
142 de Viçosa, Viçosa, MG, Brazil).

143

144 The plants were germinated from seed, planted between late October 2015 and early  
145 March 2016, in Viçosa, at the Federal University of Viçosa, MG, Brazil. The populations  
146 were raised and maintained under polycarbonate-covered greenhouse conditions,  
147 equipped with automatic environmental control using exhaust fans and evaporative  
148 cooling panels (with expanded clay wettable pads). Seeds for each *Passiflora* species  
149 were sown in 128 cell propagation plastic trays (GPlan Comércio de Produtos Agrícolas  
150 EIRELI – ME, São Paulo, SP, Brazil) filled with horticultural organic Tropstrato HT  
151 Hortaliças substrate (Vida Verde Indústria e Comércio de Insumos Orgânicos Ltda, Mogi  
152 Mirim, SP, Brazil). After germination (30-40 days), plantlets were individually  
153 transplanted to 5 L capacity plastic pots (EME-A-EME Ind. Com. Ltda., Petrópolis, RJ,  
154 Brazil) filled with horticultural substrate. Each pot received 5 g of Osmocote® Plus NPK

155 15-09-12 3-4 month controlled release fertilizer (Scotts, USA). Plants were irrigated on a  
156 daily-basis with tap water, and no phytosanitary control was applied.

157

158 For scanning, a multifunction printer (Canon PIXMA MX340 Wireless Office All-in-One  
159 Printer, model 4204B019, USA) was used. A 20 cm metallic ruler was positioned at the  
160 bottom of each scanned sheet as a size marker. Leaves were carefully detached, from the  
161 base to the tip of the shoot, and affixed to an A4 paper sheet, adaxial face down, using 12  
162 mm-double sided tape (Scotch Model 9400, 3M do Brasil, SP, Brazil). The numbers  
163 written near each leaf indicate position in the shoot, in a tip-to-base direction, starting  
164 with the youngest leaf at the tip of the shoot.

165

166 *Morphometric and statistical analyses*

167

168 All morphometric data and code used for statistical analysis is available on GitHub  
169 (Chitwood, 2016). Landmarks, as described in the text, were placed on leaves in  
170 ImageJ (Abramoff, Magalhaes, and Ram, 2004). Procrustes superimposition was  
171 performed using the shapes package (Dryden, 2015) in R (R Development Core  
172 Team, 2016) with the procGPA function using reflect=TRUE.

173

174 To isolate outlines for Elliptical Fourier Descriptor (EFD) analysis, the “Make  
175 Binary” function in ImageJ (Abramoff, Magalhaes, and Ram, 2004) was found to be  
176 sufficient to segment leaves. The wand tool was used to select individual binary leaf  
177 outlines, which were pasted into a new canvas, which was subsequently saved as an  
178 individual image, which was named by vine and node position from which the leaf  
179 was derived. The binary images were batch converted into RGB .bmp files and read  
180 into SHAPE, which was used to perform chain-code analysis (Iwata et al., 1998;  
181 Iwata and Ukai, 2002). The resulting chain-code .chc file was then used to calculate  
182 normalized EFDs. The resulting normalized EFD .nef file was then read into Momocs  
183 (version 0.2-6) (Bonhomme et al., 2014) in R. The harmonic contributions to shape  
184 were visualized using the hcontrib function. Averaged leaf outlines were calculated  
185 using the meanShapes function.

186

187 Unless otherwise noted, all visualization was performed using ggplot2 in R  
188 (Wickham, 2009). Analysis of Variance (ANOVA) was performed using the aov  
189 function fitting the model  $\text{trait} \sim \text{species} * \text{heteroblasty}$ . Linear Discriminant Analysis  
190 (LDA) was performed using the lda function and subsequent prediction of species  
191 identity or heteroblastic node position performed using the predict function with  
192 MASS (Venables and Ripley, 2002). For prediction, LDA was performed with  
193  $\text{CV} = \text{"TRUE"}$ , which is a “leave one out” cross-validation approach in which for each  
194 leaf an LDA is performed excluding the leaf after which it is assigned to the resulting  
195 LDA space it was excluded from. Hierarchical clustering was performed using the  
196 hclust function. t-distributed Stochastic Neighbor Embedding (t-SNE) was performed  
197 using the Rtsne package (Krijthe, 2015) in R with perplexity=40.

198

## 199 **RESULTS AND DISCUSSION**

200

### 201 *Heteroblastic changes in Passiflora leaf shape*

202

203 The greater than 3,300 leaves analyzed in this study come from 40 different species of  
204 *Passiflora* and were collected in order from the base of the vine onwards to the growing  
205 tip, keeping track of the node from which each leaf originated. Among the 40 *Passiflora*  
206 species sampled, the degree of heteroblastic shape change is variable (**Fig. 1**). Previously  
207 (Chitwood and Otoni, 2016a), we defined seven species groups (classes) based on their  
208 clustering in a Principal Component Analysis (PCA) and qualitative shape differences.  
209 Members of Class G, with lance-shaped leaves, generally exhibit little to no changes in  
210 leaf shape across the leaf series, and similarly Class E leaves remain round regardless of  
211 shoot position. Members of Class C and D, which are characterized by different numbers  
212 of lobes later in the series, begin with a rounder, less lobed juvenile leaf shape at the base  
213 of the vine. Members of Class A and B, with wide “bat-like” leaves begin with a rounder  
214 leaf shape that transitions to a stereotypical wide leaf (**Fig. 1**).

215

216 Generally, if heteroblastic changes in leaf shape are observed, the juvenile leaf form (at  
217 the base of the shoot) tends to be rounder and exhibit less lobing compared to adult leaves  
218 at the shoot tip (**Fig. 1B**). To substantiate this statement, we used 15 landmarks  
219 measuring vascular patterning and the position of the lobes and sinuses, and Elliptical  
220 Fourier Descriptors (EFDs), quantifying the contours of leaves, to visualize shape  
221 changes through the heteroblastic series (**Fig. 2A-B**). Landmarks are x,y coordinates that  
222 define the position of homologous points found on every leaf, such that leaf shape is  
223 represented as a set of x,y points shared between samples (Bookstein, 1997). EFDs  
224 convert shapes into chain code, a string of numbers that records pixel movements to  
225 perfectly recapitulate the shape (Freeman, 1974). The chain code is treated as a wave  
226 function and decomposed into a harmonic series using a Fourier transform (Kuhl and  
227 Giardina, 1982). The coefficients of the harmonic series represent shape and are used in  
228 subsequent analyses.

229

230 Averaged landmarked leaves (**Fig. 2C-D**) and EFD-derived outlines (**Fig. 2E**) across the  
231 leaf series compared to the first leaf show that leaves become progressively more  
232 dissected. However, most of these heteroblastic changes occur between leaves 1 and 2 at  
233 the shoot base, and the remainder of leaves later in the shoot, on average, shows little  
234 further changes in shape. This result is consistent with the previous observation  
235 (Chitwood and Otoni, 2016a) that shape features in juvenile leaves in the first nodes  
236 allow them to be correctly assigned to the predicted node at higher rates than leaves later  
237 in the series. We return to this idea later, and explore the hypothesis that across species  
238 juvenile leaves are more like each other than leaves later in the series that exhibit  
239 divergent shapes.

240

241 *Allometric changes in leaf shape across the heteroblastic series*

242

243 To explore the relative contributions of leaf sub-areas to differences in leaf shape across  
244 the heteroblastic leaf series in different species, we performed an allometric analysis.  
245 Changes in leaf shape, whether between species or within the heteroblastic series, often  
246 correlate linearly with size (Chitwood et al., 2016b); whether such relationships exist in

247 this dataset remains an open question. Procrustes-aligned leaves were divided into sub-  
248 regions (**Fig. 2F**), including the areas of the midvein and proximal vein, and distal and  
249 proximal leaf blade areas. The rationale of using Procrustes-aligned leaf shapes (which  
250 have been scaled, translated, and rotated to superimpose leaves for analysis) is to analyze  
251 the relative contributions of vein and blade area to total leaf area. When plotting the  
252 square root of each sub-region area against the square root of the overall Procrustes-  
253 aligned leaf area, clear linear relationships are observed (**Fig. 2G**). Notably, blade areas  
254 expand at a higher rate compared to vein areas as the total leaf area increases (that is, the  
255 slope of the blade sub-regions is greater than the vein sub-regions). That total area of the  
256 leaf occupied by blade expands at a faster rate than the area occupied by veins is  
257 consistent with the previous results observed in grapevines (Chitwood et al., 2016a;  
258 Chitwood et al., 2016b).

259

260 When the results are plotted for each species, the general trend of blade area expanding at  
261 the expense of vein area is observed, but there is a large amount of variation between  
262 species (**Fig. 3**). For example, *P. misera* exhibits linear relationships in which both blade  
263 regions have similar slopes that are greater than the vein regions. Contrastingly, the  
264 distribution of total leaf area for *P. caerulea* is bimodal, and differences in each  
265 population of leaves contributes to widely different slopes between the distal and  
266 proximal blade sub-regions. The distinctness of each *P. caerulea* sub-population reflects  
267 the discrete transformation of leaf shape from entire to highly dissected and palmate (**Fig.**  
268 **1A**). This is reflected when the heteroblastic node number is projected onto the plots  
269 (**Fig. 4**), revealing distinct populations of juvenile and adult leaves with different ratios of  
270 blade and vein areas that contribute to each leaf type. Other species vary in the extent that  
271 the heteroblastic series is defined by the linear allometric relationships contributing to  
272 differences in leaf shape across the leaf series.

273

274 Although overall blade sub-regions expand at faster rates compared to vein sub-regions,  
275 and this relationship is mostly linear across all species (**Fig. 2G**), species vary widely in  
276 the relative ratios of these regions across allometric lines (**Fig. 3**) and the heteroblastic  
277 series (**Fig. 4**).

278

279 *Statistical effects of species, heteroblasty, and interaction*

280

281 Previously, we demonstrated using a Linear Discriminant Analysis (LDA) that not only  
282 can the leaves of *Passiflora* species be predicted regardless of the node from which they  
283 arise, but surprisingly too that the leaf node can be predicted regardless of the species  
284 (Chitwood and Otoni, 2016a). This result is consistent with work in grapevine (Chitwood  
285 et al., 2016a; 2016b) and demonstrates that within the complex shapes of leaves, some  
286 attributes vary independently by species whereas others by node position.

287

288 To more rigorously quantify these effects in *Passiflora*, we performed an Analysis of  
289 Variance (ANOVA) for each x and y landmark coordinate as well as coefficients of the  
290 harmonic series resulting from an Elliptical Fourier Descriptor (EFD) analysis (**Fig. 2A-**  
291 **B**). Each trait was modeled by species\*heteroblasty, which takes into consideration the  
292 additive species and heteroblastic effects as well as their interaction (**Figs. 5-6**). *P. actinia*  
293 was set as the intercept, primarily because its species epithet is alphabetically first, but  
294 conveniently, the round shape of *P. actinia* leaves means that since effect sizes are  
295 compared against this species (by definition, *P. actinia* effects sizes are 0) the magnitude  
296 of leaves of other species deviating from a round shape is being measured.

297

298 The largest effect on leaf shape is species (**Fig. 5**). The strongest effect sizes are seen in x  
299 landmark coordinates of the petiolar junction in *P. micropetala* and *P. gracilis*, likely  
300 owing to the unique intersection of veins in these species. But more general trends are  
301 also observable. The x and y landmarks of the distal sinus and harmonic coefficients from  
302 the EFD analysis are widely affected in Class C (lobed species) and Classes A and B  
303 (wide, wing-shaped species), reflecting the deviation of these leaf shape classes from the  
304 round shape of *P. actinia* leaves.

305

306 Heteroblasty and interaction effects (**Fig. 6**) were about a magnitude less than species  
307 effects (compare legend between **Fig. 5** and **Fig. 6**). The heteroblasty effect is negligible  
308 compared to the interaction effect (**Fig. 6**, see top row). This does not mean there are no

309 shape attributes modulated by node position independent from species effects, as we  
310 previously showed node position can be predicted independently from species identity  
311 (albeit to a much less degree and most strongly for the first two leaves in the heteroblastic  
312 series; Chitwood and Otoni, 2016a). Rather, it indicates that heteroblastic-independent  
313 effects on leaf shape are slight compared to species and interaction effects. The strongest  
314 species x heteroblasty interaction effects are found in the EFD harmonic coefficients of  
315 *P. cincinnata*, the leaves of which are highly lobed. Generally, like species effects (**Fig.**  
316 **5**), the interaction effects are strongest for the lobed (Class C) and winged (Classes A and  
317 B) leaf shapes (**Fig. 6**).

318

319 Given that 1) we previously showed that node position can be predicted independently  
320 from species identity, although strongest for the first two leaves (Chitwood and Otoni,  
321 2016a) and 2) that the interaction effect sizes between species and heteroblasty are much  
322 stronger than heteroblasty alone (**Figs. 5-6**), we hypothesized that evolutionary  
323 differences in leaf shape between species of *Passiflora* arise within a heteroblastic  
324 context, which we explore more thoroughly, below.

325

326 *Divergent heteroblastic trajectories and similar juvenile leaf shapes*

327

328 Many pieces of evidence suggest the earliest leaf shapes in the heteroblastic series are  
329 similar across *Passiflora* species, and that leaves later in the series differ between species.  
330 When the average shape of leaves across the heteroblastic series are compared using  
331 landmarks (**Fig. 2D**) and contours derived from Elliptical Fourier Descriptors (EFDs)  
332 (**Fig. 2E**) it is evident that the more lobed leaf shape characteristic of later leaves is  
333 achieved within the first two nodes.

334

335 To test the idea that juvenile leaves at the base of the shoot are more similar between  
336 species than those later in the series, we performed a Linear Discriminant Analysis  
337 (LDA) using both landmark and EFDs to discriminate leaves from each node by species  
338 identity (**Fig. 7A**). Although there is wide variability of the ability to discriminate the  
339 leaves of each species, the LDAs using leaves from nodes 1 and 2 performed poorly in

340 their ability to discriminate leaves by species compared to subsequent nodes. From the  
341 overall average correct reassignment rate for the LDA performed for each node (see  
342 bottom of **Fig. 7A**) it is evident that leaves from nodes 1 and 2 have less distinctive  
343 features differentiating leaves from species compared to later nodes. This suggests that  
344 leaves from nodes 1 and 2 are more similar in shape between species than leaves from  
345 later nodes.

346

347 A more direct test of variability in leaf shape is to measure the standard deviation of the  
348 raw traits used to measure leaf shape. Shape features of the leaf were hierarchically  
349 clustered and the standard deviation for each across species for each node calculated (**Fig.**  
350 **7B**). Most shape features either have reduced or unchanged standard deviation values in  
351 the first 1-3 leaves of the series compared to later leaves. A minority of shape features,  
352 especially the x coordinate values for landmarks defining the petiolar junction and bases  
353 of the major veins (landmarks 1-6) have increased standard deviation values in the earlier  
354 nodes. The results suggest that generally leaf shape is less variable in leaves from the first  
355 nodes (**Fig. 7B**) consistent with the observation that juvenile leaves discriminate species  
356 less than leaves later in the series (**Fig. 7A**). Exceptionally, the landmarks defining the  
357 petiolar junction in the x coordinate direction are more variable in juvenile than adult  
358 leaves (**Fig. 7B**).

359

360 To visualize the divergent heteroblastic trajectories leading to disparate leaf shapes  
361 between *Passiflora* species, we used a t-distributed Stochastic Neighbor Embedding (t-  
362 SNE) approach to reduce the dimensionality of the data (**Fig. 8A**) (Krijthe, 2015). t-SNE  
363 separates species classes and benefits from no assumptions of linearity and reducing the  
364 data to strictly two dimensions. By doing so, each sampled vine can be visualized as a  
365 vector in two-dimensional space, with the base and tip of the vector corresponding to the  
366 first sampled node at the base of the shoot and the furthest sampled node at the tip of the  
367 shoot, respectively (**Fig. 8B**). Each vector, therefore, is a representation of the shape  
368 space traversed over nodes across the heteroblastic series.

369

370 The bases of the vectors representing each vine tend to cluster together, with similar  
371 Dimension 1 values but varying across Dimension 2. From this common region  
372 representing a shared juvenile leaf shape, the directions of each vector for different  
373 species classes vary, representing differing heteroblastic trajectories leading to disparate  
374 adult leaf shapes at nodes towards the shoot tips. To better visualize the divergent  
375 heteroblastic trajectories of each species class, each vector base was centered to the origin  
376 (**Fig. 8C**). After centering, it is apparent that different species classes vary drastically—  
377 sometimes diametrically opposed—in the direction of their heteroblastic shape changes.  
378

379 Collectively, the inability of leaves from the first nodes to successfully discriminate  
380 different species (**Fig. 7A**), the reduced variability in shape features of leaves from the  
381 first nodes (**Fig. 7B**), and the divergent heteroblastic trajectories between species classes  
382 (**Fig. 8**) demonstrate that juvenile leaves between *Passiflora* species are similar and that  
383 divergent heteroblastic trajectories are responsible for the distinctive leaf shapes between  
384 species.

385

## 386 **CONCLUSIONS**

387

388 Many explanations for heteroblasty, the changes in leaf shape and other traits across a  
389 shoot resulting from the temporal development of the shoot apical meristem from which  
390 lateral organs arise, have been proposed. The idea that juvenile and adult leaves  
391 recapitulate the ancestral and derived leaf forms across evolution (Cushman, 1902;  
392 Cushman, 1903) or that juvenile leaves result from the lack of photosynthate to complete  
393 development (Goebel, 1908) have been proposed as possible explanations for the  
394 dramatic changes in leaf shape across a shoot. In *Passiflora*, the heteroblastic series has  
395 been hypothesized to be a mechanism to avoid *Heliconius* butterflies that use leaf shape  
396 as a cue to lay eggs (Gilbert, 1982). Although we cannot distinguish between these  
397 alternatives, it is nonetheless important to quantify changes in leaf shape across the  
398 heteroblastic series, so that a rigorous understanding of how leaf shape changes manifest  
399 across vines contribute to diversity within the genus *Passiflora* (**Fig. 1**). Doing so

400 provides insight into how different leaf shapes arise within a developmental and  
401 evolutionary context.  
402  
403 Superimposing averaged leaf shapes from across the heteroblastic series, the leaves  
404 arising from the first two nodes share a similar shape and more deeply lobed leaves arise  
405 later in the series (**Fig. 2C-E**). These heteroblastic changes in leaf shape are  
406 allometrically constrained to linear relationships, and these strict linear relationships vary  
407 between species (**Figs. 2-4**). Statistically modeling species, heteroblasty, and interaction  
408 effects, species effect sizes are the largest (**Fig. 5**) followed by interaction effects  
409 approximately a magnitude less and negligible heteroblasty effects (**Fig. 6**). Considering  
410 the unique similarity in the shape of leaves arising from the first two nodes, we  
411 hypothesized that divergent leaf shapes between *Passiflora* species arising later in the  
412 series arise from a shared, juvenile leaf shape. Juvenile leaves are more often mistakenly  
413 identified between species than adult leaves found later in the shoot (**Fig. 7A**), and  
414 consistent with juvenile leaves resembling each other, the variability of most  
415 morphometric features is lower in juvenile compared to adult leaves (**Fig. 7B**).  
416 Comparing the first and last leaves of a shoot within a multivariate space, the  
417 heteroblastic trajectories of different species are divergent, originating from a similar  
418 juvenile form but traversing towards disparate shapes (**Fig. 8**). Our data show that the  
419 striking differences in leaf shape between *Passiflora* species are expressed in a  
420 developmental manner, later in the heteroblastic series, arising from a shared juvenile leaf  
421 shape.

422

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424

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428

#### 429 **Authors' contributions**

430

431 The overall project was conceived by DHC and WCO. WCO grew and scanned all  
432 plant material and DHC carried out analysis. DHC and WCO wrote the paper.

433

#### 434 **Competing interests**

435

436 The authors declare that they have no competing interests.

437

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## 593 **Figure legends**

594

595  
596 **Figure 1: The heteroblastic series in 40 *Passiflora* species.** A) Notable examples of  
597 heteroblastic changes in leaf shape in the indicated *Passiflora* species. B) Examples of  
598 changes in leaf shape across the heteroblastic series of the 40 *Passiflora* species analyzed  
599 in this manuscript, grouped by class. Leaves are scaled such that leaves in the series have  
600 the same height. Silhouettes of the first ten leaves of the series are shown. Leaves are  
601 arranged from the first leaf (at the shoot base) onwards towards the shoot tip.

602

603 **Figure 2: Morphometric methods used to study heteroblastic changes in *Passiflora***  
604 **leaf shape.** A) The 15 landmarks used for analysis. Left to right, landmark placement

605 when the distal and proximal veins l) pinnately emerge from the midvein, m) both  
606 originate from the petiolar junction, or r) the proximal vein branches from the distal. **B)**  
607 Harmonic contributions to shape resulting from Elliptical Fourier Descriptor (EFD)  
608 analysis. The harmonic rank is arranged horizontally and the amplification factor  
609 vertically. **C)** For each heteroblastic node, the mean leaf as measured with landmarks in  
610 shown in black, whereas all landmark data for leaves from the node are depicted in semi-  
611 transparent blue. **D)** The average landmark leaf from node 1 is depicted in green and  
612 superimposed upon the averaged landmark leaves from other nodes depicted in magenta.  
613 **E)** Mean leaves calculated for each heteroblastic node from the harmonic series resulting  
614 from an Elliptical Fourier Descriptor (EFD) analysis of leaf contours. The mean contour  
615 of leaves from node 1 is depicted in green and the mean contour leaves from other nodes  
616 in magenta. **F)** Sub-areas of Procrustes-aligned landmark data calculated for each leaf. **G)**  
617 Overall allometric relationships for the square root of distal blade area (light green),  
618 proximal blade area (dark green), midvein area (light brown), and proximal vein area  
619 (dark brown) plotted against the square root of overall leaf area. All areas are calculated  
620 from Procrustes-aligned landmark data as indicated in F). Heteroblastic node position is  
621 numbered “1” starting from the shoot base. Note: for convenience to the reader, panels A)  
622 and B) are recapitulated in the companion manuscript (Chitwood and Otoni, 2016a). Leaf  
623 depicting sub-areas shown in **Fig. 3** re-drawn here for convenience.

624

625 **Figure 3: Allometric changes in relative leaf areas in different *Passiflora* species.** For  
626 each species, the linear relationships between the square root of sub-areas (distal blade  
627 area, light green; proximal blade area, dark green; midvein area, light brown; and  
628 proximal vein area, dark brown) are plotted against the square root of total area for  
629 Procrustes-aligned landmark data. Fitted linear models are super-imposed with 95%  
630 confidence bands against data points. Mean leaf contours for each species are provided  
631 for reference, colored by class membership. Leaf depicting sub-areas shown in **Fig. 2** re-  
632 drawn here for convenience.

633

634 **Figure 4: Allometric changes in relative leaf areas across the heteroblastic series.**  
635 Same plots as in **Fig. 3** except colored by heteroblastic node. For some species, strong

636 linear allometric changes across the heteroblastic leaf series are observed. Heteroblastic  
637 node color scheme: shoot base, black; middle shoot, orange; shoot tip, yellow.

638 Heteroblastic node position is numbered “1” starting from the shoot base.

639

640 **Figure 5: Analysis of Variance (ANOVA) species effects.** For each *Passiflora* species  
641 separated by class (rows) and x,y landmark and Elliptical Fourier Descriptor (EFD) traits  
642 hierarchically clustered (columns) the species effect resulting from the model trait ~  
643 species\*heteroblasty is shown. Opacity indicates magnitude of the species effect and  
644 color direction (positive, magenta; negative, green). Effect sizes and directions are  
645 relative to *P. actinia* (indicated with asterisk).

646

647 **Figure 6: Analysis of Variance (ANOVA) heteroblasty and species x heteroblasty**  
648 **interaction effects.** For each *Passiflora* species separated by class (rows) and x,y  
649 landmark and Elliptical Fourier Descriptor (EFD) traits hierarchically clustered  
650 (columns) the heteroblasty and species x heteroblasty effects resulting from the model  
651 trait ~ species\*heteroblasty are shown. The heteroblasty effect is the top row and the  
652 species x heteroblasty interaction effects are the subsequent rows. Opacity indicates  
653 magnitude of the effect and color direction (positive, magenta; negative, green). Effect  
654 sizes and directions are relative to *P. actinia* (indicated with asterisk).

655

656 **Figure 7: Juvenile leaves are similar in shape across *Passiflora* species. A)** Heatmap  
657 showing the proportion of correctly assigned species from Linear Discriminant Analyses  
658 (LDAs) performed with both landmark and Elliptical Fourier Descriptor (EFD) data for  
659 each heteroblastic node. The average correct assignment across species is provided as  
660 well. Generally, the proportion of leaves correctly assigned to species increased with  
661 heteroblastic node number. Averaged contours of leaves from each species are provided  
662 for reference and colored by class. **B)** For each trait, the standard deviation across each  
663 heteroblastic node is shown. Traits are arranged by hierarchical clustering and groups  
664 corresponding to x and y coordinates of landmarks indicated. Proportion correctly  
665 assigned: low, black; middle, orange; high, yellow. Standard deviation: low, black;  
666 middle, orange; high, yellow. Class color scheme: class A, teal; class B, orange; class C,

667 lavender; class D, magenta; class E, green; class F, yellow; class G, brown. Heteroblastic  
668 node position is numbered “1” starting from the shoot base.

669

670 **Figure 8: Traversal of the heteroblastic series through t-SNE space. A)** Graph of  
671 Dimension 2 vs. Dimension 1 of leaves, colored by class, in a t-Distributed Stochastic  
672 Neighbor Embedding (t-SNE) analysis. **B)** Same data as in A) except with arrows  
673 corresponding to the leaf series collected for each plant. The first node (towards the base  
674 of the shoot) is the arrow base and indicated with a hollow, black circle. The last node  
675 (towards the tip of the shoot) is the arrow head. Arrows are colored by species class. **C)**  
676 The same arrow data as in B) except that the base of all arrows have been translated to  
677 the origin of Dimension 2 vs. Dimension 1. Arrows are colored by species class. Class  
678 color scheme: class A, teal; class B, orange; class C, lavender; class D, magenta; class E,  
679 green; class F, yellow; class G, brown.

**A***P. edulis**P. caerulea**P. racemosa***B**













